SEROLOGICAL AND MOLECULAR STUDIES OF BACTERIAL POPULATION DEVELOPED FOR BIODESULFURIZATION OF COAL HEAPS

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MUHAMMAD SALEEM

QUAID-I-AZAM UNIVERSITY
ISLAMABAD

National Institute for Biotechnology
& Genetic Engineering (NIBGE), Faisalabad
&
Quaid-i-Azam University, Islamabad, Pakistan.

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CERTIFICATE

This thesis submitted by "*Muhammad Saleem*" is accepted in its present form by the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, and Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in Biotechnology.

Internal Examiner

Dr. Ahmad M. Khalid

External Examiner

Dr. Zabta Khan Shinwari

Chairman

Dr. Ahmad M. Khalid

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DEDICATED

to

My Beloved

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VII. Development of polyclonal antisera for the identification of locally isolated acidophilic bacteria. Paper accepted for oral presentation at "4th International and 14th National Chemistry Conference", 2004 to be held at PCSIR laboratories complex, Lahore.

ABSTRACT

Polyclonal antisera against whole cells of locally isolated acidophilic bacterial isolates i.e (HC-AF2), (KC-AT2), and *Sulfobacillus thermosulfidooxidans* strains (MT13, TH1) used in coal heap biodesulfurization process were successfully raised in rabbits. Among these bacteria, KC-AT2 was observed to be highly immunogenic while TH1 was least immunogenic. The specificity of each of the developed polyclonal antisera was determined against many other acidophilic isolates (TF9, TF10, MT9, MT10, MT16, MT17) by the serological tests including agglutination reaction, fluorescent antibody labeling reaction and enzyme linked immunosorbent assay (ELISA). The polyclonal antisera against mesophilic isolates HC-AF2 and KC-AT2 were found to be more specific as they exhibited no cross reactivity with other mesophilic and moderate thermophilic acidophilic isolates. While polyclonal antisera developed for TH1 and MT13 strains showed cross reactivity against other isolates (MT9, MT10, MT16, MT17) of the same species. Population of these bacteria in coal heap set for biodesulfurization was determined by quantitative ELISA, which indicated that moderate thermophiles were dominated bacteria in leach liquor while in heap both mesophiles and moderate thermophiles were detected. PCR amplification of 16S rDNA of genomic DNA from HC-AF2 and KC-AT2 indicated that they belong to *At. ferrooxidans* (98%) and *At. thiooxidans* (95%), respectively. A phylogenetic tree of these bacteria was constructed by aligning 16S rDNA sequences of other acidophilic bacteria obtained from NCBI database using various softwares available at our laboratory. Integron linked genes were amplified in these bacteria using integron specific primers HS286 and HS287. Two types of DNA bands of approximately 396 bp and 220 bp were obtained in case of both, the HC-AF2 and moderate thermophiles. Similarly in case of mixed culture the bands of same size were obtained. However, in case of pure KC-AT2 no amplification product was observed after agarose gel electrophoresis with the same set of primers.

INTRODUCTION

CHAPTER 1

INTRODUCTION

Two decades ago coal looked like a fuel without future. Petroleum and gas were cheap and abundant and nuclear power was going to electrify the world. In recent years, however, the picture has changed almost beyond recognition. The fuel that fired the industrial revolution has been experiencing a revolution of its own, and has been restored to preponderance.

Major problem associated with direct utilization of coal is its sulfur contents that are from fraction to more than 10%. The burning of this coal on an open fire or in any traditional way, accordingly produce sulfur dioxide and trioxide as well as carbon dioxide. If these oxides of sulfur are released into the environment, they combine with water to become sulfurous and sulfuric acids and sulfates, which are corrosive to structures and potentially pernicious to living organisms. The removal of sulfur from coal before combustion is an attractive emission control strategy. Various physical, chemical and biological methods have been developed for pre-combustion desulfurization of coal (Wheelock, 1977). Among these processes, biocatalytic desulfurization of coal has been given increasing attention in the recent years due to lower capital and operational cost, desulfurization without any loss of coal, operating at relatively low temperatures, and is therefore less energy incentive than chemical processes (Kargi and Robinson, 1985). The key component of biocatalytic desulfurization is microbial population which carries out these processes. Therefore, the observation and measurement of bacterial population is of major interest to understand and control the rate of desulfurization. But unfortunately there are no specific methods present for monitoring bacterial profile during these processes. Present study was carried out to develop serological methods for monitoring specific bacterial species during coal heap biodesulfurization process. Serological methods are quick, accurate and relatively less labor intensive. These methods employ

antibodies that react with components located on the surface of the pathogen e.g. capsid protein, or cell wall or flagellar antigens. Such assays will determine the presence and quantity of these antigens. Among various serological methods, enzyme linked immunosorbent assay (ELISA) is well suited for monitoring large number of samples and the quantitative results can easily be obtained by comparisons against known concentrations of controls.

1.1 STRUCTURE OF COAL

Coal is a compact, stratified mass of fossilized plant debris interspersed with smaller amounts of inorganic matter and covered by sedimentary rocks. Genesis of coal involved decayed plant materials, which by microbial action, under the influence of heat and pressure over a prolonged period of time resulted into coal formation (Hessley *et al.*, 1986). Coal being of fossil origin, is not a homogeneous substance. The coal molecule has a three dimensional structure in which the aromatic constituents are joined by covalent or hydrogen bridge bonds as shown in figure 1.1. In addition to its varying contents of fixed carbon and hydrogen, it contains varying amounts of fixed oxygen, sulfur, nitrogen and trace minerals of which originated as constituents of the living cells that contributed to the fossilization process. Coal also contains varying amounts of minerals, which are co-deposited with the organic minerals during the process of coal formation (Couch, 1987). These minerals, predominantly metal silicates and pyrite, remain physically entrapped within the coal matrix and are largely responsible for the sulfur and ash contents of coal. Carbon bonding and the structure of coals also vary and coal is, therefore a mixture of chemical substances.

Sulfur, in coal exists in two major forms: organic sulfur and inorganic sulfur (Wheelock, 1977). Organic sulfur occurs as a part of complex macromolecular structure of coal and is either covalently bonded to carbon in the general form –R-S-S-R- and –R-S-R- or bonded as sulfate in the general form –R-O-SO_2 (Casagrande and Diefert, 1977). Organic sulfur may be as high as 50-70% of the total sulfur in coal (Attar and Hendrickson, 1982).

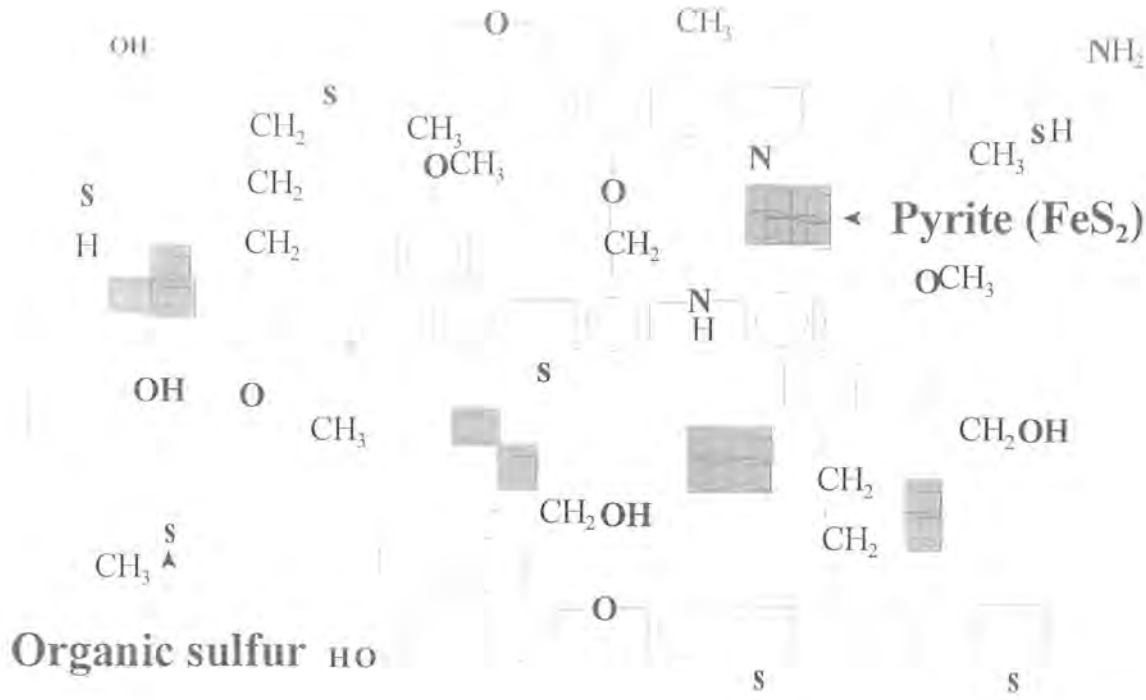

Figure1.1: Structure of Coal

Inorganic sulfur in coal is mainly pyritic (FeS_2), including pyrite and/or marcassite (Kargi, 1986). Pyrite and marcassite have the same composition but different crystal structures. Their components occur as inclusions and are not the integral part of the coal matrix. Pyrite is the most abundant and widespread sulfide mineral. The name, pyrite, is originated from a Greek word, *pur*, fire; friction causes the mineral to emit sparks. Pyrite is a very dense mineral, with a specific gravity of 4.95-5.03, a hardness of 6.0-6.5, a brass-yellow color and a metallic luster. Pyrite is insoluble in hydrochloric acid but its powder is soluble in concentrated nitric acid. Pyrite has a cubic structure but it also occurs as pyrithohedron (125-sided faces), and less commonly as octahedron. It frequently occurs in granulated massive form and sometimes as fibrous radiating nodules. Separate grains occur in many types of rock. Pyrite occurs in a wide variety of geological deposits. It occurs in large masses and veins of hydrothermal origin. Deposits of pyrite

and related minerals often contain relatively high levels of many heavy metals. Weathering of such minerals (main process being oxidation) results in the release of these heavy metals into the environment (Clarke, 1961).

1.2 COAL CLEANING STRATEGIES

The removal of sulfur from coal before combustion is an attractive emission control strategy. A variety of chemical, pre-combustion sulfur removal processes have been developed such as the TRW ferric leaching (Meyers), Battle, Kennecott processes. These, however, have been too expensive to meet practical consideration and, to large degree, their expense has been related to the relatively severe process conditions required (Table 1.1). Further more, for the production of solid fuel it appears undesirable to develop a process that chemically breaks down the coal structure itself. Therefore a biocleaning process that operates under mild conditions has been sought. The operating cost of microbial desulfurization process is lower than those of physical or chemical desulfurization methods (Detez and Barvinchak, 1979). A microbial desulfurization process represents an excellent method of coal cleaning (Ohmura *et al.*, 1989).

Table: 1.1 Comparison of cost estimates for various processes for sulfur removal from coal (Koizumi, 1994).

Process	Approximate Cost (US $ /ton)
Microbiological	10-14
Battelle hydrothermal	20
TRW ferric leaching (Meyers)	20
Kennecott oxygen leaching	22
Solvent-refined coal	45-150

1.2.1 Biocleaning of Coal

Although biotechnology involves the potential use of all living forms, but microorganisms, especially, bacteria have played a major role in this discipline. The diverse metabolic activities of bacteria, and in particular their ability to interact with the

complex organic and inorganic substrates are now being exploited in the treatment of pollution including coal pollution. Biocatalytic methods involve different types of microorganisms for removal of sulfur from coal. Microorganisms that oxidize metal sulfides, especially pyrite (FeS_2), assist in the commercial recovery of metals from sulfide ores and have been studied for desulfurization of coal (Clark *et al.*, 1993). Solubilization of minerals by microorganisms is called microbial leaching or bioleaching and the process of removing pyritic sulfur from coal is called biodepyritization.

1.2.1.1 Mechanism of Biodesulfurization

The ability of microorganisms to remove pyritic sulfur from coal via the oxidative metabolism of sulfide (S^{2-}) to soluble sulfate (SO_4^{2-}) is well established on a variety of bituminous coal samples (Dugan and Apel, 1978; Kargi and Robinson, 1985). The process requires contact either between microbe and pyrite or between a secondary oxidant such as ferric iron and pyrite. Since pyrite (FeS_2) is the major inorganic sulfur compound in coal, the desulfurization of coal is therefore regarded as oxidation of pyrite. The mechanism of microbial attack on sulfide minerals such as pyrite has long been a controversial issue. Two independently separate methods have been proposed (Silverman and Ehrlich, 1964), i.e. indirect and direct mechanism. In direct attack, the microbes attack the mineral directly by oxidizing it in its soluble form. In indirect mechanism, the microbes are responsible for generating a reagent that cause solubilization of the mineral, most commonly through oxidation. Silverman, (1967) concluded that both the mechanisms operate concurrently in the solubilization of the mineral.

1.2.1.1.1 Direct microbial oxidation

In direct microbial oxidation of metal sulfides, a physical contact exists between bacteria and the crystal lattice of susceptible metal sulfide. The oxidation takes through several enzymatically catalyzed steps.

Generally the direct mechanism is expressed by the following reaction:

$$MS + 2O_2 \xrightarrow{Bacteria} MSO_4$$

where M is a bivalent cation that could be Zn, Co, Pb, Ni, etc (Torma, 1987).

Pyrite oxidation to ferric sulfate takes place through following reactions (Silverman, 1967):

$$2FeS_2 + 2H_2O + 7O_2 \xrightarrow{Bacteria} 2FeSO_4 + 2H_2SO_4$$

$$4FeSO_4 + 2H_2SO_4 + O_2 \xrightarrow{Bacteria} 2Fe_2(SO_4)_3 + 2H_2O$$

Microbial involvement is influenced by the chemical nature of both the aqueous and solid crystal phases (Berry and Murr, 1978). Bacteria appear to attack specifically to the sulfide moiety of mineral rock surfaces, which are the regions that contain the energy supply for the bacteria. Attachment and sulfide oxidation results in pitting of the mineral surface.

1.2.1.1.2 Indirect microbial oxidation

The indirect mechanism covers the processes in which the solubilizing agent is merely produced or regenerated by microorganisms and they have only a catalytic function because they accelerate the oxidation of Fe^{2+} that takes place only slowly in the absence of microorganisms. Ferric iron, either alone or in combinations, is the most important chemical species involved in the indirect attack on sulfide minerals (Dutrizac and McDonald, 1974). Reactions to explain the involvement of ferric iron are:

$$MS + 2Fe^{3+} + H_2 + 2O_2 \longrightarrow M^{2+} + Fe^{2+} + SO_4^{2-} + 2H^+$$

$$Fe_2(SO_4)_3 + FeS_2 \longrightarrow 3Fe\,SO_4 + 2S$$

In the presence of iron oxidizing bacteria, the iron produced by these reactions can be oxidized to ferric iron, thereby establishing a ferric-ferrous cyclic process.

Thus the oxidative attack involves two steps i.e. the chemical interaction of ferric iron with the sulfide mineral (Singer and Stumm, 1970) and the regeneration of ferric iron by bacteria.

1.2.2 Advantages of Biodesulfurization

Biocatalytic desulfurization of coal has low capital and operating cost due to mild operational conditions, inexpensive reagents and simplicity of design and construction and is therefore less energy incentive than chemical processes. Being a pre-combustion desulfurization technology, it has an edge over post-combustion desulfurization technologies that it minimizes the damage of machinery due to emission of corrosive gases and also a number of different minerals present in coal are dissolved, which reduces the ash contents as well (Bhattacharya *et al.*, 1988; Anwar *et al.*, 1993). The other advantages of this technique are: it is environment friendly, ash contents of coal are reduced by more than 70%, organic sulfur and nitrogen can also be removed by this process.

1.3 MICROORGANISMS FOR BIODESULFURIZATION

Microbial removal of sulfur from coal is the result of activity of two families of microorganisms, the first functions at about 30° C i.e. *Acidithiobacillus ferrooxidans* and closely related species and the second works at elevated temperatures i.e *Sulfolobus acidocaldarious* and related species (Koizumi, 1994). Following types of acidophilic bacteria are used in biodsulfurization of coal.

1.3.1 Moderate Thermophilic Bacteria

Moderately thermophilic iron oxidizing bacteria play a vital role in those bioleaching operations in which temperature exceeds 40° C. Moderately thermophilic iron oxidizing bacteria of genus S*ulfobacillus* were first isolated from an Icelandic hot spring and a copper leaching dump in 1977 (LeRoux *et al.*, 1977). The genus *Sulfobacillus* includes species *S. thermosulfidooxidans*, *S. thermosulfidooxidans* subsp. *thermotoleras*, *S. thermosulfidooxidans* subsp. *asporogenes*. All these bacteria are Gram's positive, strictly aerobic, of complex morphogenesis, sporulating (excluding the asprogenes subspecies), facultative autotrophs developing on mineral (Fe^{+2}, S^0 and sulfide minerals) and organic substrates including yeast extract, glucose and some amino acids). At relatively low organic substrate concentration (0.05- 0.1%) they may grow heterotrophically. Their G+C contents range from 45.5-49.3 mol%. The genome size is

approximately 3.7×10^9 bp. The bacteria of the genus *Sulfobacillus* are wide spread in nature. They are common in zones of heating dumps, which implies an important role in leaching of metals. They are also able to oxidize sulfide minerals in concentrates actively, which is important for the development of tank-leaching technology. (Karavaiko *et al.*, 1987)

1.3.2 Mesophilic Bacteria

Few species of mesophilic acidophilic bacteria including species from genus *Acidithiobacillus* have been implicated as being the most significant microorganisms involved in sulfide mineral oxidation. Since its discovery in 1902 (Nathansohn, 1902), the genus *Acidithiobacillus* (formerly known as *Thiobacillus*) has come to be recognized as "the genus of convenience", into which a wide range of Gram's negative, rod-shaped, colorless, sulfur oxidizing bacteria have been placed on the basis to obtain energy to support autotrophic growth by oxidizing reduced inorganic sulfur compounds. Subsequent additions to the genus included neutrophiles, acidophiles, halophiles and moderate thermophiles, some of these species showed the ability to grow heterotrophically and mixotrophically, some were even deficient in autotrophic ability (McDonald *et al.*, 1996).

Acidithiobacilli can be classified on the basis of nutritional characteristics, deoxyribonucleic acid (DNA) base composition, and cellular fatty acid composition (Fujiman *et al.*, 1983). The acidophilic, obligate chemolithotrophs in the genus *Acidithiobacillus* include *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. *At. ferrooxidans* and *At. thiooxidans* have continued to draw much interest because of their unique physiological characteristics (e.g. autophilism and acidophilism), environmental impact (e.g. acid and metal pollution) and commercial value e.g. metal recovery by bacterial leaching and desulfurization of coal (Wakao *et al.*, 1990). For better understanding genetics, physiological and taxonomic properties, extensive and systematic collections of these species from diverse localities and habitats in the world are very important (Novo *et al.*, 2000).

These microorganisms grow best between pH 2 to 5. *At. ferrooxidans* and *At. thiooxidans* are mesophiles growing best at temperatures ranging from 25-35°C. *At. thiooxidans* utilizes inorganic sulfur as a source of energy whereas *At. ferrooxidans* can utilize Fe^{2+} as a sole source of energy.

1.3.3 Heterotrophic Bacteria

Chemolithotrophic iron oxidizing bacteria are often contaminated with acidophilic heterotrophic microorganisms. Chemolithotrophic iron oxidizing bacteria and heterotrophic acidophiles can form a highly stable mixed cultures. Many are adept scavengers and rely to a greater or lesser extent on carbon originating as leakage or lysis products from chemolithotrophic acidophiles. Obligatory acidophilic heterotrophs include archaea, bacteria, fungi, yeasts and protozoa. Some prokaryotic acidophilic heterotrophs have a direct role in the dissimilatory oxido-reduction of iron (Pronk and Johnson, 1992). These include the iron-oxidizer *Ferromicrobium acidophilus* (Johnson and Roberto, 1997) which appears to use the energy from iron-oxidation to support growth, and various *Acidiphilium*-like isolates which can use ferric iron as terminal electron acceptor. Many acidophilic archaea are obligate heterotrophs, including *Sulfolobus acidocaldarius*, early reports of this archaean being a facultative chemolithotroph are now thought to be due to the inadvertent use of mixed cultures of *S. acidocaldarius* and another extreme thermophile (possibly *Sulfolobus metallicus*). Some data have indicated that the presence of heterotrophic acidophilic bacteria may enhance the rate of sulfide mineral oxidation by iron-oxidizing acidophiles (Wichlaez and Thompson, 1988); one way in which this may occur is by the heterotrophic bacteria metabolizing organic materials that accumulate in leachate liquors and inhibit the growth of iron oxidizers. The two characterized species of the moderately thermophilic heterotrophic archaean *Picrophilus* have the lowest recorded pH optima for growth (pH 0.7) of all known acidophilic microorganisms (Schleper *et al.*, 1995).

1.4 IDENTIFICATION OF MICROORGANISMS

Although biodesulfurization is relatively slow process, its rate can be improved by monitoring the microbial population in field trails. The methods that are usually used to

identify microorganisms include morphological characteristics, differential staining, biochemical tests, serological analysis, phage typing, amino acid sequencing, fatty acid profiles, flowcytometry, DNA base composition, DNA finger printing, ribosomal RNA sequencing, polymerase chain reaction, and nucleic acid hybridization (Tortora, 1997). But the identification and especially the enumeration of bacteria in leaching system is difficult task because of the presence of mineral particles. Also it is difficult to obtain growth of leaching bacteria on defined solid media (Harrison, 1984), although the media, which allow the growth of a variety of iron-oxidizing acidophiles, have been developed (Johnson, 1995). Even with this development it is still difficult to distinguish bacteria by colony morphologies alone and incubation time for the development of colonies is usually on the order of one week or more (Hallberg and Lindstrom, 1996). In addition, the failure of many bacteria to form colonies is a widely acknowledged problem when using plate-counting procedures (Pace *et al.*, 1986). Therefore, other quicker means of identification and enumeration of leaching bacteria have been developed. Serological tests involving the reactions of microorganisms with specific antibodies are useful in the identification of microorganisms. These tests can differentiate not only among microbial species but also among strains within species. Antibodies generated against whole cell of species can be used for determining the involvement of this species in the desulfurization of coal. Jerez and Arredondo, (1991) showed that approximately 10^3-10^4 cells of *Leptospirillum ferrooxidans* could be rapidly counted in leaching system by using antibodies.

1.4.1 Serology

The studies involving the reactions of antiserum with the antigen outside the animal body come under the heading of serology. All the serological reactions have the same underlying principle i.e. specificity of the reaction of an antibody with an antigen that elicits its formation (Somasegaran and Hoben, 1985). Antigen antibody reactions are highly specific in that the antibody reacts only with antigen that elicited its formation. Although monoclonal antibodies are more specific and presumably more reliable but when polyclonal antibodies serve the same purpose without being a threat to specificity, they are preferred.

1.4.2 Development of Antibodies for the Identification of Microorganisms

A wide range of vertebrate species can be used for the production of antibodies. However, the following basic points should be kept in mind before selecting an animal species for immunization:

I Quantity of antigen available

II Quantity of serum required

III Type of antibodies needed

IV The species from which antigens will be isolated

The most commonly used animal systems are hamsters, guinea pigs, mice, and rabbits. Rabbits are the best choice for the production of polyclonal antisera (Harlow and Lane, 1988). Their use has got many advantages such as easy to keep and handle. In addition, they can be safely and repeatedly bled without being too harmful to the animal. Moreover, the antibodies produced are well characterized and easily purified. Enough amount of high-titered serum can be obtained for relatively small amounts of antigen used for immunization. With other small animals, the amount of serum achieved is relatively low. Thus, rabbits are the best choice for the production of polyclonal antiserum (Catty, 1988; Ball *et al.*, 1990). The minimum number of animal used should be two because even in genetically identical animals, different antibodies will be produced with a single preparation of antigen. The preferable number of animals is three to four.

1.4.3 Immune System and Different Stages of Immune Response

1.4.3.1 Definition

The term immunity is derived from the Latin word *immunitas*, which referred to the exemption from various civic duties and legal prosecution offered to Roman senators during their tenures in office. Historically, immunity meant protection from disease, and more specifically, infectious disease. The cells and molecules responsible for immunity constitute the immune system (Abbas *et al.*, 1998).

Immune responses are processes in which animals form specifically reactive proteins (antibodies) and cells in response to a great variety of foreign organic macromolecules (Pelczar *et al.*, 1986). Immune response is found only in vertebrates. It is

the basis of internal defense mechanism of an animal system which is not only against infections by pathogens but also against cancerous host cells.

1.4.3.2 Different Stages of Immune Response

When an animal has been exposed to an antigen, antigen-presenting cells (APCs), T-cells and B-cells act in concert to stimulate the production of antigen-specific antibodies (Harlow and Lane, 1988; Long and Kindt, 1988).

1.4.3.2.1 Interaction between an antigen (Ag), an antigen presenting cell (APC) and a T cell:

When an antigen is introduced into the body. APCs, such as B cells, interact with it, ingest it and breakdown or process it (Fig 1.2A). The processed antigen is then returned to the surface of APC where it is presented by the cell's major histocompatibility molecule (MHC) (Long, 1988; Long and Kindt, 1988). A T cell with a receptor specific for both the processed antigen and MHC molecule (Fig 1.2B) on the APC interacts with APC (Fig 1.2C) (Ezquerra and Coligon, 1988). A trimolecular complex is formed between the T cell antigen receptor, the processed antigen and the MHC molecule on the APC (Fox, 1988). This complex triggers the APCs and T cells to produce a variety of soluble factors such as interleukin 1 (IL-1) (Fig 1.2D) and interleukin 2 (IL-2) (Fig 1.2E) which stimulate T cell proliferation and differentiation.

1.4.3.2.1 Interaction between an antigen, a B cell and an activated T cell:

A B cell, with a surface antibody specific for the antigen, interacts with the antigen (Fig 1.3A). The antigen is taken up and processed by the B cell (Fig 1.3B). The antigen is presented by the B cell's MHC molecule (Fig 1.3C). Properly activated T cells bind to antigen presenting B cells and produce IL-4 and IL-5, which stimulates B cell multiplication and differentiation. T cells secreted IL-5, along with other signals; allow B cells to differentiate into either short-lived (3-4 days) plasma cells that secrete large amounts of antigen or long-lived memory cells (Fig 1.3D). Memory B cells are primed to respond to further antigen stimulation and do not secrete but rather express antibody on their surfaces (Harlow and Lane, 1988).

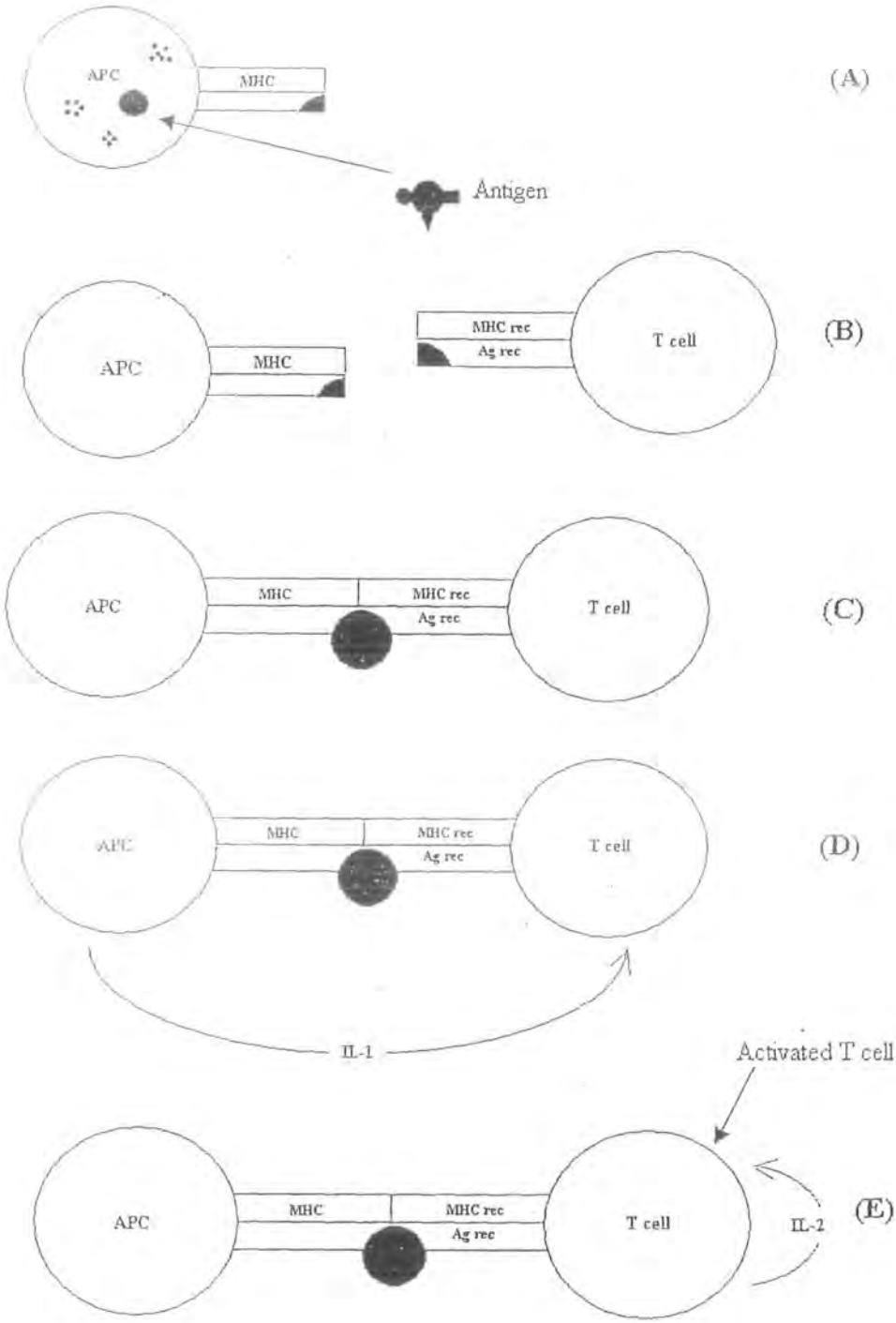

Figure 1.2: Interaction between Ag, APC and a T cell.

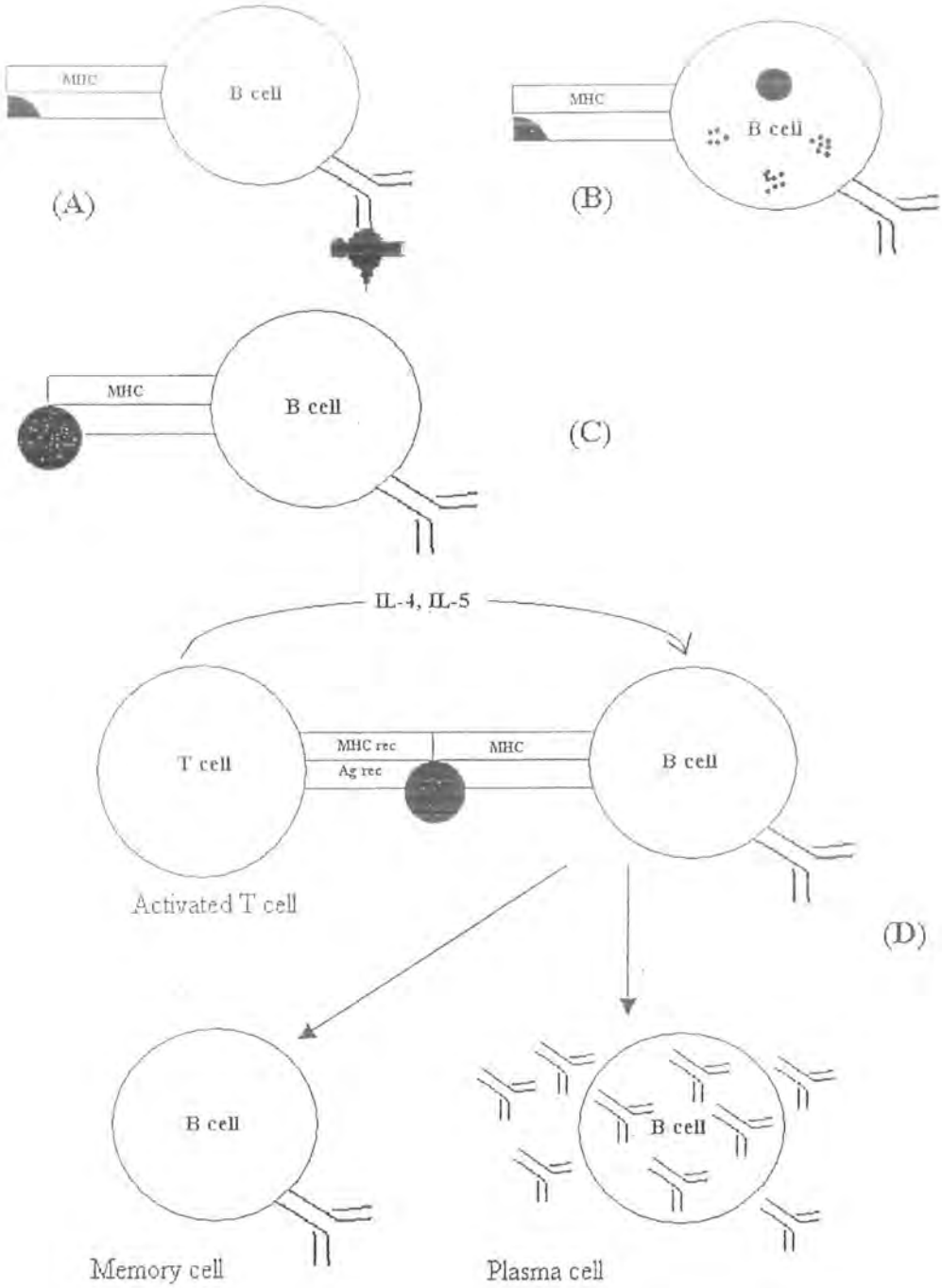

Figure 1.3: Interaction between Ag, a B cell and an activated T cell.

1.4.4 Description of Antigen and Antibody

An antigen is a molecule that evokes a specific immune response upon introduction into an animal. The response may be in the form of antibody production, cell-mediated immunity or tolerance. Antigen should also react observably with an antibody it has caused to be produced. The term antigen is also used more broadly to denote mixtures of molecules, whole microorganisms or cells used as an immunizing entity or as a complex target for antibody binding in immunoassay.

By definition, antibodies are immunoglobulin molecules produced in direct response to an antigenic stimulus and they should combine specifically, non-covalently and reversibly with an antigen that elicited their formation. The term antibody is also used to refer to a homogeneous or heterogeneous population of antibodies, which are in principle polyclonal antibodies (Abbas *et al.*, 1998).

1.4.5.1 Structure of Antibody

A monomeric immunoglobulin (Ig) molecule is composed of two identical heavy (H) and two identical light (L) polypeptide chains that are linked together by disulfide bonds as shown in figure 1.4. Both H and L chains are organized into domains that are defined by homology and are approximately 110 amino acid in length. Each domain forms a conserved structure, known as the antibody fold, which is stabilized by an internal disulfide linkage that forms a loop of about 65 amino acids. Digestion of IgG with papain yields three fragments, as shown in figure 1.5. Two of these fragments, each with a heavy and light chain, are equal in size and are antigen-binding (F_{ab} fragment). The third fragment, consisting of antibody heavy chain only, does not bind antigen and crystallizes at 4°C (F_c fragment). The region between F_{ab} and F_c that gives flexibility to antibody molecule, needed for F_{ab} segment to operate independently of one another, is called hinge region (Mernaugh *et al.*, 1993). In an antibody molecule, variable regions of heavy and light chains are paired with each other to form a site that recognizes and bind to an antigen, called an antigen binding site or paratope. Each antibody has two or more such sites, a paratope has the capability to recognize and bind a single epitope. Antibody affinity relates to the exactitude of stereo-chemical fit of an antibody-combining site to its complementary antigenic determinant (Jerne *et al.*, 1974).

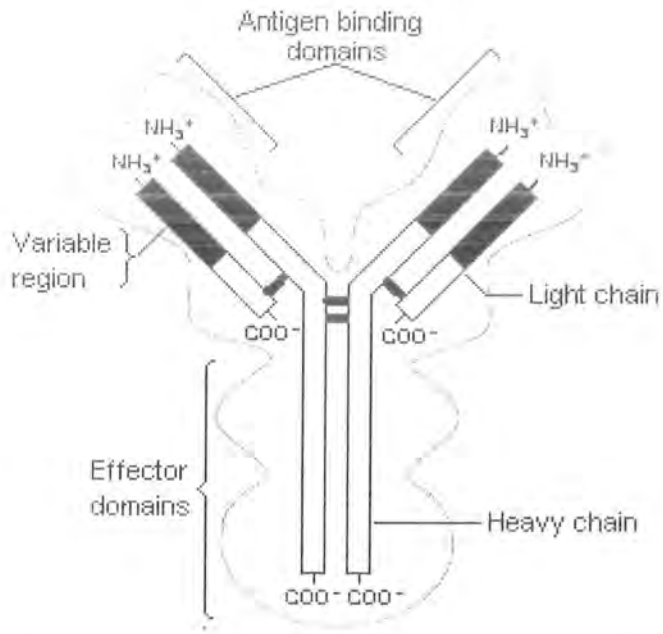

Figure 1.4: A monomeric immunoglobulin molecule (Kennedy *et al.*, 1986)

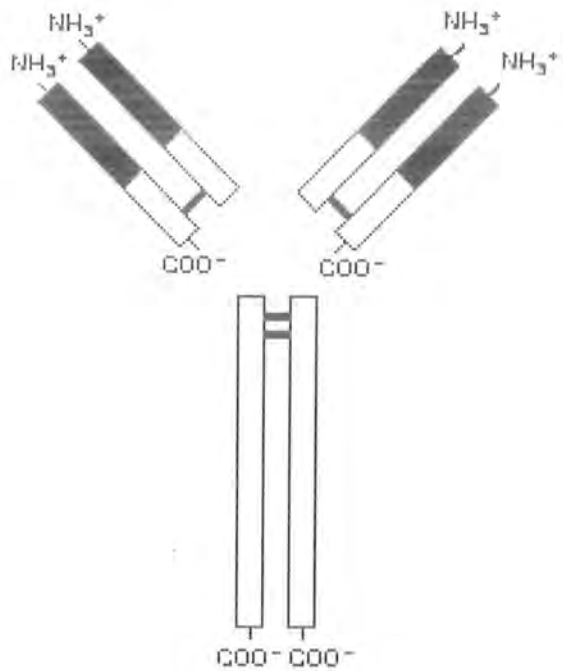

Figure 1.5: IgG molecule after hydrolysis with papain.

1.4.6 Antigen-Antibody Reaction

Each antibody molecule is specific to the antigenic determinants to which it binds. The specificity of an antiserum by contrast, reflects the much specificity of the constituent antibodies. However, some antigenic determinants are shared between molecules especially if there are similar molecules of related species. In this case some antibodies, induced in response to one antigen may bind to another antigen and are then said to be cross-reacting and the containing antiserum is said to lack specificity or be cross reactive. The reaction of an antigen with antibody results in the formation of an antigen-antibody complex (AgAb):

$$Ag + Ab \rightleftharpoons AgAb \qquad (1)$$

AgAb is formed by non-covalent interactions such as hydrogen bonds, electrostatic charges and coulombic, hydrophobic, and van der Waal's forces (Sheriff *et al.*, 1987; Harlow and Lane, 1988; Van Regenmortel, 1988). The reaction is reversible and the affinity or the strength of the reaction is affected by many factors, such as temperature, pH, and solvent conditions (Harlow and Lane, 1988). Because the reaction is non-covalent and neither reactant is altered, the reaction is, in theory, a reversible one. All of the methods used to quantify the antigen-antibody reaction depend upon the ability to measure AgAb (Golub, 1987). Avidity is the measure of overall stability of the antigen-antibody complex. Since avidity describes the complete reaction, this value ultimately determines the success of all immunochemical techniques (Harlow and Lane, 1988). The more the number of paratopes on an antibody, the greater is its avidity. However, there is a possibility that a paratope of an antibody exhibits low affinity of an antigen but such weak bonding is subsidized by the rest of the paratopes attached to nearby epitopes, thus the interaction between an antigen and antibody is stabilized.

1.4.6.1 Affinity of Antigen-Antibody Reaction

Because the formation of AgAb can be treated as a chemical reaction between two species and because the reaction is reversible, the affinity or strength of the reaction can be determined. According to the law of mass action, the rate of a reaction is

proportional to the concentration of the reactants. By applying the law of mass action to equation (1), we obtain:

$$Ag + Ab \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AgAb \qquad (2)$$

$$k_a [Ab] [Ag] = k_d [AgAb]$$

Where [Ag] and [Ab] are the concentrations of free antigen and antibody; [AgAb] is the concentration of AgAb complex; k_a and k_d and are the association and dissociation constants, respectively.

From equation (2), we can write the equilibrium constant for the reaction

$$K = \frac{k_a}{k_d} = \frac{[AgAb]}{[Ab][Ag]}$$

Affinity is the sum of the non-covalent attractive and repulsive forces, stabilizing the complex and is therefore the same as the equilibrium constant (Golub, 1987).

These equations are only approximations of the actual conditions as antibodies are extremely heterogeneous. For monoclonal antibodies, these equations come very close to representing the actual interactions.

1.5 IMMUNOLOGICAL METHODS FOR IDENTIFICATION OF MICROORGANISMS

Immunological methods are widely used to detect antigens in clinical, agricultural and environmental samples. Accurate diagnosis depends on the affinity and specificity of the antibody preparation used, and high affinity antibodies are essential for the detection of very small amounts of antigens. These antibodies react with components located on the surface of the pathogen e.g. capsid protein, or cell wall or flagellar antigens. These methods determine the presence (and quantity) of these antigens. Hallberg and

Lindstrom, (1996) have reported that when antiserum was generated against whole cell of *At. caldus* KU. It recognized protein antigens common to cell lysates of the three *At. caldus* strains KU, BC13, and C-SH12 but did not recognized the whole cell lysates of C-SH12. Difference in the lipopolysaccharide (LPS) of strain C-SH12 and those of other two *At. caldus* strains were found, and the anti-KU antiserum did not recognized the LPS from strain C-SH12. This data indicated this *At. caldus* isolate belongs to a serotype different from that of strains KU and BC13. (Hallberg and Lindstrom, 1996). Immunological methods may also be combined with other tests (e.g. culture, dye permeability stains, nucleic acid based tests) to confirm the identity and viability of positive samples. The serological methods usually used for the identification are agglutination, precipitation, immunofluorescence, enzyme linked immunosorbent assay (ELISA), and membrane immunoblot. (Somasegaran and Hoben, 1994)

1.5.1 Agglutination Reactions

The process in which antigens are linked together by their corresponding antibodies is called agglutination. When an antigen is particulate (e.g. a cell), it settles to the bottom of the container and forms a visible pellet. If the cells have been reacted with antibody, the pattern of settling will be altered and the cells will agglutinate. This agglutination can produce various forms of AgAb complex, ranging from large clumps to fine material with the appearance of ground glass (Golub, 1987). The agglutination reaction depends upon a firm structural relationship between an exposed antigen and the bacterium. Linus Pauling's lattice hypothesis is the widely accepted concept for explaining the agglutination reaction. He proposed that the antibody is bivalent and that the antigen-antibody complexes are molded into a lattice or framework of alternating antigen-antibody particles (Somasegaran and Hoben, 1994).

1.5.2 Fluorescent Antibody Labeling Reactions

Fluorescent-antibody (FA) technique is a versatile serological test for identification of microorganisms. This technique involves the fluorescent dyes such as fluorescein isothiocynate (FITC), lissamine, and rhodamine etc. These chemical dyes have the property of fluorescing when excited by near UV light. Polyclonal antibodies

developed in rabbits can be conjugated to these fluorescing chemical dyes or fluorochhromes. The chemical dye that is most commonly used for labeling the specific antibody is FITC. FITC is a small organic molecule and is typically conjugated to proteins via primary amines (i.e lysine). Usually 3-6 FITC molecules are conjugated to each antibody molecule. FITC gives an apple-green fluorescence upon irradiation with blue light. In practice, a smear of cells is made on microscopic slide, and this smear is allowed to react with specific antibody labeled with FITC. These procedures are sensitive and very specific (Tortora, 1997). There are two types of fluorescent antibody techniques, namely the direct and indirect immunofluorescence. In direct method, the specific antiserum is conjugated and is used as a stain in the procedure. Where as in the indirect method the unconjugated specific antibody is first reacted with the antigen smear, and after sufficient time is allowed for antigen-antibody reaction, the smear is washed free of excess antiserum. The above complex is then stained with the FITC-labeled secondary antibody.

Polyclonal antisera, produced against whole cells of *Acidithiobacillus thiooxidans*, *At. ferrooxidans* and *Leptospirilum ferrooxidans*, gave highly specific reactions when cross reacted with 23 strains of acidophilic bacteria using an immunofluorescence (IF) staining technique. Strains of identical serotype exhibited maximum cross-reaction whereas strains of different serotypes reacted only weakly. Lipopolysaccharides (LPS) examined by SDS-PAGE showed different, serotype-specific migration patterns indicating their rough or smooth character. LPS patterns may therefore be used for serological classification of acidophilic bacteria. By immunoblot staining surface antigens of four strains were identified. (Koppe and Harms, 1994)

1.5.3 Enzyme-linked Immunosorbent Assay (ELISA)

Of the wide range of immunoassays available, enzyme-linked immunosorbent assay (ELISA) is well suited for testing large numbers of samples, and quantitative results are readily obtained by comparisons against known concentrations of control antigens. ELISA formats can be devised for different requirements, such as testing large numbers of samples, the detection of very small amounts of antigens, or for rapid 'on-site' assays that require little expertise or facilities to perform (Martins *et al.*, 1998).

ELISA is a useful and powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine and culture supernatant. ELISA methods combine the specificity of antibody molecules with the amplification of antibody-antigen interactions by enzyme catalysis, and therefore can detect very small amounts of pathogen. There are many different ELISA methods, and both qualitative and quantitative results can be obtained (Tijssen, 1993).

The use of the enzyme linked immunosorbent assay (ELISA) techniques since its first description in 1971 (Engvall and Perlmann, 1972) is widely distributed for detecting antigens and antibodies. Briefly, the assay involves: 1) the adsorption of antigen or antibody to a solid phase; 2) the addition of sample; 3) incubation and washing steps; 4) addition of enzyme labeled antigen or antibody, and 5) the addition of the enzyme substrate and incubation, 6) colorimetric measurement of colored product.

The first ELISA technique for detecting antibodies against a bovine haemoparasite was described in 1976 by Purnell, for *Babesia divergens*. Barry *et al.*, (1982) described the first ELISA for detecting antibodies to *B. bovis* showing agreement of more than 95% with the indirect fluorescent antibody test (IFAT) in a comparative study. Also the assay was able to detect *B. bovis* antibody 14 days after experimental infection showing more sensitivity than the IFAT.

1.5.3.1 Advantages of ELISA

ELISA methods are the simplest and least labour intensive of the available immunoassays. Moreover, relatively unskilled operators with little training can do rapid tests. These methods are largely unaffected by contaminants in concentrated samples. In comparative tests, a rapid ELISA test for *Cryptosporidium* in faecal samples was shown to give equivalent results to immunofluorescence assay (IFA) (Dagan *et al.*, 1995; Graczyk *et al.*, 1996). The assays can be readily standardized, and optimized to minimise variation both between and within assays (Tijssen, 1993).

1.6 MOLECULAR ANALYSIS FOR IDENTIFICATION

Bacteria are vital components of global ecology, but we have very little information on how bacteria interact with each other in natural communities. Among the

various components used for taxonomy, only chromosomal DNA and RNA are unaffected by growth conditions. The amount of these molecules will fluctuate with growth rate, but the composition is invariant. Thus the nucleic acids offer the only standard molecules by which the widest range of microorganisms (and higher eukaryotes) can be compared and classified. Constitutively synthesized proteins are also very useful in this respect but individual proteins may not be distributed universally (Brian and Fergus, 1986). Ribosomal RNA sequence analysis has been extensively used to study phylogenetic relationship between bacteria (Woese, 1987), as well as for taxon identification (Woese *et al.*, 1985). A significant achievement of molecular evolution studies has been the recognition of three domains, the Bacteria, the Archaea and the Eucarya, each containing two or more kingdoms (Woese *et al.*, 1990).

1.6.1 Importance of rRNA studies

Ribosomal RNA is an invaluable procedure for extending classification, established by DNA re-association and phenotypic studies, to more distant organisms, and is being used to provide a comprehensive view to the relationship among all prokaryotes. Of the three methods, sequencing 5S RNA and cataloging 16S RNA molecules have the advantage of providing information for individual organisms that can be processed using estimates of similarity and clustering algorithms. Some 400 bacterial species have now been characterized by 16S rRNA cataloging (Stackebrandt and Woese, 1984).

1.6.2 16S rRNA sequence analysis

16S rDNA sequence-based bacterial identification is the most advanced and accurate as it is based on highly conserved sequence stretches. It is more reliable and precise compared to error-prone conventional morphological and metabolic identification techniques. This conserved sequence code can be used even by users with limited expertise in the field of bacterial systematics. The primary structure of 16S rRNA contain stretches of sequences conserved to varying degree and their positions are mostly known. The information gained from 16S rRNA gene sequence comparisons can be used to deduce detailed phylogenetic relationships based on evolution (Woese *et al.*, 1985). The

highly conserved portions of 16S rRNA genes are ideal for designing primers that will amplify small subunit rRNA genes from all three domains of life (Bacteria, Archaea, and Eucarya). At the other extreme, primers can be designed to highly variable regions of 16S rRNA genes and thus amplify only a particular species or genus in a mixture of microorganisms. Using conserved primers, the16S rRNA gene can be easily amplified by PCR not only from pure cultures but also directly from the environmental samples (Giovannoni *et al.*, 1990; Olsen *et al.*, 1986; Pace *et al.*, 1986; Ward *et al.*, 1992).

1.6.4 Microbial Population Studies by Integron Profile Analysis

Integron profile analysis may be useful in the identification of bacterial population developed during biodesulfurization of coal heaps. By observing the integron profile of separate and mixed cultures at various time intervals during biodesulfurization, we can observe if there is any change occurring in the microbial population of heap. Integrons are gene acquisition and expression systems. The units of DNA captured by integrons, gene cassettes, are the simplest known mobile elements and consist of only a gene and a recombination site known as a 59-be (59-base element). Cassettes are inserted into or excised from integrons by a site-specific recombination reaction catalyzed by integrase, IntI, (Collis *et al.*, 2002). Multiple insertion events lead to the formation of multicassette arrays, which in chromosomal integrons may contain over 150 cassettes. In such arrays, 59-be recombination sites flank essentially all cassette-associated genes. While these sites are variable in terms of both their sequence and length, they do have a number of common features, including a conserved sequence of about 25 bp at each end that forms imperfect inverted repeats (Hall and Collis, 1995). In the present study integrity of genetic make up of bacteria used for coal heap biodesulfurization will be studied through integron profile screening.

OBJECTIVES

Biocatalytic desulfurization of coal has been given increasing attention in the recent years due to lower capital and operational cost, desulfurization without any loss of coal, operating at relatively low temperatures and environment friendly. But Upscaling of these processes upto commercial level require the optimization of all the parameters affecting them. One of the key parameter affecting these processes is microbial population involved. So there is need to monitor the bacterial populations during desulfurization processes because the observation and measurement of bacterial population is very important to understand and control the rate of desulfurization. Serological and molecular analysis can play a vital role in this regard. The present study was conducted to analyze the bacterial population developed during biodesulfurization of coal heaps, through serological and molecular approaches. The specific objectives of present work are:

1. Development of polyclonal antibodies against acidophilic moderate thermophilic bacteria *Sulfobacillus thermosulfidooxidans* TH1 and MT13 (local isolate) and mesophilic bacteria *Acidithiobacillus ferrooxidans* (strain HC-AF2) and *Acidithiobacillus thiooxidans* (strain KC-AT2).
2. Determination of specificity and cross reactivity of developed polyclonal antibodies through various serological tests like agglutination, ELISA and FA staining.
3. Application of the above antibodies to the samples taken from heap at various time intervals for the quantification of specific bacterial strains in heap.
4. Phylogenetic studies of locally isolated coal desulfurizing bacteria strains through 16S rDNA amplification.
5. Integron profile study of separate and mixed culture of moderate and mesophilic bacteria used in coal biodesulfurization.

MATERIALS & METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 MICROORGANISMS

Microorganisms used in the present study were local isolates from **NIBGE Culture Collection**, isolated from their natural habitats (Table 2.1).

Table 2.1: Bacterial species used in the present study.

Sr. No.	Isolate	Source	Notes
1	***Sulfobacillus thermosulfidooxidans*** Strains MT13, MT9, MT10, MT16 & MT17 TH1	Uranium mines, Dera Ghazi Khan, Punjab Standard strain	Isolated and purified by Dr. M. A. Ghauri by growing on FeTSB (Johnson *et al.*, 1987) solid medium.
2	***Acidithiobacillus ferrooxidans*** Strain HC-AF2 ***Acidithiobacillus ferrooxidans*** Strains TF9, TF10	Coal mines at Hernai, Balochistan Uranium mines, Dera Ghazi Khan, Punjab	Isolated and purified by Mr. Munir A. Anwar & Dr. M. A. Ghauri by growing on FeTSB solid medium.
3	***Acidithiobacillus thiooxidans*** Strain KC-AT2	Coal mine site at Khushab, Punjab	Isolated and purified by Mr. Munir A. Anwar by growing on Gelrite (Khalid *et al.*, 1993) medium

2.2 GROWTH MEDIA FOR ACIDOPHILIC MICROORGANISMS

Both solid and liquid media were employed for the growth of *Sulfobacillus thermosulfidooxidans*, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*.

2.2.1 Solid Media

2.2.1.1 Solid Medium for *Acidithiobacillus ferrooxidans* and *Sulfobacillus thermosulfidooxidans*

(FeTSB medium)

FeTSB medium, developed by Johnson *et al.* (1987), enabled simultaneous growth of acidophilic iron-oxidizers and acidophilic heterotrophic bacteria. This medium consists of three solutions that were prepared separately and were mixed together after sterilization.

The composition of these solutions is as follows:

Solution I (Ferrous sulfate solution)

Ferrous sulfate solution (20% w/v) was prepared in distilled water, acidified to pH 2.0 with 0.5 M sulfuric acid and then filter-sterilized using 0.2 µm nitrocellulose membrane filter and was stored in a pre-autoclaved bottle of suitable size at 4° C.

Solution II (Basal Salt-Tryptone Soya Broth Solution)

This solution was prepared by dissolving following compounds in distilled water

$(NH_4)_2SO_4$	1.8 g/L
$MgSO_4.7H_2O$	0.7 g/L
TSB	0.35 g/L

The pH was adjusted to 2.0 using sulfuric acid. This solution was autoclaved at 121° C and 15 psi for 15 minutes.

Solution III (Gelling solution)

Gelrite solution (0.5 w/v) was prepared and sterilized by autoclaving at 121°C and 15 psi for 15 minute.

These solutions i.e. solution I, solution II, and solution III, were combined in the ratio 1:14:5, respectively, and poured into pre-sterilized petri dishes. The petri plates inoculated with *At. ferrooxidans* were incubated at 30° C, where as those inoculated with *S. thermosulfidooxidans* were incubated at 45° C.

2.2.1.2 Solid Medium for *Acidithiobacillus thiooxidans*

The solid medium for the growth of *Acidithiobacillus thiooxidans* was consisted of following three solutions that were prepared separately and mixed together after sterilization.

Solution I (Sodium thiosulfate solution)

10% w/v Sodium thiosulfate solution (pH 4.0) was prepared in distilled water and then filter-sterilized using 0.2 µm (Millipore) nitrocellulose membrane filter and was stored in a pre-autoclaved bottle of suitable size at 4° C.

Solution II (Basal Salt Solution)

1X basal salt solution was prepared by dissolving following compounds in distilled water:

$MgSO_4.7H_2O$	0.05 g/L
$(NH_4)_2SO_4$	0.015 g/L
KCl	0.005 g/L
KH_2PO_4	0.005 g/L
$Ca(NO_3)_2$	0.001 g/L

The pH was adjusted to 4.4 using dilute NH_4OH. This solution was autoclaved at 121° C and 15 psi for 15 minutes.

Solution III (Gelling solution)

Gelrite solution (0.5 w/v) was prepared. It was sterilized by autoclaving at 121° C and 15 psi for 15 minute.

These solutions i.e. solution I, solution II, and solution III, were combined in the ratio 1:6:3 respectively, and poured into pre-sterilized petri dishes. The petri plates inoculated with *At. thiooxidans* were incubated at 30° C.

2.2.2 Liquid Media

2.2.2.1 Liquid Medium for Growing *Acidithiobacillus ferrooxidans*

Iron medium

The liquid medium for the growth of iron-oxidizing *Acidithiobacillus ferrooxidans* was prepared according to the composition described by Leathen *et al.*, (1956) and modified by Postgate, (1966). Its composition is as follows:

Solution A (Basal salt solution)

It contains the following basal salts:

$MgSO_4.7H_2O$	0.50 g/L
$(NH_4)_2SO_4$	0.15 g/L
KCl	0.05 g/L
KH_2PO_4	0.05 g/L
$Ca(NO_3)_2$	0.01 g/L

A stock solution (10X) was prepared and stored at room temperature.

The pH was adjusted to 1.8-2.0 using 1.0 M H_2SO_4. Aliquots of basal salt solution were diluted 10 times and sterilized by autoclaving at 121° C and 15 psi for 15 minutes.

Solution B

Solution B of iron medium is 0.5 M ferrous sulfate solution and is used as energy source for iron-oxidizing *Acidithiobacillus ferrooxidans*. Ferrous sulfate solution (0.5 M) was prepared by dissolving 139 g $FeSO_4.7H_2O$ in 1litre of distilled water having pH 1.8-2.0. Solution was sterilized by using 0.2 μm (Millipore) nitrocellulose membrane filter and was stored in a pre-autoclaved bottle of suitable size at 4° C.

To prepare the growth medium for *At. ferrooxidans*, appropriate volume of ferrous sulfate (Solution B) was dispensed in basal salt solution (Solution A) to give final ferrous ion (Fe^{2+}) concentration 50 mM. After growth on iron medium, *At. ferrooxidans* was inoculated (10%) in a medium containing finely ground (<61 μm) pyrite. The composition of medium was same as that of solution A (basal salt solution) in iron medium, but instead of using solution B (0.5 M $FeSO_4$ solution), 0.35% pyrite was added as substrate for the growth of *At. ferrooxidans*.

2.2.2.2 Liquid Medium for Growing *Acidithiobacillus thiooxidans*

Sulfur medium

Elemental sulfur medium was prepared with same basal salt solution as described earlier. pH of the medium was adjusted to 2.8-3.0 using 0.5 M sulfuric acid. Finely ground sulfur, at a concentration of 1% (w/v) was added as an energy source for *Acidithiobacillus thiooxidans*. Before adding to autoclaved medium, finely ground sulfur was steam sterilized.

2.2.2.3 Liquid Medium for Growing *Sulfobacillus thermosulfidooxidans*

The liquid media for growth of *S. thermosulfidooxidans* is same as for *Acidithiobacillus ferrooxidans* as described in section 2.2.2.1 but with addition of 0.03% TSB or yeast extract.

2.2.3 GROWTH OF MICROORGANISMS

After purification on solid media *S. thermosulfidooxidans*, *At. ferrooxidans* and *At. thiooxidans* were grown in liquid medium for serological as well as molecular studies. *S. thermosulfidooxidans* and *At. ferrooxidans*, were grown in basal salt solution containing 0.35% (w/v) finely ground (<61 μm) pyrite as energy source. pH of the medium was adjusted to 2.0 with sulfuric acid. After sterilization, the flasks were inoculated (10%) and incubated on an orbital shaking incubator at 150 rpm. *S. thermosulfidooxidans* were incubated on 45° C while *At. ferrooxidans*, were on 30° C. The growth was confirmed by complete oxidation of ferrous iron to ferric iron.

At appropriate intervals, 1 mL aliquots from the test flasks were titrated against 1.0 mM $KMnO_4$ solution to calculate the amount of ferrous iron oxidized to ferric iron. The end point of the titration was the appearance of a slightly pink color.

The stochiometery of the ferrous iron and permanganate reaction is:

$$Fe^{2+} \longrightarrow Fe^{3+} + e^-$$

$$MnO_4^- + 8H^+ + 5e^- \longrightarrow Mn^{2+} + 4H_2O$$

It means 1 mole of MnO_4^- oxidizes 5 moles of Fe^{2+}.

At. thiooxidans were grown in basal salt solution supplemented with elemental sulfur (1% w/v) as energy source at pH 2.8-3.0 and temperature 30° C on an orbital shaking incubator at 150 rpm. Growth of *At. thiooxidans* was confirmed by sulfur oxidation, which was monitored by reduction in pH in the test flasks against a control. The decrease in pH was due to the production of sulfuric acid from the oxidation of sulfur according to the following chemical reaction:

$$S + \tfrac{1}{2} O_2 + H_2O \longrightarrow H_2SO_4$$

2.2.3.1 Harvesting of Cells

After obtaining rich growth, whole contents of flasks were filtered through Whatman No. 1 filter paper to remove pyrite or sulfur particles under sterilized conditions. After filtration the cell mass was harvested by using centrifuge (Beckman J2-HS), with JA-14 rotor at 10,000 rpm for 10 minutes at 4° C.

2.2.3.2 Washing of Cells

The cell pellet was washed twice with autoclaved distilled water having pH adjusted to 2.0 using 1 M sulfuric acid to remove jarosites, and once with autoclaved distilled water. Finally the pellet was resuspended in 10 mL of previously mentioned basal salt solution (Section 2.2.1.1). Washed cells were stored at 4° C for further studies.

2.2.4 Maintenance of Pure Cultures

In order to keep the microorganisms alive, subculturuing was done routinely after each 20-25 days in fresh autoclaved respective liquid medium using the same culture conditions.

2.3 SEROLOGICAL STUDIES

Rabbit antiserum developed against different bacterial strains (TH1, MT13, HC-AF2 and KC-AT2) was the basic reagent for strain identification utilizing serological techniques. Therefore antigens of these bacteria were prepared and then used for the development of antisera in rabbits.

2.3.1 Antigen Preparation

Rich growth of bacteria was taken for preparing antigens from these bacterial species. After washing, the cell pellet was resuspended in filter-sterilized saline (NaCl; 0.85% w/v) and cell concentration was adjusted to approximately $1x10^9$ cells/mL i.e. absorbance at 600 nm (A_{600}) was adjusted approximately to 0.45 using double beam spectrophotometer (Cary1, Varian, Australia). For different strains, different cell concentrations were used for the preparation of antigens according to the response of rabbits against these strains.

2.3.1.1 Heat Inactivation

The cell suspension was transferred to sterile serum bottles and closed with rubber septum. A small gauge (about 23G) needle was inserted through the septum to act as an air and steam vent. The antigens were heat treated for one hour at 100° C to inactivate any remaining flagellar and internal antigens. Heat inactivation was accomplished by partly immersing the serum bottle in boiling water or by subjecting it to heat in a steam bath. After heat inactivation, 1 mL of 1% filter-sterilized merthiolate (Thimrosal, Sigma) per 100 mL of antigen suspension was added as a preservative (i.e in ratio of 1:10000) and then stored at 4° C.

2.3.2 Immunizing the Rabbits

Four to five months old, healthy, albino rabbits were used, in duplicate, for each strain for the production of polyclonal antibodies. Each rabbit was labeled with an ear tag and was pre-tested for the presence of antibodies against the bacterial species, which is being injected. Individual records of all treatments to which each rabbit was subjected were maintained. Various schedules for stimulating antibody production and recovering blood samples were reported (Schmidt *et al.*, 1968; Dudman, 1964). The schedule used in present study is mentioned in table 2.2.This schedule employed two different routes of injection.

I Intravenous injections (IV)

Intravenous injections were given to rabbits in the marginal vein of one ear (Figure 2.1). Shaving a small section of the ear with a razor blade exposed the marginal ear vein. The shaved area was swabbed with 70% ethanol and antigen was injected with a 1-3 mL syringe fitted with a narrow gauge (26G) needle. As the schedule called for several consecutive injections, the first injection was delivered at the distant end of the ear and progressed towards the base of the ear with each successive injection.

II Subcutaneous injection (SC)

Subcutaneous booster injections were given to maintain the antibody titter. The antigen was injected under the skin in the shoulder area. The syringe used was fitted with a 26 gauge needle.

Table 2.2: Injection and blood collection schedule.

Day	Procedure	Needle used
1	0.5 mL intravenously **(IV)**	26 G
2	1.0 mL **(IV)**	26 G
3	1.5 mL **(IV)**	26 G
4	1.5 mL **(IV)**	26 G
5	2.0 mL **(IV)**	26 G
6	2.0 mL **(IV)**	26 G
16	Test bleed (3 mL) Titer determination	25 G
18	Inject 1 mL **SC** & 1 mL **IV** (booster injection)	26 G
26	Test bleed (3 mL) Titer determination	25 G
28	Inject 1 mL **SC** & 1 mL **IV** (booster injection)	26 G
38	Test bleed (3 mL) Titer determination	25 G
40	Inject 1 mL **SC** & 1 mL **IV** (booster injection)	26 G
48	Test bleed (3 mL) Titer determination	25 G
50	Cardiac bleed (20mL) Inject 20 mL saline intraparetonealy **(IP)** Titer determination	18 G 25 G

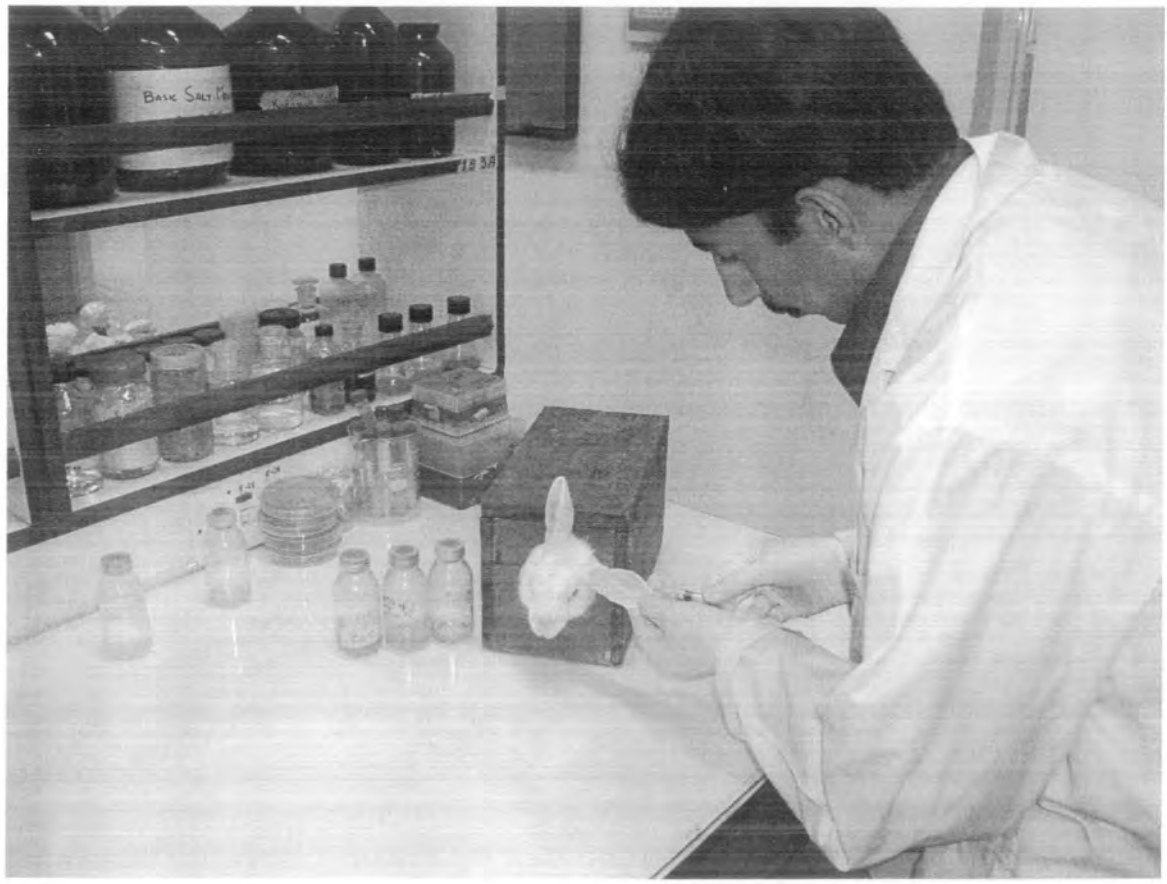

Figure 2.1 Intravenous (IV) injection to a rabbit

2.3.3 Trail Bleeding for Titer Determination

Ten days after the last injection, the rabbits were test bled. For test bleeding, the blood was drawn from the ear. A small area along the marginal vein was shaved and swabbed with 70% alcohol. The blood (3mL) was collected using 3 mL disposable syringe equipped with 25 G needle. The collected blood was transferred to a sterile screw-capped test tube.

2.3.3.1 Collection of Antiserum

The blood, obtained by trail bleeding was allowed to clot at room temperature for about two hours. After the blood had been clotted, the clot was separated from inner walls of the test tube using a wooden applicator stick. It was refrigerated overnight. If the clot was not tight enough after 12 hours at 4° C, refrigeration was continued for another 12 hours or more. After the blood had been clotted and refrigerated, the clear serum was decanted into microcentrifuge tubes minimizing the carry over of red blood cells. These tubes were centrifuged at 5,000 rpm for 15 minutes at 4° C to clear the serum of red blood cells. Clear serum supernatant was transferred to suitable sized vials. Merthiolate was added to each vial as a preservative and vials were adequately labeled. Then these were stored at 4° C.

2.3.4 Agglutination Reactions

The titer of the antiserum may be determined using the microtiter trays, test tubes, and microscope slides methods. The scheme for the antiserum titer determination in wells of microtiter tray is as follows:

2.3.4.1 Preparation of Serial Dilutions of Antiserum

Ten test tubes (15 mL) were arranged in a row on a test tube rack. They were labeled 1 to 10. In tube 1, 4.8 mL of filter-sterilized saline was pipetted. In tubes 2 to 10, 1.0 mL saline was taken. (0.2 mL) of the stock antiserum was accurately added into tube 1. The serum and saline were mixed thoroughly, but gently to avoid frothing using a vortex mixer. Thus tube 1 contained antiserum of a 1/25 dilution. 1.0 mL of diluted serum from tube 1 was transferred to tube 2 and mixed well. The serum dilution in tube 2

was thus 1//25 x 1/2 = 1/50. The dilution was repeated down the series by transferring 1.0 mL of the diluted antiserum successively from the previous tube to the next until tube 10. Tube 10 contained serum dilution of 1/12800. Table 2.3 shows antiserum dilutions and corresponding serum titer.

Table 2.3: Antiserum dilutions and antiserum titer

Tube No.	Antiserum dilution	Antiserum titer
1	1:25	1:50
2	1:50	1:100
3	1:100	1:200
4	1:200	1:400
5	1:400	1:800
6	1:800	1:1600
7	1:1600	1:3200
8	1:3200	1:6400
9	1:6400	1:12800
10	1:12800	1:25600
11	Serum-saline control	-----
12	Serum-antigen control	-----

2.3.4.2 Performing Agglutination Reaction in Microtiter Trays

A microtiter tray having 96 wells was used to perform agglutination reaction. Starting with the highest dilution (tube 10) and accurate micropipette, 100 µL of the diluted antiserum was placed into well A-10 of the plastic agglutination tray. From the antiserum of the next antiserum dilution (Tube 9), 100 µL was placed into well A-9 of the agglutination tray. Same procedure was repeated until all the dilutions of the antiserum have been dispensed into the respective wells of Row-A of the agglutination tray. Then 100 µL of the homogeneous antigen was dispensed into each of the wells from A-1 to A-10, avoiding touching the antiserum in the well or the wall of the well with the tip of the

antigen pipette. In each well, the antigen-antiserum mixture was stirred using a clean glass applicator, avoiding spillage into neighboring wells.

2.3.4.3 Controls

I Serum-saline control

To prepare serum-saline control, 100 µL of serum of 1/25 dilution was placed into well A-11. Then 100 µL of saline was added to it.

II Antigen-saline control

100 µL of saline was placed into a well A-12 and 100 µL of antigen was added to it. This served as the antigen-saline control.

2.3.4.4 Incubation

All the wells were sealed using a cellophane tape. The microtiter tray was incubated in an incubator at 52° C for 4 hours and then transferred to a refrigerator before reading the reactions.

2.3.4.5 Recording the Titer

Serum titer is the reciprocal of the highest serum dilution at which positive agglutination occurs (Somasegaran and Hoben, 1985). After incubation at 52° C for 4 hours and refrigeration for two hours at 4° C, the microtiter trays were ready to be observed. Some times refrigeration was done for 24 hours to get distinct results. Positive agglutination was appeared as granular clumps with clear supernatant. Negative agglutination was indicated by settling of the cells to the bottom of the well and turbid supernatant. The antigen-saline control helped in distinguishing between positive and negative agglutinations. Further confirmation of the positive reaction was made by gently stirring the reactants in the well with a sterile loop. Stirring caused the granular clump to float in clear supernatant.

2.3.4.6 Giving Subcutaneous Booster Injections

In case of low titer in the initial trial bleeding (less than 1:1600), a booster injection of 2.0 ml. antigen was given subcutaneously (SC) immediately after the titer

determination. After ten days, rabbit was again test bled until the desired titer was reached. After that the blood was taken by cardiac puncture.

2.3.5 Collection of Blood by Cardiac Puncture

When the titer was satisfactory, the rabbit was bled from the heart by cardiac puncture on a bleeding rack (Figure 2.2). The rabbit was tied on the bleeding rack. The area above the sternum was shaved using razor blade and swabbed with 70% alcohol. About 20 mL of blood was extracted with a large syringe fitted with 21-gauge needle. The blood was transferred into a sterile, screw-capped falcon tube of 50 mL capacity. After the blood has been clotted and refrigerated, the serum was decanted and centrifuged at 5,000 rpm for 10 minutes at 4° C to clear the serum of red blood cells. The clear supernatant was transferred into other screw caped tube. Antisera from rabbits receiving the same antigen were pooled and stored at 4° C after titer determination.

2.3.6 Maintaining the Level of Immunoglobulins

The level of immunoglobulins in the rabbit was maintained by booster injections 7 days after each bleeding. However in such cases, subcutaneous injections of sterile saline were made to replenish the liquid level in the animal each time after the blood had been taken. The volume of saline injected was equal to the volume of blood taken from the rabbit.

2.4 DETERMINATION OF CROSS REACTIVITY OF DEVELOPED POLYCLONAL ANTIBODIES

In order to check whether or not polyclonal antiserum developed against one strain reacts with antigens of other strains, following three antigen-antibody reactions were used.

1. Agglutination reaction
2. Enzyme linked immunosorbent assay (ELISA)
3. Fluorescent antibody labeling reaction

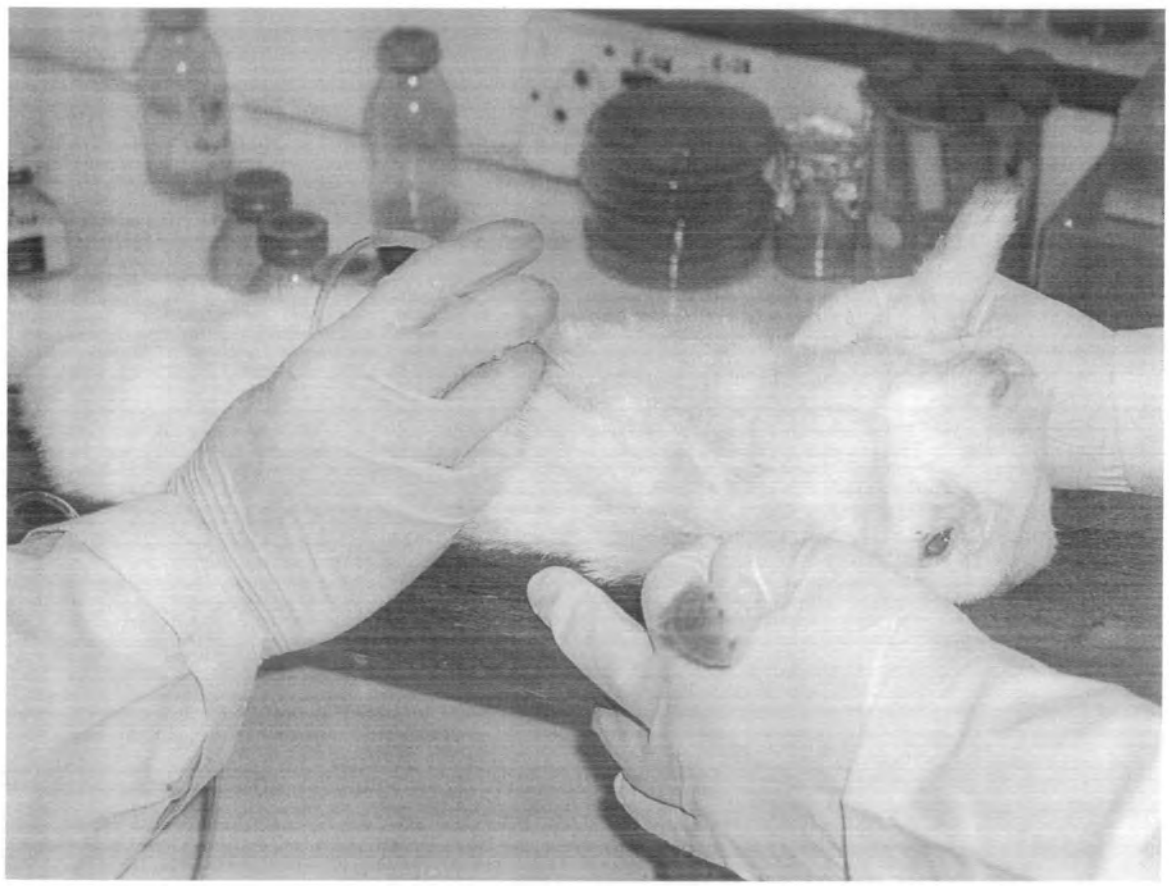

Figure 2.2: Collection of blood from rabbit by cardiac puncture

2.4.1 Agglutination Reaction

The agglutination reactions to check cross reactivity were performed in the same manner as for titer determination discussed earlier.

2.4.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a colorimetric enzyme immunoassays used to detect antigens. Several variations of the assay have been developed. In this case, indirect ELISA was used that does not require the conjugation of primary (rabbit) antibodies with an enzyme. The primary antibodies bound to the antigens in the wells react with alkaline phosphatase conjugated secondary antibodies that bind specifically to the primary antibodies.

For ELISA, microtiter plates having 96 wells were used. Specificity & cross reactivity of each strain specific antisera was determined in separate plates.

The procedure used for the indirect ELISA is as follows:

1) The bacterial antigens (100 µL) were pipetted into each well of polystyrene microtiter plate. Six replicates of each antigen were used. Saline was also pipetted into the six wells as negative control.
2) Added 200 µL of coating buffer (appendix I) in each well. Covered the plate with transparent tape to prevent evaporation.
3) Incubated the plates at 4° C for 12-16 hours for the attachment of antigens to solid phase.
4) Emptied the wells by turning the plate upside down. Filled all the wells with phosphate buffered saline tween20 (PBST), (appendix II) contained in a squeezed bottle. Allowed soaking for three minutes and turned the plate over again. Repeated the washing three times and shake out residual liquid in the wells.
5) To block the nonspecific binding sites, added 200 µL blocking buffer (1% BSA in PBS) to each well and incubated at 37° C in a moist chamber for one hour.
6) Repeated the washing as described in step 4.

7) Added 100 µL of appropriately diluted antisera (1: 1600 in PBS) into each well containing antigens and saline and incubated the plate at 37° C for one hour.
8) Repeated the washing as in step 4.
9) Added 100 µL of diluted (1:4000 in PBS) secondary antibody, goat anti rabbit alkaline phosphatase conjugate (GAR.AP) to each well and incubated at 37° C for three hours.
10) Repeated the washing as described in step 4.
11) Added 100 µL of freshly prepared enzyme substrate solution (appendix III) to each well. Incubated at 37° C in dark for 20-60 minutes or until color was developed in +ve control.
12) Stopped the reaction by adding 50 µL of 3M NaOH/well.
13) The intensity of color was noted by taking absorbance at 405nm in Microplate reader (EL_x 800 UV, Biotech. Instruments. Inc., USA).

2.4.3 Fluorescent Antibodies Preparation

Certain chemical dyes (FITC, lissamine and rhodamine) have the property of fluorescing when excited by near UV light. Polyclonal antibodies developed in rabbits can be conjugated to these fluorescing chemical dyes or fluorochhromes. In the present study, fluorescien isothiocynate (FITC) was used to label the developed antibodies. This dye has an apple-green fluorescence upon irradiation with blue light.

2.4.3.1 Preparation of Globulin Fraction

Serum collected from rabbit contained many kinds of proteins such as albumen, L, B, Y globulin etc. Protein that acts, as antibody is a globulin called immunoglobulin (Ig). IgGs were separated by "salting out" with saturated cold ammonium sulphate (3.9M) solution. Cold 3.9 M ammonium sulfate solution was added drop wise with constant stirring in a 1:1 ratio to the volume of serum placed at crushed ice to precipitate the serum globulins. The mixture was left overnight at 4° C. Globulins were separated by centrifugation at 10,000 x g for 30 minutes. The supernatant was discarded and pellet was dissolved in enough saline to bring the volume back to the original serum volume.

Precipitation and centrifugation procedure was repeated three times, which was enough to render the globulins completely white and free of hemoglobin. The desalting of globulins was done through dialysis against 0.85% saline (pH 8.0) in a cold room with constant stirring. When dialysis had completed, the protein was kept at –20° C.

2.4.3.2 Conjugation of Globulins with Fluorescent Dye

Protein concentration of the dialyzed globulin solution was determined by Bradford method (appendix IV) and was adjusted to 1% (10 mg mL^{-1}) by the addition of 0.85% saline. For conjugation 4.0 mL of 0.15 M sodium phosphate buffer (pH 9.0), (appendix V) was added to 10 ml of 1% globulin solution. Fluorescien isothiocynate (FITC), at the rate of 0.05 mg per mg of protein, was dissolved in 0.1 M sodium phosphate buffer (pH 8.0), (appendix V) separately. FITC solution and globulins solutions were mixed and pH of the solution was adjusted to 9.5 with 0.1 N NaOH and the volume was made up to 20 mL with phosphate buffered saline (PBS). The conjugation was allowed to proceed for eight hours at room temperature under constant stirring.

2.4.3.3 Purification of Conjugated Antibodies

Conjugated FAs were separated from unreacted FITC by column chromatography or dialysis. In chromatographic method, the glass column was washed with distilled water and clamped in a stand to keep the column straight. Sephadex G25 (15 g) was soaked in 500 mL suction flask for 30 to 60 minutes. Then sephadex was poured gently into the column and the flow rate of PBS was adjusted at 40 drops/minutes by moving the lower valve of column. The flow of PBS through the sephadex was allowed until the level of PBS reached 1cm high from the sephadex surface. The mixture of FITC and FAs was added into the column. The first dark yellow fraction (FAs) was collected in three parts (initial portion, middle portion and last portion) in three tubes separately.

2.4.3.4 Staining of Cultures with Fluorescent Antibodies (FAs)

A smear of cell suspension was made on a glass slide. Smear was air-dried, heat fixed, covered with gelatin rhodamine conjugate and dried for one hour in an incubator at

60^0 C. A drop of homologous FAs was placed on the slide and incubated the slide for 20 minutes in moist chamber. Extra FAs were drained and slide was washed by placing in PBS for 10 minutes. Then air-dried and mounted with mounting solution (1:9 PBS – glycerol). Cover the plate with coverslip and observe it under UV microscope (ZIESS, West Germany).

2.5 ANALYSIS OF SPECIFIC BACTERIAL SPECIES IN HEAP

This analysis was carried out to determine different bacterial species in the liquid as well as solid samples taken from heap.

2.5.1 Bacterial Analysis in Leach Liquor

Liquid samples were taken regularly from the effluent coming out of the heap and the population of specific bacterial species was monitored by ELISA. For this purpose, exactly 25 mL of each sample taken from heap was centrifuged to get cell pellet. The pellet was washed, first with autoclaved water, and then with 0.85% saline. Finally, the cell pellet was suspended in 1.5 mL saline and the absorbance was taken at 600 nm. The cells were heat treated at 100^0 C for 20 minutes. After cooling, merthiolate was added as preservative. Then these samples were subjected to ELISA. The procedure used for ELISA was the same as discussed earlier in section 2.4.2.

2.5.2 Analysis in Solid Coal of Heap

For quantitative analysis of specific bacteria in heap, solid coal samples were taken from five different places of heap i.e top side (TS), mid side (MS), mid centre (MC), bottom centre (BC) and bottom edge (BE).

A known amount (500 g) of each coal sample was taken in a separate 2 L flasks and 300 mL of 1% Tween20 solution was added to each flask. The flasks were agitated and allowed the coal to settle down for an hour. Then separated the supernatant in another flask and added 200 mL of 1% Tween20 in the coal. After agitation, again allowed the solid particles to settle down for an hour. Then the supernatant was collected in the same flask in which earlier one was collected. Filtered the supernatant through whattman filter paper to remove residual solid particles. Centrifuged it at 10,000 rpm for ten minutes.

Discarded the supernatant and washed the pellet with autoclaved distilled water having pH 2. Again filtered the suspension to remove remaining pyrite particles and centrifuged to get pellet. Finally the cell pellet was suspended in 5 mL of 0.85% saline solution. Then the absorbance of each sample was taken at 600 nm. Finally these samples were heat treated at 100° C for 20 minutes. After cooling, merthiolate was added to each sample as preservative. Then samples were subjected to ELISA.

2.6 MOLECULAR STUDIES

Molecular studies involved two parts.

1. PCR amplification of 16S rDNA gene of HC-AF2 and KC-AT2 for phylogenetic analysis.
2. Integron profile study of heap inoculum by using integron specific primers.

2.6.1 Total Genomic DNA Extraction

For molecular studies total genomic DNA was extracted from the bacterial strains as specified below.

1. From pure cultures of HC-AF2, KC-AT2 and MT13 grown in shake flasks under sterile conditions.
2. From cultures of HC-AF2, KC-AT2 and moderate thermophiles grown separately, in fermentors for heap inoculum under non-sterilized conditions.

Methodology:

1. Bacterial cultures were centrifuged for 10 minutes at 10,000 rpm to get cell pellet. 10-20 mg cell pellet/tube was taken in several 1.5 mL eppendorf tubes. 500 µL TE buffer and 200 µL of lysozyme (15 mg/mL of TE buffer) were added in each tube. Then these were incubated at 37° C for 24 hours.
2. 50 µL RNase (10 mg/mL) was added in each eppendorf tube and mixed gently by inverting the tube for several times. After mixing, tubes were incubated again at 37° C for 24 hours.

3. 30 µL SDS (10%) was added in each tube and incubated them at 70° C for 30 minutes. Then 10 µL Proteinase K (20 mg/mL) was added in each tube and incubated again at 30° C for 24 hours.
4. Tubes were centrifuged at 10,000 rpm for 10 minutes to remove cell debris and the supernatant was collected in fresh tubes.
5. 690 µL chloroform: isoamyl alcohol (24:1) was added in supernatant and mixed gently by inverting the tubes to form an emulsion.
6. The tubes were centrifuged at 13,000 rpm for 15 minutes.
7. Aqueous layer was taken in a new tube and discarded the remaining chloroform phase.
8. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added in each tube and centrifuged for 15 minutes. Aqueous layer was taken in fresh tube and 70 µL 3M Na-acetate and 400 µL of isopropanol was added in it.
9. Each tube allowed to stay at –20° C for 1 hour.
10. Centrifuged at 14,000 rpm for 10 minutes and discarded the supernatant. Washed the pellet with 70% ethanol to remove the bases.
11. Suspended the pellet in appropriate volume of deionized H_2O.
12. Quality of DNA was checked by running on 1% agarose gel.

2.6.2 Agarose Gel (1%) for DNA Electrophoresis

1. Adequate volume of TAE buffer (1.0X) was prepared to fill the electrophoresis tank and to prepare the agarose gel.
2. For 1% agarose gel, desired amount of agarose was taken in a flask containing TAE buffer (1.0X) and was melted in microwave oven.
3. Melted agarose was cooled to 45-55° C and poured onto the gel-casting tray. A comb was inserted into it.
4. Air bubbles were removed, if any, underneath the comb or on the surface of gel and the gel was allowed to solidify at room temperature.
5. After solidification of gel, the comb was removed carefully to avoid tearing of wells.

6. The gel casting tray, containing gel, was placed in electrophoretic tank, having 1.0X TAE buffer in it. Ethidium bromide (2.0 µL) was added to the tank.
7. DNA samples were mixed with appropriate volume of loading dye and loaded into the wells with a micropipette.
8. Voltage was set at 70 V. Movement of dye indicated the migration of DNA from anode to cathode through gel.
9. When dye covered the distance sufficient for separation of DNA fragments, the power supply was turned off.
10. DNA fragments were visualized under UV light and photographed.

2.6.3 Protocol for Amplification of 16S rDNA

Polymerase chain reaction (PCR) amplification of 16S ribosomal DNA of HC-AF2 and KC-AT2 was carried out using the following primers:

Forward primer (FD1) AGAGTTTGATCCTGGCTCAG

Reverse primer (rP1) ACGG(ACT)TACCTTGTTACGACTT

Reaction mixture (50 µL) contained:

1.	Nanopure water	34.75 µL
2.	1X PCR buffer	5.0 µL
3.	$MgCl_2$	6.0 µL
4.	dNTPs	1.0 µL
5.	FD1	1.0 µL
6.	rP1	1.0 µL
7.	Taq DNA polymerase,	0.25-1 µL
8.	Template	1.0 µL

PCR profile

Amplification was carried out in thirty cycles. Each cycle was comprised of 30 seconds at 95° C, 40 seconds at 55° C and 2 minutes at 72° C. The final extension was for 10 minutes at 72° C. The amplified PCR product was run on 1.5% agarose gel.

2.6.3.1 Phylogenetic Analysis of HC-AF2 and KC-AT2

Amplified 16S rDNA product was got sequenced commercially (Genelink™, Hawthorne, New York). The gene sequences were compared with others in the GenBank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov). Gene sequences of 16S rDNA of selected organisms were obtained from GenBank and aligned with gene sequence of our isolates using CLUSTALX. The aligned sequences were used to construct a distance matrix (Jukes and Cantor, 1969), after the generation of 100 bootstrap sets that was subsequently used to construct a phylogenetic tree by neighbor-joining method (Saitou and Nei, 1987), using Treecon for windows (Van de Peer and De Wachter, 1993).

2.6.4 Recovery of Different Genes Using Integron Specific Primers

Integron linked genes were recovered by PCR amplification using integron specific primers.

The primers to conserved sequences were HS286 (5′ GGGATCCTC(GC) GCT(GT)GA(GA)CGA(AC)TTGTTAG(GCA)C 3′) and HS287 (5′ GGGATCCGC (GC)GCT(GT)A(AGCT)CTC(GCA)(GA)(GA)CGTTAG(GC)C 3′). These primers target the flanking regions of 59-base element sites (stokes *et al.*, 2001). The underlined sequence is a *BamH1* linker that is not complementary to 59-base element sequences. Reaction mixtures consisted approximately of 5ng of template DNA, 100pmol of each of the primer, 200nM deoxynucleoside triphosphate (dNTP) mix, 2mM $MgCl_2$ and 1U of Taq DNA polymerase.

Reaction mixture (50µL) comprised of:

1. Nanopure water 36.0 µL
2. 1X PCR buffer 5.0 µL
3. $MgCl_2$ 4.0 µL
4. dNTPs 1.0 µL
5. HS286 1.0 µL
6. HS287 1.0 µL
7. Taq DNA polymerase 1.0 µL
8. Template 1.0 µL

PCR profile

Amplification was carried out with the following cycling program: 3 minutes at 94° C for 1 cycle, 30 seconds at 94° C, 30 seconds at 55° C, 2 minutes and 30 seconds at 72° C for 35 cycles, and 5 minutes at 72° C for 1 cycle.

RESULTS

CHAPTER 3

RESULTS

The results obtained during the present study are presented below.

3.1 GROWTH OF MICROORGANISMS

As described in section 2.1, the local bacterial isolates from NIBGE culture collection were subjected to serological and molecular analysis for present study. These local bacterial isolates involved HC-AF2, TF9, TF10 (strains of *Acidithiobacillus ferrooxidans*), KC-AT2 (a strain of *Acidithiobacillus thiooxidans*), MT9, MT10, MT13, MT16, MT17 (strains of *Sulfobacillus thermosulfidooxidans*) and TH1 (reference strain of *S. thermosulfidooxidans*). Both solid and liquid media were employed for the growth of microorganisms.

3.1.1 Growth of *S. thermosulfidooxidans*

Both solid and liquid media were employed for the growth of *S. thermosulfidooxidans*.

3.1.1.1 Growth of *S. thermosulfidooxidans* on Solid Medium

FeTSB medium developed by Johnson *et al.*, (1987) was employed as a solid medium for the growth of *S. thermosulfidooxidans*. After spreading, the petri plates were incubated at 45° C. After three days of incubation dark brown colonies were appeared on solid medium. A single colony was picked, using a platinum loop, and inoculated in liquid medium for *S. thermosulfidooxidans* (iron medium) for inoculum preparation to conduct serological and molecular studies.

3.1.1.2 Growth of *S. thermosulfidooxidans* in Liquid Medium

Iron medium (Leathen *et al.*, 1956; Postgate, 1966) was employed as a liquid medium for the growth of *S. thermosulfidooxidans*. A single colony grown on solid medium was inoculated and the flasks were incubated at 45° C on an orbital shaker.

Observing turbidity in flasks and typical reddish-brown coloration indicated growth of bacteria. For further confirmation of growth, aliquots from flasks were titrated against 1.0 mM $KMnO_4$ solution, which indicated the amount of ferrous iron oxidized to ferric iron by *S. thermosulfidooxidans*. Growth was obtained after three days of incubation.

After growth on iron medium, fresh bacterial culture was inoculated (10%) in basal salt solution containing finely ground pyrite (0.35%). The flasks were incubated again at 45° C. Bacterial growth was indicated by observing turbidity in medium and by the appearance of yellowish green coloration due to the formation of Fe^{+2} and Fe^{+3} by the oxidation of pyrite. Confirmation of growth of *S. thermosulfidooxidans* was done by titrating aliquots from flasks against 1.0 mM $KMnO_4$ solution. Rich growth was obtained after seven days of incubation. After rich growth the flasks were removed from shaker and stored at 4° C for further studies.

3.1.2 Growth of *At. ferrooxidans*

3.1.2.1 Growth of *At. ferrooxidans* HC-AF2 on Solid Medium

FeTSB solid medium developed by Johnson *et al.*, (1987) was employed as a solid medium for the growth of *At. ferrooxidans* HC-AF2. After spreading, the petri plates were incubated at 30° C. After three days of incubation, dark brown colonies appeared on solid medium. A single colony was picked, and inoculated in liquid medium for *At. ferrooxidans* (iron medium) for inoculum preparation.

3.1.2.2 Growth of *At. ferrooxidans* HC-AF2 in Liquid Medium

Iron medium (Leathen *et al.*, 1956; Postgate, 1966) was employed as a liquid medium for the growth of *At. ferrooxidans*. A single colony grown on solid medium was inoculated and the flasks were incubated at 30° C on an orbital shaker.

Growth of bacteria was indicated by observing turbidity in flasks and typical reddish-brown coloration. For the confirmation of growth, aliquots from flasks were titrated against 1.0 mM $KMnO_4$ solution, which indicated the amount of ferrous iron oxidized to ferric iron by *At. ferrooxidans*. Growth was obtained after two days of incubation.

After growth on iron medium, fresh bacterial culture was inoculated (10%) in basal salt solution containing finely ground pyrite (0.35%). The flasks were incubated at 30° C on an orbital shaker. Bacterial growth was indicated by observing turbidity in medium and by the appearance of yellowish green coloration. Confirmation of growth of *At. ferrooxidans* HC-AF2 was done by titrating aliquots from flasks against 1.0 mM $KMnO_4$ solution. Rich growth was obtained after seven days of incubation. When rich growth was obtained the flasks were removed from shaker and stored at 4° C for further studies.

3.1.3 Growth of *At. thiooxidans*

3.1.3.1 Growth of *At. thiooxidans* KC-AT2 on Solid Medium

Sodium thiosulfate was used as energy source for *At. thiooxidans* growth on solid media. After spreading, the petri plates were incubated at 30° C for three days. After three days of incubation, off white colonies appeared on solid medium. A single colony of *At. thiooxidans* KC-AT2 was picked with a platinum loop and inoculated in liquid medium for inoculum preparation.

3.1.3.2 Growth of *At. thiooxidans* KC-AT2 in Liquid Medium

For the growth of *At. thiooxidans* KC-AT2 in liquid medium, elemental sulfur medium was used whose composition was same as that of iron medium but instead of ferrous sulfate, finely ground elemental sulfur (1%) was added as energy source and pH was adjusted to 2.8-3.0. Flasks were incubated at 30° C on orbital shaker. Growth of *At. thiooxidans* KC-AT2 was indicated by observing turbidity in liquid medium and reduction of pH against control. Rich growth was obtained after five days.

SEROLOGICAL STUDIES

3.2 AGGLUTINATION REACTIONS

In the present study, the somatic agglutination reactions were carried out to determine the antibody titer in the rabbit antisera against each bacterial strain. Agglutination reactions were performed in microtiter trays as discussed in section 2.3.4. The antiserum titer against *S. thermosulfidooxidans* MT13 strain reached to a value of 1:3200 after two booster injections. This was indicated by visible agglutination (granular clump) in wells of microtiter plate having antisera dilution upto 3200. The controls did not show any agglutination as in well having antisera and saline the solution was transparent while the well containing antigens and saline showed the settling of antigens at bottom and turbid supernatant.

The rise in antisera titer against *S. thermosulfidooxidans* TH1 strain was slow and required four booster injections for reaching upto 1:3200.

In case of *At. thiooxidans* KC-AT2, the antibody titer value easily reach up to 1:3200 after early six doses, which indicates that response of rabbits against this strain is very high.

However in case of *At. ferrooxidans* HC-AF2, the response of rabbits was again slow and booster injections were needed to raise the antisera upto 1:3200.The two rabbits used for each strain showed almost same level of antibodies in serum.

3.3 CROSS REACTIVITY

Cross reactivity refers to the ability of an individual antibody combining site to react with more than one antigenic determinant or the ability of a population of antibody molecules to react with more than one antigens. In order to check whether or not antiserum developed against one strain reacts with antigen of other strains of same species or different species, three types of serological tests were performed i.e agglutination reaction, enzyme linked immunosorbent assay (ELISA) and fluorescent antibody labeling test.

3.3.1 Cross Reactivity of Polyclonal Antisera Developed Against Antigens from *At. ferrooxidans* Strain HC-AF2

The results of antigen-antibody reactions to check cross reactivity of antiserum developed against antigens from *At. ferrooxidans* HC-AF2 are shown in table 3.1. It is evident from the results mentioned in table 3.1 that antibodies developed against *At. ferrooxidans* HC-AF2 were very specific and did not cross react with antigens of other bacterial strains except little cross reactivity with species of *Acidithiobacillus*. From the ELISA results, it was noted that *At. ferrooxidans* HC-AF2 strain has almost 20% cell surface antigens common with *At. thiooxidans* KC-AT2 isolated from Khushab coal mines and 10% cell surface antigens common with *At. ferrooxidans* TF9 and *At. ferrooxidans* TF10 isolated from uranium mines DG khan. The agglutination test showed that this strain has no cross reactivity with all other strains. In case of fluorescent antibody labeling test, dull green color was observed with KC-AT2 antigens which was rated as (1+) while with +ve control (HC-AF2) the color was brilliant yellow green (4+).

Table 3.1: Reactivity of antisera developed against *At. ferrooxidans* HC-AF2

Bacterial strains	Agglutination reaction	Fluorescent antibody labeling	ELISA
At. ferrooxidans HC-AF2	+ve	4+	100%
At. ferrooxidans TF9	-ve	ND	10%
At. ferrooxidans TF10	-ve	ND	10%
At. thiooxidans KC-AT2	-ve	1+	20%
S. thermosulfidooxidans TH1	-ve	0	0
S. thermosulfidooxidans MT13	-ve	0	0
S. thermosulfidooxidans MT9	-ve	ND	4%
S. thermosulfidooxidans MT10	-ve	ND	0
S. thermosulfidooxidans MT16	-ve	ND	0
S. thermosulfidooxidans MT17	-ve	ND	5%

(ND=Not determined)

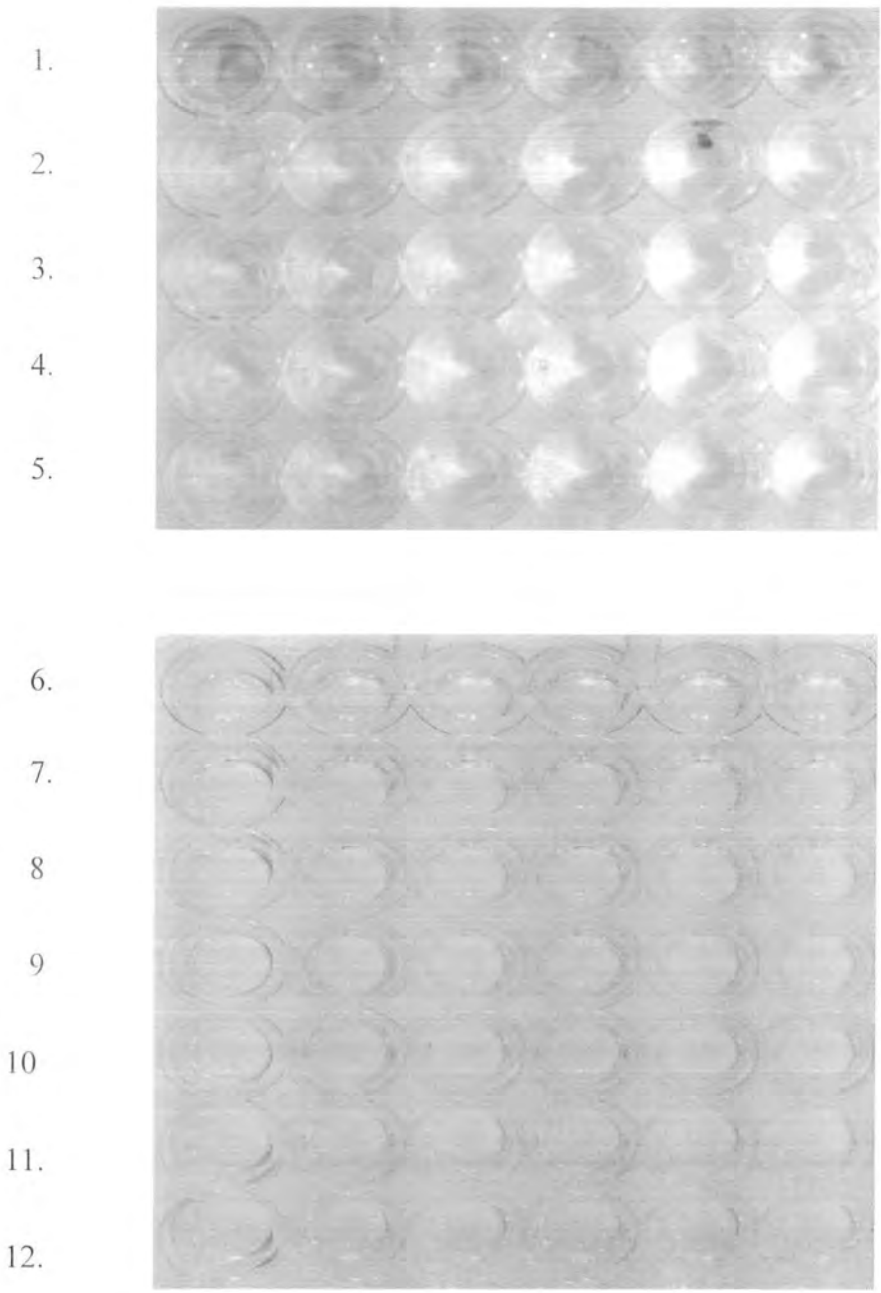

Figure 3.1: Enzyme-linked immunosorbent reaction of polyclonal antibodies developed for *At. ferrooxidans* HC-AF2 with antigens from different bacterial isolates. Rows: (1) +ve control, (HC-AF2 antigens), (2) –ve control (without antigens) (3) TH1, (4) MT13, (5) KC-AT2, (6) +ve control, (7) TF9, (8) TF10, (9) MT9, (10) MT10, (11) MT16, (12) MT17

3.3.2 Cross Reactivity of Polyclonal Antisera Developed Against Antigens from *At. thiooxidans* Strain KC-AT2

The table 3.2 is showing the results of cross reactivity of antiserum developed against antigens from *At. thiooxidans* KC-AT2. ELISA results indicated that *At. thiooxidans* KC-AT2 strain has almost 28% cell surface antigens common with *At. ferrooxidans* HC-AF2 isolated from Hernai coal mines. 7% cell surface antigens similarity of KC-AT2 was also observed with *At. ferrooxidans* TF10 isolated from uranium mines DG khan. Antisera against *At. thiooxidans* KC-AT2 also showed a some reaction with antigens of moderate thermophilic strains MT9 and MT10. The agglutination test indicated that this strain has cross reactivity with HC-AF2 strain of *At. ferrooxidans*. In case of fluorescent antibody labeling test, dull green color was observed with antigens from HC-AF2, which was rated as +1 while with +ve control (KC-AT2) the color was brilliant yellow green.

Table 3.2: Reactivity of antisera developed against *At. thiooxidans* KC-AT2

Bacterial strains	Agglutination reaction	Fluorescent antibody labeling	ELISA
At. thiooxidans KC-AT2	+ve	4+	100%
At. ferrooxidans HC-AF2	+ve	1+	28%
At. ferrooxidans TF9	-ve	ND	0
At. ferrooxidans TF10	-ve	ND	7%
S. thermosulfidooxidans TH1	-ve	0	0
S. thermosulfidooxidans MT13	-ve	0	0
S. thermosulfidooxidans MT9	-ve	ND	10%
S. thermosulfidooxidans MT10	-ve	ND	16%
S. thermosulfidooxidans MT16	-ve	ND	0
S. thermosulfidooxidans MT17	-ve	ND	0

(ND=Not determined)

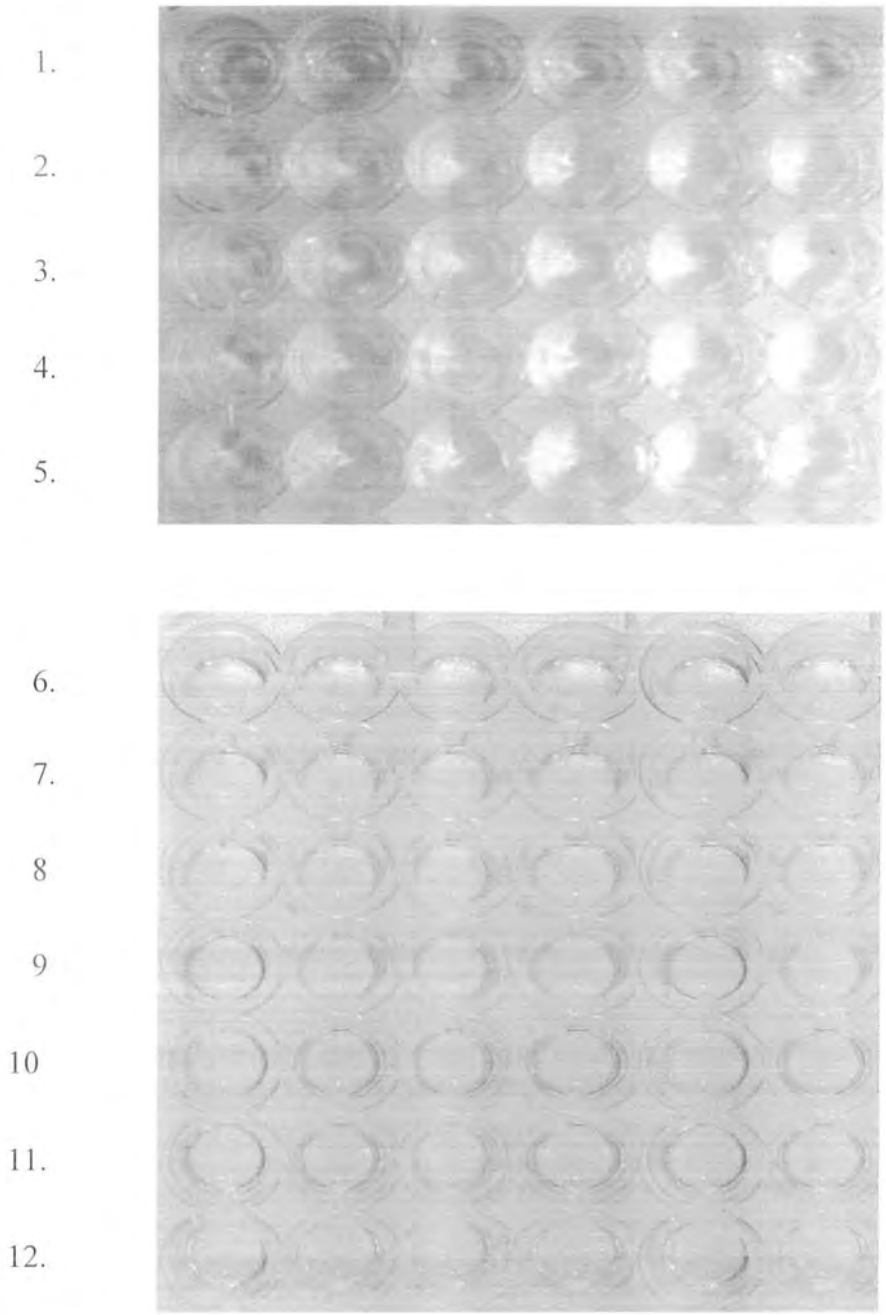

Figure 3.2: Enzyme-linked immunosorbent reaction of polyclonal antibodies developed for *At.thiooxidans* KC-AT2 with antigens from different bacterial isolates. Rows: (1) +ve control (KC-AT2 antigens), (2) –ve control (without antigens) (3) HC-AF2, (4) TH1, (5) MT13, (6) +ve control, (7) TF9, (8) TF10, (9) MT9, (10) MT10, (11) MT16, (12) MT17

3.3.3 Cross Reactivity of Polyclonal Antisera Developed Against Antigens from *Sulfobacillus thermosulfidooxidans* Strain TH1

The results of antigen-antibody reactions to check cross reactivity of antiserum developed against antigens from *S. thermosulfidooxidans* strain TH1 are shown in table 3.3. Strong reaction was observed among the antisera developed against *Sulfobacillus thermosulfidooxidans* and locally isolated moderate thermophilic strains MT13 and MT10. The other moderate thermophilic strains MT9, MT16 and MT17 were also found to have more than 50% cell surface antigens common with *S. thermosulfidooxidans*. However, it was found that *S. thermosulfidooxidans* has no antigenic similarity with mesophilic strains *At. ferrooxidans* HC-AF2, *At. thiooxidans* KC-AT2, *At. ferrooxidans* TF9 and *At. ferrooxidans* TF10. The agglutination test also showed that this strain has cross reactivity with MT9, MT10, MT13, and MT16. However, no agglutination was observed with antigens from MT17 strain. In case of fluorescent antibody labeling test, yellow green color was observed with antigens from MT13 and also from +ve control (TH1), which was rated as 2+.

Table 3.3: Reactivity of antisera developed against *S. thermosulfidooxidans* TH1

Bacterial strains	Agglutination reaction	Fluorescent antibody labeling	ELISA
S. thermosulfidooxidans TH1	+ve	2+	100%
At. ferrooxidans HC-AT2	-ve	0	0
At. ferrooxidans TF9	-ve	ND	0
At. ferrooxidans TF10	-ve	ND	0
At. thiooxidans KC-AT2	-ve	0	0
S. thermosulfidooxidans MT13	+ve	2+	90%
S. thermosulfidooxidans MT9	+ve	ND	65%
S. thermosulfidooxidans MT10	+ve	ND	95%
S. thermosulfidooxidans MT16	+ve	ND	56%
S. thermosulfidooxidans MT17	-ve	ND	50%

(ND=Not determined)

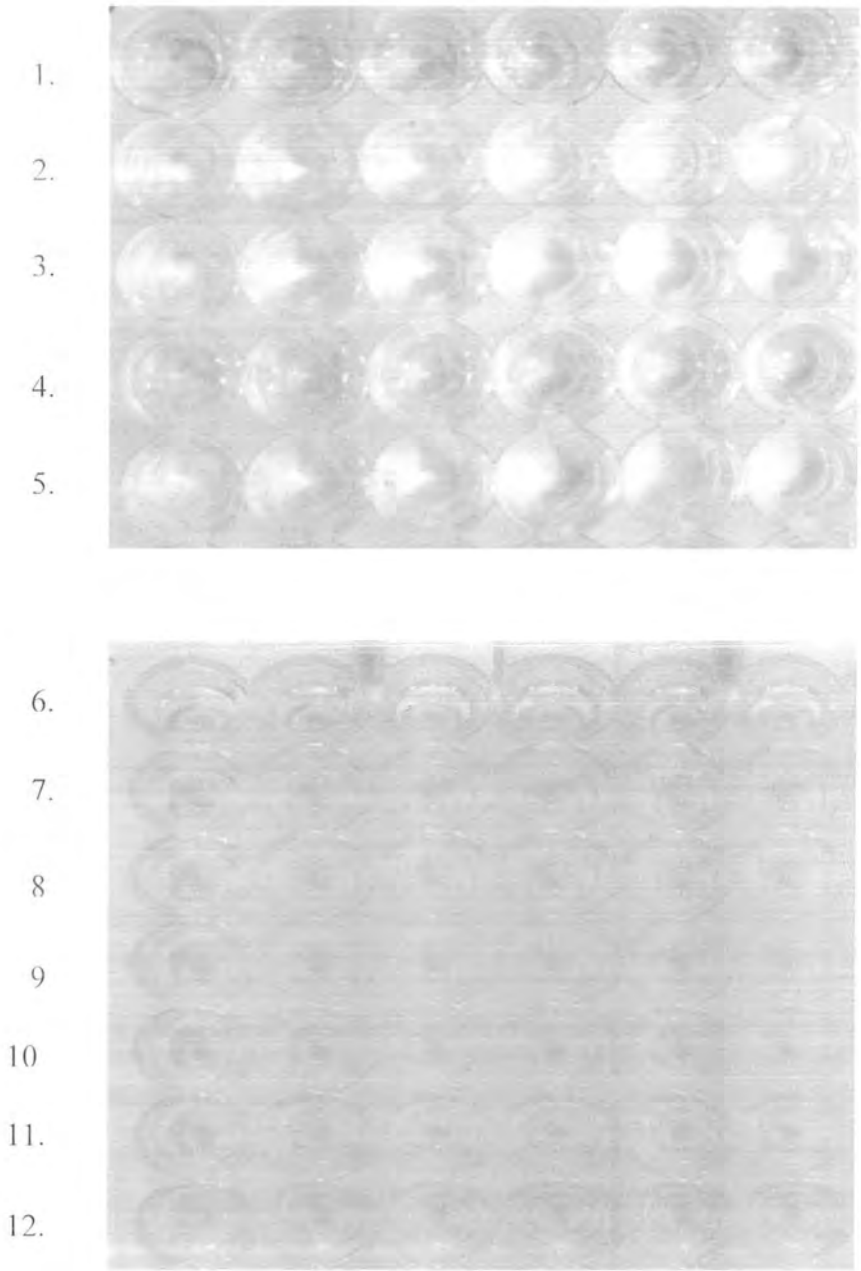

Figure 3.3: Enzyme-linked immunosorbent reaction of polyclonal antibodies developed for *S. thermosulfidooxidans* TH1 with antigens from different bacterial isolates. Rows: (1) +ve control (TH1 antigens), (2) –ve control (without antigens) (3) HC-AF2, (4) MT13, (5) KC-AT2, (6) +ve control, (7) TF9, (8) TF10, (9) MT9, (10) MT10, (11) MT16, (12) MT17

3.3.4 Cross Reactivity of Polyclonal Antisera Developed Against Antigens from *Sulfobacillus Thermosulfidooxidans* Strain MT13

The table 3.4 is showing the results of cross reactivity of antiserum developed against antigens from *S. thermosulfidooxidans* strain MT13. *S. thermosulfidooxidans* MT13 strain gave strong positive reaction with other moderate thermophilic strains MT9, MT10, MT16, MT17 and with standard strain TH1. This strain was also found to have no antigenic similarity with mesophilic strains *At. ferrooxidans* HC-AF2, *At. thiooxidans* KC-AT2, *At. ferrooxidans* TF9 and *At. ferrooxidans* TF10. The agglutination test also showed that this strain has cross reactivity with TH1, MT9, MT10 and MT17 strains. However, no agglutination was seen with antigens from MT16 strain. In case of fluorescent antibody labeling test, yellow green color was observed with antigens from TH1and also from +ve control (MT13) that was rated as 2+.

Table 3.4: Reactivity of antisera developed against *S. thermosulfidooxidans* MT13

Bacterial strains	Agglutination reaction	Fluorescent antibody labeling	ELISA
S. thermosulfidooxidans MT13	+ve	2+	100%
At. ferrooxidans HC-AT2	-ve	0	0
At. ferrooxidans TF9	-ve	ND	0
At. ferrooxidans TF10	-ve	ND	0
At. thiooxidans KC-AT2	-ve	0	0
S. thermosulfidooxidans TH1	+ve	3+	93%
S. thermosulfidooxidans MT9	+ve	ND	70%
S. thermosulfidooxidans MT10	+ve	ND	95%
S. thermosulfidooxidans MT16	-ve	ND	50%
S. thermosulfidooxidans MT17	+ve	ND	50%

(ND=Not determined)

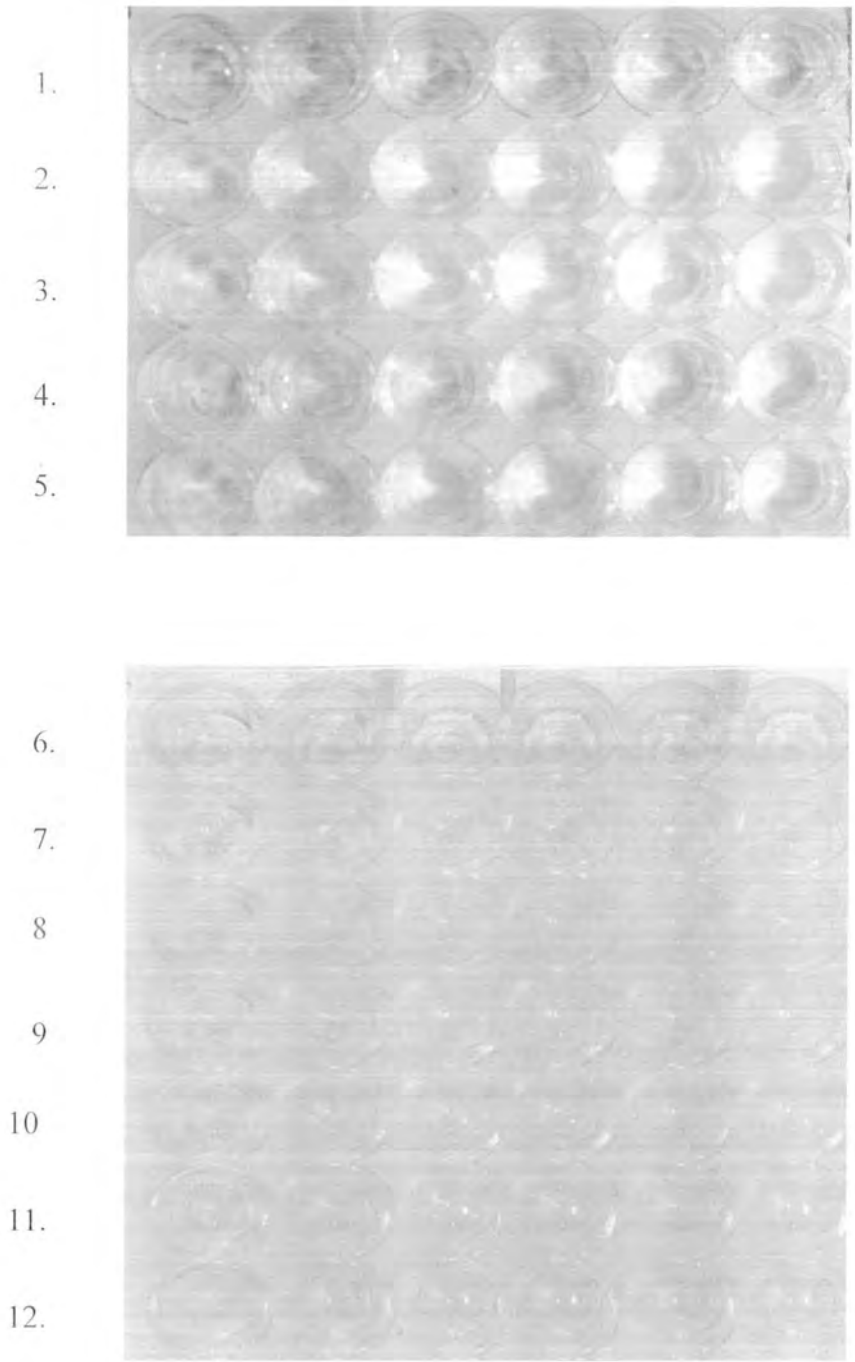

Figure 3.4: Enzyme-linked immunosorbent reaction of polyclonal antibodies developed for *S. thermosulfidooxidans* MT13 with antigens from different bacterial isolates. Rows: (1) +ve control (MT13 antigens), (2) –ve control (without antigens) (3) HC-AF2, (4) TH1, (5) KC-AT2, (6) +ve control, (7) TF9, (8) TF10, (9) MT9, (10) MT10, (11) MT16, (12) MT17

3.4 Quantitative Analysis of Specific Bacterial Species in Leach Liquor

Quantitative ELISA results indicated that moderate thermophiles dominated in heap leach liquor, though *Acidithiobacillus ferrooxidans* were also found in leach liquor coming from the heap. However, no *Acidithiobacillus thiooxidans* were found in leach liquor (Table 3.5).

Table 3.5: Bacterial quantification in leach liquor

Sample #	**Days**	**Moderate thermophiles cells/ml**	***At.ferrooxidans* HC-AF2 cells/ml**	***At. thiooxidans* KC-AT2 cells/ml**
Initial inoculum	0	5×10^3	2.4×10^4	2.4×10^4
1	7	1.16×10^6	ND	ND
2	14	9.2×10^6	6×10^4	ND
3	21	2.4×10^6	ND	ND
4	30	2.4×10^6	5.4×10^4	ND
5	37	1.32×10^6	5.9×10^4	ND
6	45	4.28×10^6	ND	ND
7	54	8.68×10^6	ND	ND
8	67	8.48×10^6	ND	ND
9	77	5.6×10^6	ND	ND
10	85	1.44×10^7	ND	ND
11	93	5.12×10^6	ND	ND

3.5 Quantitative Analysis of Specific Bacterial Species in Heap

The figure 3.5 is showing the population of specific bacterial species at five different locations of heap and the figure 3.6 is showing the locations from where the samples were taken.

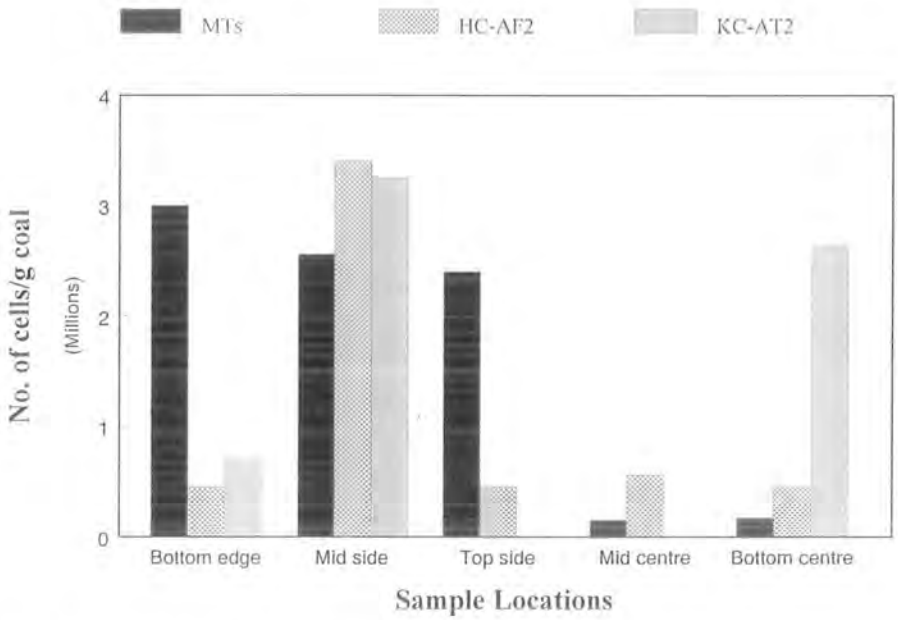

Figure 3.5: Bacterial quantification in heap

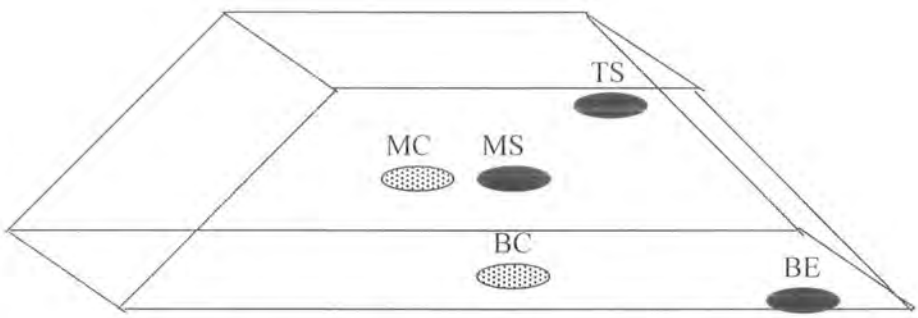

Figure 3.6: Location of sampling sites in 20-ton coal heap set for coal biodesulfurization (sites on the periphery, sites inside the heap)

3.6 MOLECULAR STUDIES

3.6.1 DNA Isolation

Total genomic DNA was isolated from pure cultures of *At. ferrooxidans* HC-AF2, *At. thiooxidans* KC-AT2 and *S. thermosulfidooxidans* MT13 and also from the cultures of the same bacteria grown in fermentors under non-sterilized conditions. The DNA bands obtained on agarose gel are shown in figure 3.7. The sizes of the total genomic DNA of all strains were observed to be approximately of equal size.

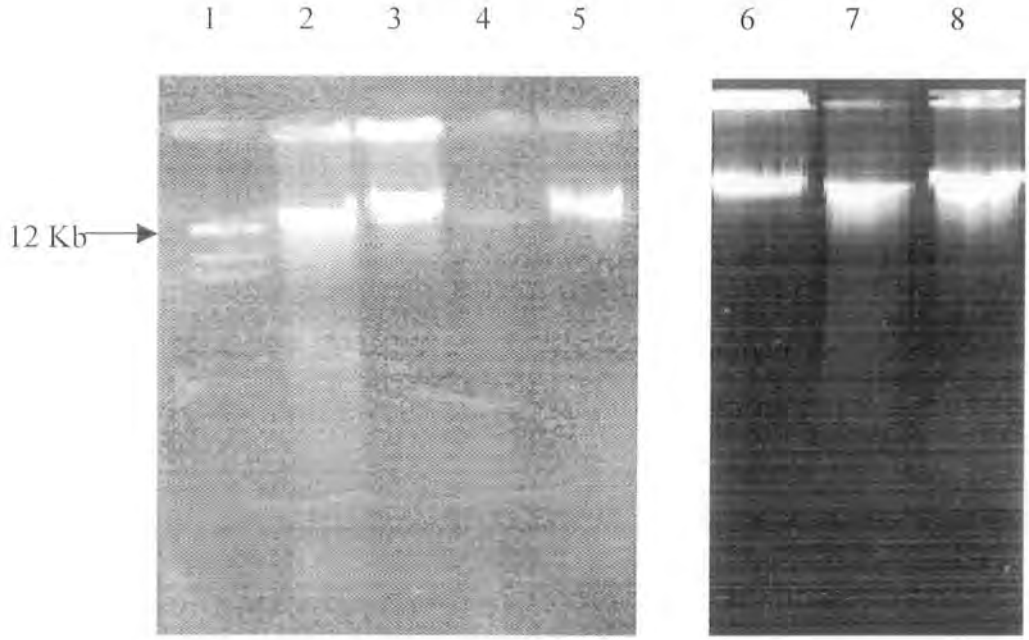

Figure 3.7: Genomic DNA bands on agarose gel: (1) 1 Kb ladder, (2) Mix culture of HC-AF2, KC-AT2 & moderate thermophiles, (3) HC-AF2 grown in fermentor, (4) KC-AT2 grown in fermentor (5) moderate thermophiles grown in fermentor, (6) HC-AF2 pure culture, (7) KC-AT2 pure culture (8) *S. thermosulfidooxidans* MT13

3.6.2 PCR Amplification of the 16S rDNA and Sequencing

DNA samples from HC-AF2 and KC-AT2 were amplified using FD1 and rp1 primers and PCR product of approximately 1600 bp was obtained (Fig 3.8). Purified PCR product was got sequenced and the sequences obtained were very fine and only few Ns were present in some sequences (section 3.8). Partial sequence of the 16S rDNA showed

that HC- AF2 isolate has homology with *At. ferrooxidans* species and KC-AT2 has homology with *At. thiooxidans*.

Figure 3.8: PCR amplification for 16S rDNA (1) 1 Kb ladder, (2) HC-AF2, (3) KC-AT2

3.6.4 Partial 16S rDNA Sequences (forward) of HC-AF2 and KC-AT2

Following are the sequences of *At. ferrooxidans* HC-AF2 & *At. thiooxidans* KC-AT2 obtained by using FD1 primer.

***At. ferrooxidans* HC-AF2**

5'.....TAGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGGTAACAGG
TCTTCGGATGCTGACGAGTGGCGGACGGGTGAGTAATGCGTAGGAATCTGTC
TTTTAGTGGGGGGACAACCCAGGGAAACTTGGGCTAATACCGCATGAGCCCT
GAGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCTAAGAAGAAGGAGCCTA
CGTCCGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCGGTA
GCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATTTTTCGCAATGGGGGCAACCCTGACG
AAGCAATGCCGCGTGGATGAAGAAGGCCTTCGGGTTGTAAAAGTCCTTTCGT
GGAGGACGAAAAGGTGGGTTCTAATACAATCTGCTATTGACGTGAATCCAAG
AAGAAACACCGGTTACTCCGTGCCAGCAGCCGCGGTAATACGGGGGGTGCA
AGCGTTAATTC GGAAT... ... .3'

***At. thiooxidans* KC-AT2**

5'.....TTAGACGCTGGCGGCATGCCTAACACATGCAAGTCGAACCGAGGCAGN
CCTTCGGATGCTGACGAGTGGCGGNCGGGTGAGTAATGCGTAGGAATCTGCC
TTTGAGTGGGGGACAACCCAGGGAAACTTGGGCTAATACCGCATAAGCCCTG
AGGGGGAAAGCGGGGGATCTTCGGACCTGGCGCTGGAAGAGGAGCCTACGT
CTGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACNATCGGTAGCT
GGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACTGGCCCAGACTCC
TACGGTTAGGCACGCAGTGGGGAATTTTTCGCAATGGGGGCAACCCTGACGA
AGCAATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTAAAAGTCTTTCGTGG
AGGACCAAAAGGNGGGTGCTATAACGCCTGCTGTTGACGTGAATCCAAAGAA
GAAACACCGTTTACTTC... ... .3'

3.6.3 Nucleotide Sequence Accession Numbers

Accession numbers of the partial 16S rDNA sequences of the isolates described in this study are given below along with the closest matching sequences already in the database. The sequences of moderate thermophiles TH1 & MT13 were taken from data base for phylogenetic tree.

Isolate	Nearest relatives	Identity (%)
HC-AF2 (AY437843)	*At. ferrooxidans* (AJ295655)	98
KC-AT2 (AY437844)	*At. thiooxidans* (B-S3) (X75269)	95

3.6.4 Phylogenetic Analysis of HC-AF2 and KC-AT2

Partial 16S rDNA sequences were obtained from the NCBI (www.ncbi.nlm.nih.gov) site and they were aligned with our isolates to generate a phylogenetic tree.

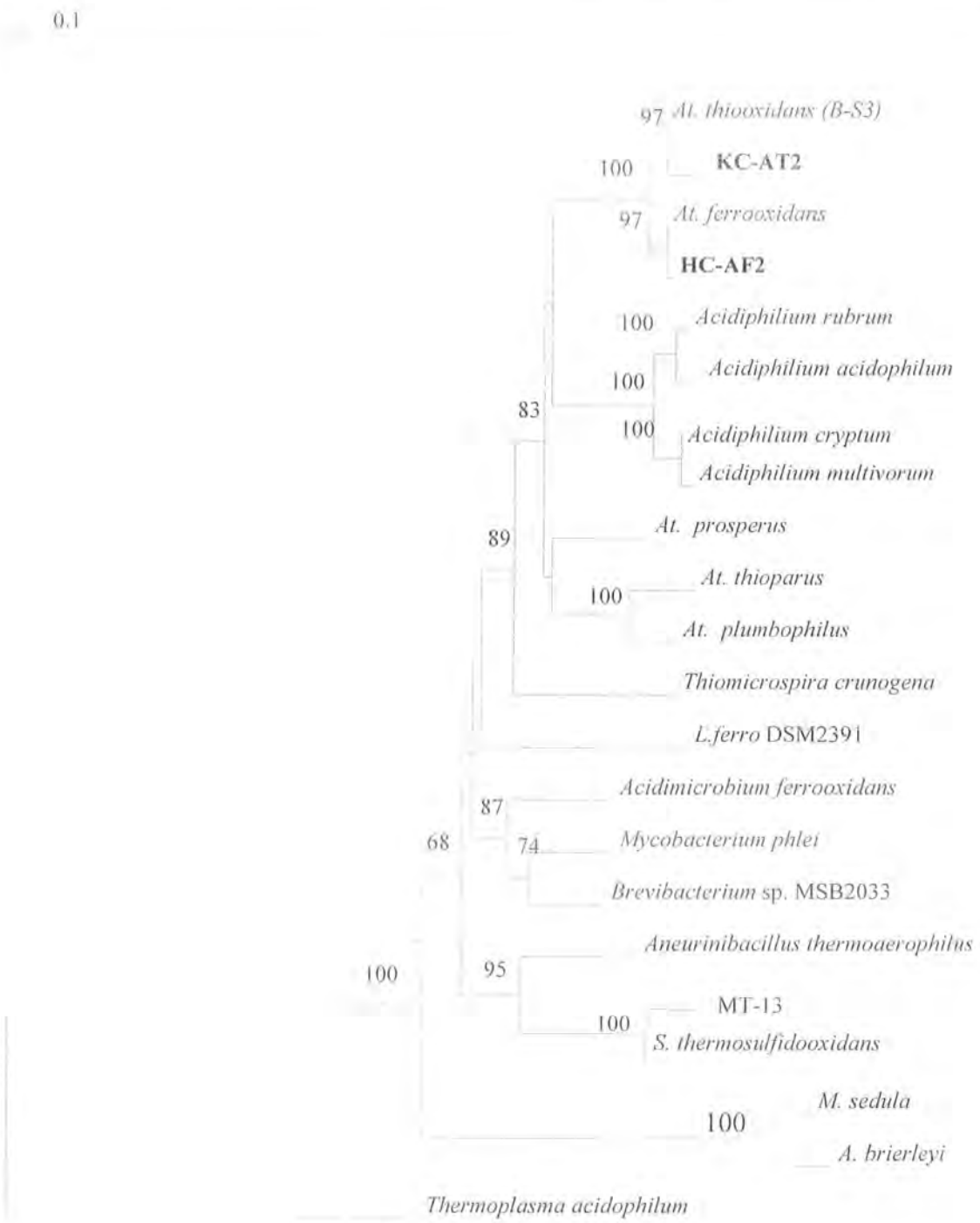

Fig.3.9. The inferred relationship based on partial 16S rDNA sequence, of the locally isolated HC-AF2 and KC-AT2 (in bold) to other bacteria. The tree was rooted with *E.coli*. Scale bar represents the number of inferred nucleotide substitution per site. Bootstrap values (100 replicates) are shown at the nodes.

3.9 Integron Profile of Inoculum Prepared for Heap

Integron profile of inoculum was studied by PCR amplification of integron-linked genes using HS286 and HS287 primers. The results indicated that amplification was much clear at 5 times dilution of genomic DNA although bands were observed in concentrated DNA and at its 10 times dilution also. The approximate sizes of observed the bands were 396 & 220bp. Integrons bands were not observed in case of *At. thiooxidans* KC-AT2.

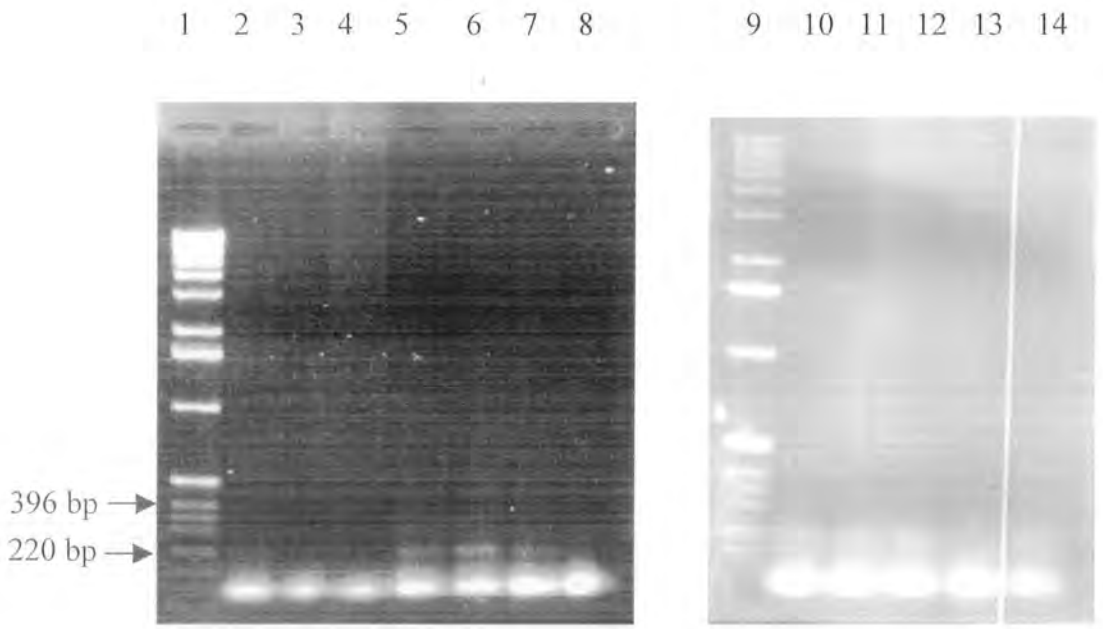

Figure 3.10: PCR amplification for integron-linked genes. (1) 1 Kb ladder, (2) Mix culture of HC-AF2, KC-AT2 & moderate thermophiles (MTs), (3) HC-AF2 grown in fermentor, (4) KC-AT2 grown in fermentor (5) MTs grown in fermentor, (6) Mix culture (5 time dilution), (7) HC-AF2 (5 time dilution), (8) KC-AT2 (5 time dilution), (9) 1 Kb ladder, (10) MTs (5 time dilution), (11) Mix culture (10 time dilution), (12) HC-AF2 (10 time dilution), (13) KC-AT2 (10 time dilution), (14) MTs (10 time dilution),

DISCUSSION

CHAPTER 4

DISCUSSION

Microbiological accelerated oxidation of pyrite and other sulfide minerals is important in both environmental and applied microbiology and also from economic point of view. Few species of acidophilic bacteria including *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Sulfobacillus thermosulfidooxidans* have been implicated as being the significant microorganisms involved in the sulfide mineral oxidation during coal desulfurization and metal leaching from ores. Monitoring of microbial population is very important to improve the biocatalytic desulfurization as well as other bioleaching processes at pilot and commercial scale. Serological tests can play an important role in identification and enumeration of specific bacterial populations during these processes. Thus the present study has significant importance regarding the improvement in large-scale biocatalytic sulfide mineral oxidation processes.

Local isolates from the NIBGE culture collection were used in the present study. These bacteria have been isolated from their natural habitats and included *At. ferrooxidans* strains, HC-AF2, TF9, & TF10, *S. thermosulfidooxidans* strains TH1, MT13, MT9, MT10, MT16 & MT17 and *At. thiooxidans* KC-AT2. *At. ferrooxidans* and *S. thermosulfidooxidans* are capable of oxidizing ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). Iron tryptic soya broth (FeTSB) medium, developed by Johnson *et al.*, (1987) was used as solid medium for their growth and to check the purity of culture (Johnson and Kelso, 1983). Growth was observed as dark brown colonies due to jarosites produced from the oxidation of ferrous ions by the bacteria. In addition to dark brown colonies, characteristic of chemolithotrophic growth, white colonies are also obtained on this medium if the culture is contaminated with heterotrophs. However, no such colonies were observed in this case confirming the purity of our culture. Single, isolated colony was picked from the plate and inoculated into iron medium (Leathen *et al.*, 1956; Postgate,

1966), consisting of basal salt solution and 0.5 M of ferrous sulfate. From iron medium, these bacteria obtain ferrous iron (Fe^{2+}) as substrate and oxidize it to ferric iron (Fe^{3+}) due to which the color of the growth media becomes reddish brown after growth. The number of cells obtained on iron media was not enough for antigen preparation. Therefore, to obtain rich growth, the cells were inoculated into the liquid medium containing pyrite (FeS_2) as energy source instead of ferrous sulfate. Pyrite is better substrate as *Acidithiobacilli* & *S. thermosulfidooxidans* utilize it as energy source and convert it into ferrous sulfate through its oxidation. The ferrous ions are oxidized to ferric ions by bacteria and energy for growth is obtained from this step also. The ferric ions, thus produced, react chemically with pyrite to produce more ferrous ions. Excess of ferric ions have inhibitory effect on the growth of bacteria, which is alleviated in pyrite containing medium as no accumulation of ferric ions takes place. After obtaining rich growth in pyrite containing liquid medium, the cultures of *At. ferrooxidans* and *S. thermosulfidooxidans* were filtered, washed and centrifuged. The cell pellet was suspended in 0.85% saline and after adjusting the desired cell concentration the cell suspension was stored at 4° C to avoid contamination.

The other bacterium used in this study i.e *Acidithiobacillus thiooxidans* KC-AT2 obtains energy from the oxidation of various reduced sulfur compounds, such as elemental sulfur, sulfide and thiosulfate etc. during its chemolithotrophic growth. However it is unable to oxidize Fe^{2+} as energy source. Jones and Starkey, (1961) demonstrated that cultures of *Acidithiobacillus thiooxidans* produced a wetting agent that permitted wetting of sulfur thereby facilitated bacterial oxidation. Moreover they decreased the pH of growth medium due to the production of sulfuric acid from the oxidation of sulfur. Growth of KC-AT2 on solid medium containing sodium thiosulfate as energy source was observed as off white colonies. Colonies of fungi or any other type of bacteria were not observed on the plates confirming the purity of our culture. Single, isolated colony was picked from solid media plate and inoculated into liquid medium containing elemental sulfur as an energy source. After obtaining rich growth, the cells were filtered to remove solid sulfur particles and centrifuged. The cell pellet was suspended in 0.85% saline and after adjusting the desired cell concentration the cell suspension was stored at 4° C to avoid contamination.

The optimum concentration of cells for antigen preparation is reported to be 1 X 10^9 (~$O.D_{600}$ = 0.45) in literature (Somasegaran and Hoben, 1994). However, in the present study it was observed that, with reported concentration of cells, the response of rabbits was low for iron oxidizing bacteria. Therefore higher cell concentration (~1.8 x 10^9 cells/ml) was used for antigen preparation to get optimum titer of antibodies in the rabbit sera. In the present study, the somatic agglutination reactions of whole bacterial cells with the homologous antiserum were used for titer determination. However in case of *At. ferrooxidans* HC-AF2 and *S. thermosulfidooxidans* TH1, the antigen antibody reaction was slow as the agglutination occurred by incubating the reaction mixture at 52°C for 4 hours and then leaving tubes for overnight at 4° C instead of one hour at later temperature. This might be due to low affinity of antibody for antigen. Among all the four strains injected to the rabbits for antibodies production, *Acidithiobacillus thiooxidans* KC-AT2 strain was found to be most immunogenic as rabbit response against this strain was very quick and antibody titer value easily reached to 1:3200 by early doses of antigens. When booster injections were given later on for maintaining the antibody titer, the titer value reached to 1:6400. *S. thermosulfidooxidans* strain MT13 was also considered to be reasonably immunogenic as for this strain the titer value reached to 1:3200 after two booster doses of antigens. The strain *S. thermosulfidooxidans* TH1 was observed to be poorly immunogenic as rabbit response against this strain was very slow and many booster doses were given in order to get the proper titer of antibodies in the serum. The slow response might be due to the inability of this antigen to stimulate both cellular as well as humoral immune system. At present there is not enough information available concerning the serology of these acidophilic bacteria especially moderate thermophiles. No report is available in literature regarding the serological characterization of *S. thermosulfidooxidans*.

Cross reactivity refers to the ability of an individual antibody combining site to react with more than one antigenic determinant or the ability of a population of antibody molecules to react with more than one antigen. Cross-reactions arise because the cross-reacting antigen shares an epitope in common with the immunizing antigen or because it has an epitope that is structurally similar to one on the immunizing antigen (multispecificity). Three of the serological tests i.e agglutination, enzyme linked

immunosorbent assay & fluorescent antibody labeling reactions were performed to check cross reactivity of antiserum developed against one strain with antigen prepared from another one. Among these tests, enzyme-linked immunosorbent assay was observed to be the more appropriate as from this test we can estimate the %age similarity among the epitopes at different antigens while agglutination some times gives false positive results as in case of cross reactivity discussed in table 3.3 & 3.4. Though, the immunofluorescence is as sensitive as ELISA but it is time consuming and it might not be suitable for testing large number of samples.

From serological reactions, *S. thermosulfidooxidans* strain MT13 and reference strain TH1 were found to have same serotype. On the basis of results shown in table 3.3 & 3.4 it can be inferred that strains MT9, MT10, MT16 & MT17 of *S. thermosulfidooxidans* have also most of the cell surface antigens common with those of MT13 and TH1. These observations indicate that all these strains are closely related to each other and have similar serotype. On the other hand, no cross reactivity was observed when antisera developed against MT13 and TH1 were reacted with mesophilic strains KC-AT2, HC-AF2, TF9 and TF10. Thus indicating that all these mesophilic strains belong to different serotypic group and no cell surface antigens are common among these strains. Similar results were obtained when antisera developed against KC-AT2, HC-AF2 were reacted with moderate thermophilic strains MT9, MT10, MT13, MT16, MT17 & TH1, confirming the above observation. Only about 25% antigenic determinants were found to be common among mesophilic strains *At. ferrooxidans* HC-AF2 & *At. thiooxidans* KC-AT2 isolated from two different locations. Antisera developed against *At. ferrooxidans* HC-AF2 strain show only small positive reaction when reacted with antigens from TF9 & TF10 indicating them to be distinct strains having different serotype among the same species. These results are in accordance with those obtained by Halberg and Lindstrom (1996) who reported that multiple serotypes of biomining microorganisms such as *At. ferrooxidans* or *At. caldus* exist.

Compared to pervious quantitative indirect methods using counting of cells in suspensions (Monroy *et al.*, 1994) and qualitative methods using a combined immunofluorescence-DNA-fluorescence staining procedure (Muyzer *et al.*, 1987) the enzyme linked immunosorbent assay performed in microtiter plates during this study

allowed the direct quantification of specific bacterial strains in leach liquor as well as in solid coal samples taken from heap at regular time intervals. ELISA results indicated that moderate thermophiles dominated in the leach liquor as compared to *At. ferrooxidans* & *At. thiooxidans* (Table 3.5). These moderate thermophiles were also detected in solid coal samples taken from different locations of heap, significant population was in the bottom edge, mid side and top side samples (Figure 3.5 & 3.6). Although temperature conditions in the inner portion of heap seem to be favourable for the growth of moderate thermophiles, but their low population density was observed in that region probably due to less availability of oxygen. On the other hand, we were unable to find the mesophilic bacteria *At. thiooxidans* KC-AT2 in the leach liquor which indicates that this bacterial strain has more affinity (adhesion) with solid coal as compared to leach liquor as we found this strain in the solid samples. However, this strain was not detected in samples taken from topside and mid-centre. Unexpectedly, the highest population of this strain was found at bottom centre of the heap where the temperature range was 60-70° C. It was also confirmed by spreading the aqueous extract from these coal samples on solid media containing sodium thiosulfate as energy source. The cells of other mesophile, *At. ferrooxidans* HC-AF2, were found in some liquor samples, but considerable population of this bacterial species was detected in solid coal samples indicating its adhesion to pyrite crystals in the coal. The overall bacterial population was higer towards peripheries (i.e. mid side, bottom edge and top side) while less in central portion (i.e. bottom centre and mid centre) of heap (Fig. 3.5). This might be due to elevated temperature and less availability of oxygen in the centre of heap. So, providing aeration can increase the bacterial population in the centre.

The 16S rDNA amplification was done to confirm the identity of our locally isolated strains being used in coal biodesulfurization studies because at present 16S rDNA sequence-based bacterial identification is the most advanced and accurate as it is based on highly conserved sequence stretches. It is more reliable and precise compared to error-prone conventional morphological and metabolic identification techniques. 16S rDNA sequence analysis of the two bacterial strains HC-AF2 & KC-AT2 used in the coal heap biodesulfurization showed that HC-AF2 belongs to *At. ferrooxidans* and has 98% 16S rDNA similarity with *At. ferrooxidans* (AJ295655) isolated from uranium piles by

Selenska-pobel *et al.* (2001) and KC-AT2 strain belong to *At. thiooxidans* and has 95% 16S rDNA similarity with *At. thiooxidans* (B-S3)(X75269) isolated by Goebel and Stackebrandt in 1994. So these results confirmed the identification done by conventional methods.

Integrons seem to be very efficient gene acquisition and expression systems making them a good alternative to traditional methods for gene isolation. Comparison of integron linked genes in HC-AF2 and moderate thermophiles and inoculum containing both of these strains along with KC-AT2 on agarose gel have shown that equal size integron-linked genes are present in all of the three samples Presence of integrons in these species has also been reported by other investigators (Ghauri *et al.*, 2003; Rowe-Magnus and Mazel, 2001). However no bands were observed in case of KC-AT2 strain indicating that this strain apparently has no integron linked genes. Further separation of these multiple genes and sequencing can lead to the identification of proteins coded by these genes.

The present study indicates that serological reactions could be very useful for qualitative as well as quantitative analysis of bacterial populations. By indirect ELISA we can easily quantify large number of samples in a very short time. So the method developed for the quantification of bacteria in this study has significant importance for monitoring population of specific bacterial species during the bioleaching processes and ultimately leads to the improvement of these processes.

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CHAPTER 5

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APPENDICES

APPENDIX I

Coating Buffer (pH 9.6)

(Carbonate-bicarbonate buffer, 0.05 M)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.20 g

Dissolve in 1 liter of distilled water; store at 4° C for not more than two weeks.

APPENDIX II

Phosphate Buffer Saline (PBS)

NaCl	8.5 g
Na_2HPO_4 (anhydrous)	1.08 g
$NaH_2PO_4.2H_20$	0.31 g
Merthiolate	0.10 g

Dissolve in 1 liter distilled water and store at 4° C.

Phosphate Buffer Saline Tween 20 (PBST)

For PBST, dissolve 0.5 mL Tween 20 in 1 liter PBS and store at 4° C.

APPENDIX III

Enzyme Substrate Solution for ELISA

Enzyme substrate buffer

(Diethanolamine Buffer, 10%)

Diethanolamine	97 mL
NaN_3	0.2 g
$MgCl_2.6H_2O$	0.1 g

Dissolve in 800 mL of distilled water and adjust the pH to 9.8 with HCl. Adjust the volume to 1 liter with distilled water. Store at room temperature in an amber bottle.

To make enzyme substrate solution, dissolve

p-nitrophenol phosphate	5 mg
Enzyme substrate buffer	5 mL

Immediately before use, dissolve 5 mg of p-nitrophenol phosphate in 5 mL enzyme substrate buffer at room temperature. Store p-nitrophenol phosphate at –20° C in the dark until use.

APPENDIX IV

Bradford's Reagent

The assay reagent was made by dissolving 100 mg of Coomassie blue G-250 in 50 mL of 95% ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and was made upto one liter with distilled water. It was stirred overnight. The reagent was then filtered through Whatman No. 1 filter paper before storage in an amber bottle at room temperature.

APPENDIX V

Phosphate Buffer, 0.15 M (pH 9.0)

Na_2HPO_4 (anhydrous) 21.3 g

Dissolve in 800 mL of distilled water. Adjust the pH to 9.0. Dilute to 1000mL distilled water.

Phosphate Buffer, 0.1 M (pH 8.0)

Na_2HPO_4 (anhydrous) 14.2 g

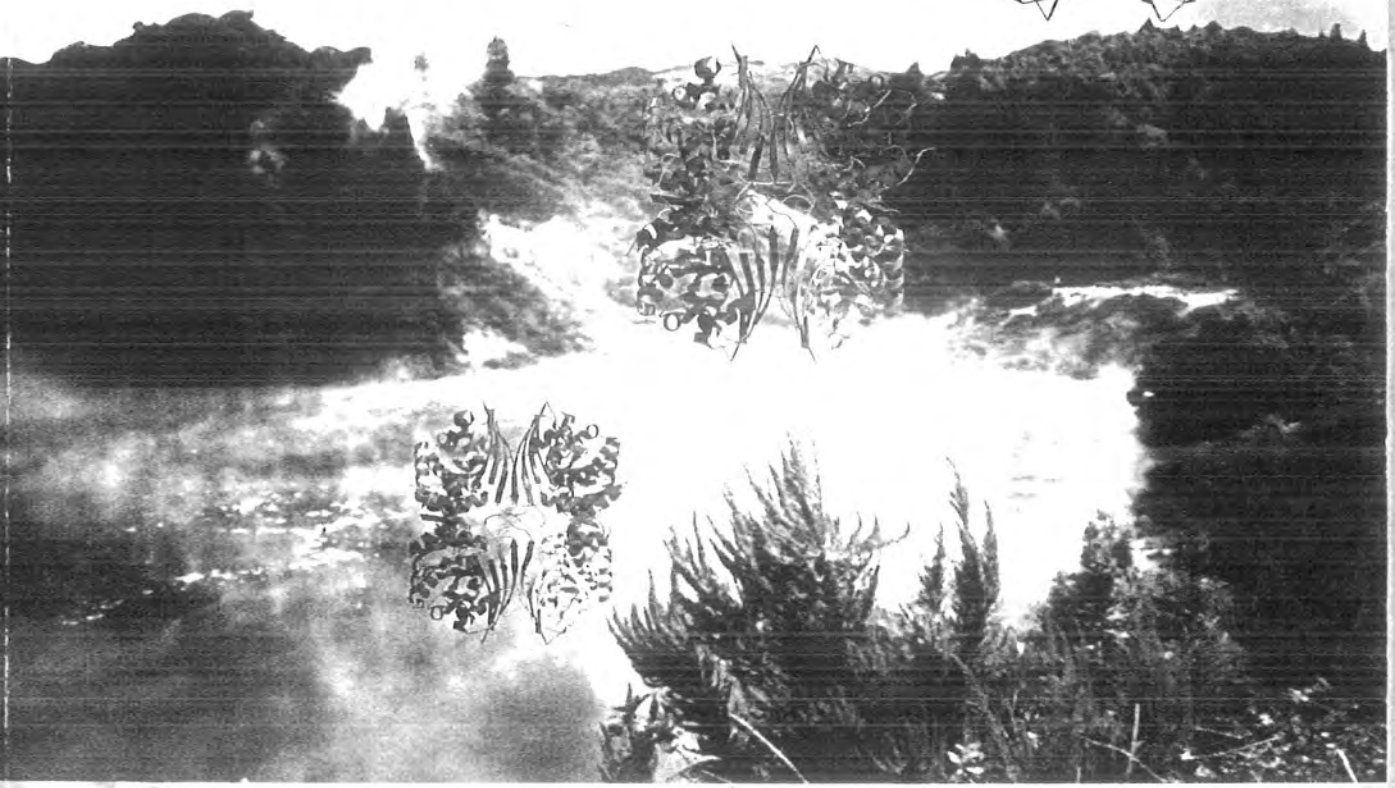
Dissolve in 800 mL of distilled water. Adjust the pH to 9.0 by the dropwise addition of 1N HCl. Dilute to 1000mL distilled water.

APPENDIX VI

INTERNATIONAL CONFERENCE

Thermophiles 2003

September 15 – 19



UNIVERSITY of EXETER

2003

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P1.40: DEVELOPMENT OF POLYCLONAL ANTIBODIES AGAINST MT13 STRAIN OF *SULFOBACILLUS THERMOSULFIDOOXDANS* AND DETERMINATION OF THEIR SPECIFICITY

Saleem, M; Anwar, M. A; Ghauri, M. A; Akhtar, K; Rehman, M; Hafeez, F. Y. and Khalid, A. M.

Bioprocess Technology Division, National Institute for Biotechnology and Genetic Engineering, P. O. Box 577, Jhang Road, Faisalabad, Pakistan.

The identification of bacteria in a leaching system is very critical and difficult task. Quicker and accurate means of identification of bacteria have been reported (1). In the present study, polyclonal antisera were raised in rabbits against whole cells of locally isolated moderate thermophilic acidophilic MT13 strain of *Sulfobacillus thermosulfidooxidans* (Ghauri et al., 2003) and its specificity was determined against various mesophilic and moderate thermophilic isolates. To get higher titer of antibodies 1.8 x 10^9 cells/ml were used for antigen preparation. The production of antibodies was slow and titer value reached to 3200 in about two months thus indicating slow release of antibodies when rabbit is used as a model system in this case. The antigen antibody reaction was also slow as the agglutination reaction occurred by incubating the reaction mixture at 52°C for 4 hours and then leaving tubes for overnight at 4°C instead of one hour at later temperature. This might be due to low affinity of antibody for antigen. The developed antisera were highly specific for MT13 strain as no cross reactivity was observed when tested against other strains of the same species and bacteria belonging to *Acidithiobacillus thiooxidans and Acidithiobacillus ferrooxidans.* It is postulated that MT13 has unique serotype amongst the tested bacterial groups. This was further confirmed by making fluorescent antibodies (FAs) and applying them to all the above-mentioned cultures. No detectable fluorescence was observed except in the case of MT13 cultures thus confirming earlier observation of antibodies specificity.

(1). Halberg, K. B. and Lindstrom, E. B. *Appl. And Environ. Microbiol.* **62:11** (1996) 4243-4246
(2). Ghauri, M. A; Khalid, A. M; Grant, S; Heaphy, S. and Grant , W. D. *Extremophiles* (2003) (in Press)

DEVELOPMENT OF POLYCLONAL ANTIBODIES AGAINST MT13 STRAIN OF *SULFOBACILLUS THERMOSULFIDOOXDANS* AND DETERMINATION OF THEIR SPECIFICITY

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Bioprocess Technology Division,
National Institute for Biotechnology and Genetic Engineering, P. O. Box 577, Jhang Road, Faisalabad, Pakistan.

BACKGROUND

Thermophilic acidophilic bacteria are able to oxidise reduced iron and sulfur compounds and possess great potential for coal desulfurization and bioleaching of mineral sulfide ores at high temperature. However, the identification and enumeration of specific acidophilic bacterial species in a leaching system are difficult tasks because of the presence of mineral particles. Also, it is difficult to obtain growth of leaching bacteria on defined solid media because incubation time for the development of colonies is usually on the order of one week or more. It is also difficult to distinguish bacteria by colony morphology. Different methods like morphological characteristics, differential staining, biochemical tests, serological analysis and molecular analysis are used for the identification of bacteria. Among these methods, serological tests have some advantages that these are quick and more specific.

In the present study, serological test was developed for the identification and quantification of moderately thermophilic bacteria *Sulfobacillus thermosulfidooxidans* MT13 in bioleaching system. For this purpose polyclonal antibodies were developed against MT13 bacteria in rabbits. The cross reactivity of these antibodies with other moderate thermophilic bacteria like TH1 and mesophilic bacteria like *Acidithiobacillus ferrooxidans* C2 strain and *Acidithiobacillus thiooxidans* TT2 Strain was also checked.

METHODOLOGY

For polyclonal antibodies production the rabbits were used as model system. For antigen preparation *sulfobacillus thermosulfidooxidans* MT13 strain was grown on basal salt media. After rich growth the cells were suspended in 0.85% saline. The antigens were prepared by steaming 1.8 x 10^9 cells/ml for one hour. Higher concentration of cells was used to get high response.

Rabbits were immunized with antigens and the blood serum was tested for specific antibodies by agglutination reaction (Fig1, Table1). When the antibody titer value reached to 3200 then the blood was taken and the serum was separated. Methiolate was added in it as a preservative. The cross reactivity of these antibodies was checked by agglutination and indirect ELISA methods. The secondary antibodies used in ELISA were commercial IgG goat anti-rabbit alkaline phosphatase conjugate (GAR AP). These results were further verified by making fluorescent antibodies (FAs).

For FAs preparation, immunoglobulins were purified from serum by ammonium sulfate precipitation and the salt was removed by dialysis. Then these immunoglobulins were conjugated with fluorescien isothiocyanate (FITC) dye with FITC concentration 0.03mg/mg of protein. The unconjugated dye was separated by passing the mixture through sephadex column (Fig 2). The FA staining of the cultures was done and the fluorescence was checked under UV microscope.

RESULTS

The developed antibodies gave no cross reactivity with mesophilic strains C2 and TT2 during agglutination reaction indicating that these mesophiles belong to different serotype than MT13. ELISA also showed similar results because no color was developed in microtiter plate wells having antigens from mesophiles (Fig3). However, color was seen in wells having antigens of TH1 cells along with +ve control indicating that MT13 and TH1 bacteria have almost same serotype (Table 2).

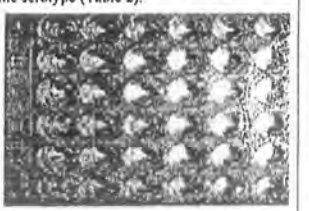
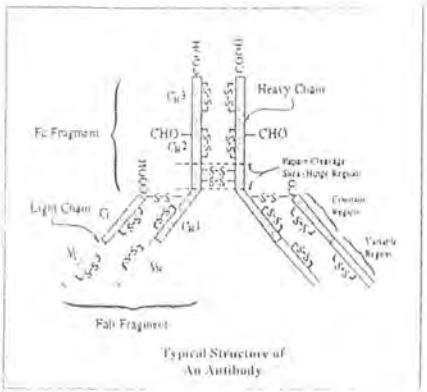


Fig:3 Cross reactivity of MT13 with other bacteria checked by ELISA. Wells A have +ve control, B: -ve control, C: C2, D: TH1, and E: TT2

Table2: Results of Antigen-antibody reaction of polyclonal antiserum developed against MT13 strain

Strains	Techniques		
	Agglutination	ELISA	FA staining
MT13	+ve	100%	+
C2	-ve	0%	0
TT2	-ve	0%	0
TH1	+ve	90%	+



Typical Structure of An Antibody

Table1 Injection Schedule

Days	Procedure
1	0.5 ml (IV)
2	1.0 ml (IV)
3	1.5 ml (IV)
4	1.5 ml (IV)
5	2.0 ml (IV)
6	2.0 ml (IV)
16	Test bleed (5 ml) Titer determination
18	Inject 2.0 ml SC (booster)
26	Test bleed (5 ml) Titer determination
28	Inject 2.0 ml SC (booster)
38	Test bleed (5 ml) Titer determination
40	Inject 2.0 ml SC (booster)
48	Test bleed (5 ml) Titer determination
50	Cardiac bleed (20ml) Inject 20 ml saline (IP) Agent titer determination Inject 2.0 ml SC (booster)

Fig:1 Intravenous injection of antigens to rabbit

Fig:2 Purification of FAs by column chromatography

CONCLUSIONS

- Serological reactions are important tools for quick and accurate identification of bacterial strains.
- From various antigen-antibody reactions it is clear that MT13 strain has no serotypic homology with mesophilic strains C2 and TT2.
- MT13 bacteria have some serotypic homology with moderate thermophilic strain TH1.

This work is part of M. Phil research project presented at "International Conference Thermophiles 2003" University of Exeter, United Kingdom (15-19 September 2003).

APPENDIX VII

APPENDIX-VII

DEVELOPMENT OF POLYCLONAL ANTISERA FOR THE IDENTIFICATION OF LOCALLY ISOLATED ACIDOPHILIC BACTERIA

Muhammad Saleem*, Munir A. Anwar, Muhammad A. Ghauri, Kalsoom Akhtar, Moazur Rehman, Fauzia Y. Hafeez, & Ahmad M. Khalid*.

Bioprocess Technology Division, National Institute for Biotechnology and Genetic Engineering, P. O. Box 577, Jhang Road, Faisalabad, Pakistan.
Presenting author: saleemraza_5@yahoo.com
Corresponding author: amkhalid@nibge.org
Fax: +92-(41)-651472
Phone No. +92-(41)-651472

Acidophilic bacteria are able to oxidize reduced iron and sulfur compounds and possess great potential for coal desulfurization and bioleaching of mineral sulfide ores. However the identification and enumeration of specific acidophilic bacterial species in a leaching system is difficult task because of the presence of mineral particles. Moreover, it is difficult to obtain growth of leaching bacteria on defined solid media because incubation time for the development of colonies is usually on the order of one week or more. Therefore, some quicker and accurate means of identification of bacteria should be developed. In the present study, polyclonal antisera against whole cells of locally isolated acidophilic bacteria including *Acidithiobacillus ferrooxidans* (HC-AF2), *Acidithiobacillus thiooxidans* (KC-AT2), *Sulfobacillus thermosulfidooxidans* strains TH1 and MT13 were raised in rabbits. Their specificity was determined against each other and various other acidophilic isolates by the serological tests such as agglutination and ELISA. The antigen antibody reaction was observed to be slow as the agglutination reaction occurred by incubating the reaction mixture at 52°C for 4 hours and then leaving tubes for overnight at 4°C instead of one hour at later temperature. This might be due to the low affinity of polyclonal antisera for their antigens. The polyclonal antisera against mesophilic strains HC-AF2 and KC-AT2 observed to be more specific as it gave no cross reactivity with other mesophilic and moderate thermophilic acidophilic isolates when tested by indirect ELISA. While polyclonal antisera developed for TH1 and MT13 strains showed cross reactivity against other moderate thermophilic acidophilic isolates.

Paper accepted for oral presentation at "4th International and 14th National Chemistry Conference", 2004.

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  JOURNAL   Unpublished
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  AUTHORS   Saleem,M., Ghauri,M.A., Anwar,M.A., Akhtar,K. and Khalid,A.M.
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  TITLE     Direct Submission
  JOURNAL   Submitted (14-OCT-2003) Bioprocess Technology, National Institute
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