

Studies on Microbial Remediation of Bauxite Residue Sites

*A thesis submitted in fulfillment of the
requirement for the award of the degree of*

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**

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March, 2009

CERTIFICATE

Certified that the thesis “**Studies on microbial remediation of bauxite residue sites**” which is submitted by Mr. Pankaj Krishna, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate’s own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis “**Studies on microbial remediation of bauxite residue sites**” submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. M. Sudhakara Reddy, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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ACKNOWLEDGEMENT

In pursuit of this academic endeavor, I feel that I have been singularly fortunate because inspiration, guidance, direction, cooperation, love and care - all came in my way in abundance and it seems almost an impossible task for me to acknowledge the same in adequate term.

Yes, I shall be failing in my duty if I do not record my profound sense of indebtedness and heart felt gratitude to my guide, Dr. M Sudhakara Reddy, Associate Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University (TU), Patiala, who guided and inspired me in pursuance of this work. His association with this endeavor of mine will remain a beacon light to me through out my life.

I am very thankful to Dr. N. Das, Assistant Professor and Head, DBTES, TU, Patiala for providing suggestions, for-thoughts and taking interest in the progress of this work since inception. I express my gratitude to Dr. Susheel Mittal, Dean (Research and Sponsored Projects), Thapar University, Patiala, for his encouragement and support during my research work in the Institute. I wish to express my thanks to Dr. Abhijit Mukherji, Director, Thapar University, Patiala, for providing infrastructure and helping me towards the smooth and timely completion of my research work.

I hereby take this opportunity to express my deep sense of gratitude to my revered teachers, Dr. Dinesh Goyal, Dr. N Tejo Prakash, Dr. Sanjai Saxena and Dr. Abhijit Ganguli, DBTES, TU, Patiala, for their meticulous guidance, keen interest, invaluable suggestion, constant support and encouragement during the course of my study. The personal and professional guidance that I received from them would be cherished life long. My gratitude is due to all faculty members for help and suggestions during my experimental work.

I express my regards to Dr. Anil Kumar Datta, DBTES, TU, Patiala, along with team members of Tissue Culture Centre, TIFAC-CORE, TU, Patiala for their constant help and providing the necessary facilities.

I am especially indebted to Dr. M. Krishnamohan, Birla Institute of Scientific Research, Jaipur and Dr. Banwari Lal, The Energy Research Institute, Delhi for their cooperation and timely help.

The cooperation received through my colleagues namely Bella, Ramesh, Diwakar, Achal, Giri, Santosh, Purnima, Kamal, Machy, Neetu, Siddharth and Charu are thankfully acknowledged. I owe a word of thanks to all the research scholars and Mr. Om Prakash, Jai Prakash, Babban (Ram Neval), Lallan and Lab assistants for their help. I also thank those who could not find a separate name but helped me directly or indirectly.

I would like to convey my thanks to Mr. Manmeet Singh and Mr. Aniruddh Handa for their timely help and cooperation.

I owe my personal reckoning of gratitude and benevolence to my family for their love, care, patience, inspiration, encouragement; motivation and constant driving force, which has enabled me to complete my task. A sincere token of gratitude is also paid to my friends Arvind, Arun and Anita for all their amicable endeavors and sustained encouragement for my success.

I wish to express my gratitude and thanks to DST and TIFAC-CORE, India for providing me financial support in the form of fellowship to accomplish my work.

Date :

Place :

Pankaj Krishna

The following publications are the outcome of the present research work:

- Pankaj Krishna, M. Sudhakara Reddy and S. K. Patnaik (2005) *Aspergillus tubingensis* reduces the pH of the bauxite residue (red mud) amended soils. *Water Air Soil Pollution*, 167: 201-209.
- Pankaj Krishna, Amita Arora and M. Sudhakara Reddy (2008) An alkaliphilic and xylanolytic strain of actinomycetes *Kocuria sp.* RM1 isolated from extremely alkaline bauxite residue sites. *World J Microbiol Biotech*, (DOI 10.1007/s11274-008-9801-8)
- Pankaj Krishna, A. Giridhar Babu and M. Sudhakara Reddy (2008) Bacterial diversity of bauxite residue (red mud) sites of alumina industrial plants. (*Submitted*)
- Pankaj Krishna and M. Sudhakar Reddy (2008) Strategies of red mud bacterial isolates to inhabit in highly alkaline habitat. (*manuscript*)

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Abbreviations

bp	Base pair
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
g	Gram
hr	Hours
IPTG	Isopropyl- β -thiogalactoside
kb	Kilo base
L	Litre
MES	2-(Morpholino)-ethanesulfonic acid monohydrate
mg	Milligram
mL	Milliliter
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
TABS	<i>N</i> -Tris(hydroxymethyl)methyl-4-aminobutanesulfonic acid
TAPS	<i>N</i> -[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid
Tris	Tris-(hydroxymethyl-) aminomethane
X-Gal	5-Bromo-4-chloro-3-indolyl- β -D-galactoside
μ g	Microgram
μ l	Microlitre
μ S cm ⁻¹	Microsiemens per cm

Chapter 1

Introduction

To meet the ever-growing demands of materials, natural resources are being exploited to the maximum extent. As a result, there is depletion of valuable resources as well as an accumulation of different types of wastes. Red mud (bauxite residue) is one such waste, produced during aluminum extraction from bauxite ore in the Bayer process (Evans, 1993). Bauxite ore is the only economic ore of aluminum. It contains 30-54% alumina (Al_2O_3), the rest being a mixture of silica, iron oxides, and titanium oxide. Aluminum metal is commercially produced through two main process steps. In first step, alumina is obtained by the Bayer process (Chinloy and Holzwarth, 1984) and then the alumina is electrolyzed by Hall and Heroult (Hall, 1889) method to yield aluminum metal. In the Bayer process, bauxite is washed with a hot solution of sodium hydroxide, at 175 °C (called digestion). This converts the alumina to aluminium hydroxide, which dissolves in the hydroxide solution. The other components of bauxite do not dissolve and are filtered from the solution as solid impurities (clarification). The mixture of solid impurities is called bauxite residue (red mud) (Fig 1.1; Fig 1.2 a and b), and presents a disposal problem.

The use of NaOH in the Bayer process results in the bauxite residue being extremely saline, sodic and alkaline (Wong, 1990). In addition to these extreme properties, bauxite residue is generated in very large quantities in each year, and occupies huge area of land. The major components in the red mud are iron oxide, silica, un-reacted alumina and residual NaOH as Na_2CO_3 , as well as alkali bound in the form of sodalite, ferrite etc. The residual alkali content makes the red mud alkaline with a pH from 9 to 13. It is disposed into red mud ponds. By the middle of the twentieth century it was established that red mud is a pollutant, and has a detrimental effect on the environment and human health (Thakur and Das, 1994).

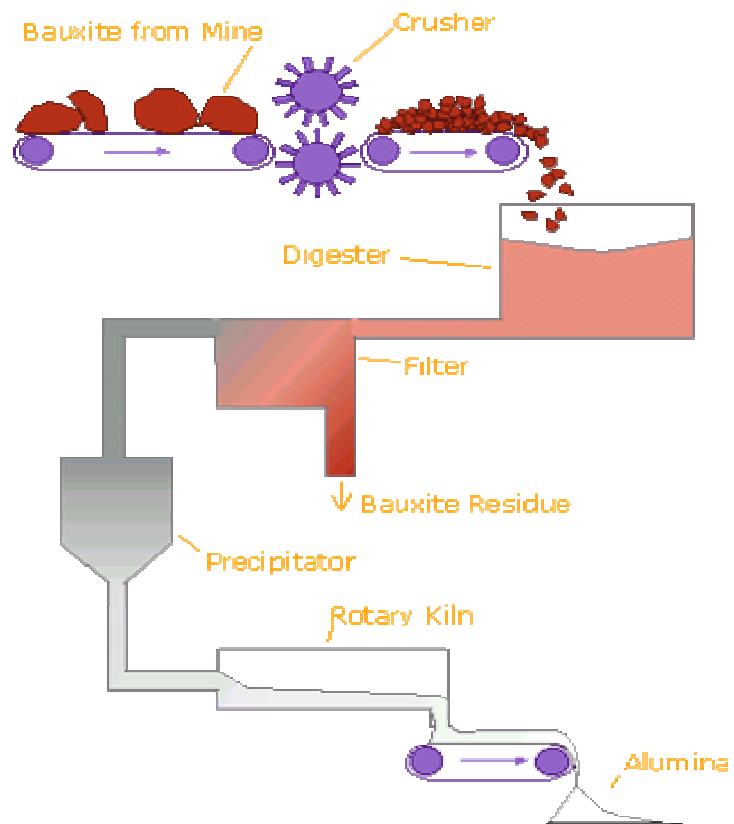


Fig 1.1: Schematic diagram of the red mud production during alumina extraction



Fig 1.2 (a & b) Red mud pond of National Aluminum Company (NALCO), Damanjodi, Orissa, India

Introduction of Bayer's process resulted in the deposition of its waste and the problem of red mud has grown along with increasing demand and manufacturing of alumina. This waste material has been accumulating at an increasing rate throughout the world. Worldwide production of alumina was estimated at 52.5 million metric tons in 2003. An estimated 70 million metric dry tons of residue is produced globally per year and is disposed on land in large residue disposal areas (tailings dams), either as wet slurry or de-watered/ dry-stacked (TAA, 2000).

Disposal of any solid waste is associated with availability of space/ real estate near industry, cost of disposal and the pollution caused by it. Obviously these three problems are also associated with red mud disposal, and the problem has become more acute with increasing amounts of red mud, shortage of real estate around the industry and environmental awareness of the society. Disposal of waste adds to the cost of production in the range of 2-5%. Dewatering techniques for red mud slurries were developed and red mud was dumped on real estate, in the form of heaps or ponds. In India, the slurry is pumped (Fig 1.3) out to nearby estate dug into ponds where it is left for sun drying (Thakur and Das, 1994) (Fig 1.4). Simultaneously it is deeply felt by the scientists and technologists that a good amount of metal value is disposed as a waste for which mining and transport cost have been paid. Hence soon after the acceptance of Bayer process in industry, scientists and technologists directed their efforts in possible utilization and reclamation of red mud. The stream water used by human beings and animals is also spoiled due to leaching out of alkali from red mud soil. The vegetation cover can considerably checks such type of potential damages. Because of the extreme alkalinity and salinity, the microbial growth is also a limiting factor in red mud sites. It is necessary to reclaim such sites that are depleted of the nutrients due to industrial wastes by using different treatments such as leaching out alkali and soluble salts, adding soil conditioners and microbial inoculants, plant growth as well as development can be improved on such sites.



Fig 1.3: Disposal of red mud in the form of wet slurry to nearby artificial impoundment at NALCO, Damanjodi, Orissa, India



Fig 1.4: Red mud ponds are left open for sun drying, leaving cracks after being dried at NALCO, Damanjodi, Orissa, India

Reclamation of red mud ponds are difficult because of high pH, high concentration of soluble ions such as sodium and carbonate which are toxic and competitively inhibit the uptake of nutrients in plants and microbes. Without drainage and costly chemical neutralization, the exchangeable sodium percentage, pH and soluble aluminum concentrations remain high. These properties when coupled with lack of nutrients and anoxic conditions prevent the vegetation to establish. Similarly when exposed to air, these barren surfaces dry up and become source of highly alkaline wind blown dust, which causes severe health problems.

Efforts were also made to establish vegetation on red mud lakes by chemical neutralization. Reduction of alkalinity of red mud may be achieved by the use of strong acid (sulphuric acid). It is not economically viable due to high requirement of acid to neutralize the alkalinity of the red. Gypsum (CaSO_4) and copperas (FeSO_4) were suggested as ameliorants for red mud; the addition of these acidic wastes at 8% released Na and Ca from red mud and reduced the pH to about 8.5 (Stewart *et al.*, 1995). The nutrient concentrations in red mud grown plants tend to be low, but can be increased to some extent by addition of ameliorants such as gypsum (CaSO_4) and sewage sludge (Wong and Ho, 1994). Adequate growth of *Bermuda* grass is possible when sufficient gypsum was added to lower the pH to 8.3. It has been found that fly ash and coarse texture red mud can be mixed 20:80 to reduce the pH to 7.0-8.0. After addition of NPK fertilizer, it became suitable for growth of Rhodes grass (Eastham *et al.*, 2006). Sewage sludge added to coarse structure red mud dikes in Soviet Union allowed adequate growth of a number of species. However, the surface pH was only 7.8 indicating that excessive leaching has taken place. Reaction of bauxite residue with seawater results in neutralization of alkalinity through precipitation of Mg-, Ca-, and Al- hydroxide and carbonate minerals. Reaction with seawater produced a residue pH of 8.0 to 8.5 (Menzies *et al.*, 2004).

Currently, remediation of various wastes is based on several processes like physical, chemical, biological, either singly or in combination. However, some uneconomical physicochemical methods are slowly losing ground due to inherent problems of secondary contamination and non-sustainable control of contaminants (Vidali, 2001). Bioleaching requires the presence of microorganisms able to proliferate in extreme ecosystems (strongly acidic pH, highly oxidizing conditions, high concentration of metal ions in solution, high alkalinity) and draw their energy from oxidation of mineral sulfides. In fact, extraction of metals by biological

solubilization has been extensively exploited and used for several years in biohydrometallurgy. Because of the inherent problem with the chemical amelioration, biological treatment is gaining ground and with a recent estimate of Environmental Protection Agency (EPA) approximately 32% of remediation is achieved by biological means.

Despite the extreme alkalinity and salinity, bacteria have been isolated from bauxite residue (Williams and Hamdy, 1982; Agnew *et al.*, 1995) and bacterial induced pH reduction of bauxite residue has been made (Williams and Hamdy, 1982). Thus it may be possible to induce the indigenous bacteria in bauxite residue to produce acidic compounds and hence neutralize red mud. Valarie (1999) suggested that pH reduction of red mud would be possible by bacterial metabolism. Ward and Koch, (1996) have reported the potential use of bauxite residue for recovery of aluminium by chemical or biological leaching (*Penicillium simplicissimum*). Edwards *et al.* (1999) suggested of using bauxite residue as a lime substitute for treating acid soils as well as acid mine drainage sites and to enhance minerals deficient soils. Anand *et al.* (1996) used *Bacillus polymyxa* for the removal of calcium and iron from bauxite ore.

Microbial remediation is accomplished with the help of diverse group of microorganisms, particularly indigenous bacteria present in soil. Moreover, metabolic diversity of bacterial flora is well-established phenomenon, and a consequence of this metabolic diversity is degradation of various biohazardous or persistent anthropogenic compounds by microbial activities. Enhanced activity of indigenous bacteria help in bioremediation of contaminated sites. An advantage of inoculation of indigenous bacteria in contaminated sites is that it may not show deterrent effect on the indigenous microbial community. Microbiological analysis of red mud might provide the clue for the selection of microbes for remediation or other biotechnological applications. But there are very few reports pertaining to the microbiology of the red mud. There is only one published report stating the isolation of bacteria from red mud. Less than ten research publications in last 30 years, based on the microbial remediation of bauxite residue have been published. It shows that ample work can be done to exploit the microbes inhabiting in this extreme conditions. These indigenous microbes can be used to rehabilitate the waste site for sustainable environment. No reports are available regarding the microbial diversity and community structure of the red mud. Keeping

these points in view the following objectives have been formulated to study the microbes in the red mud.

Aim of present study

1. To study the diversity of microbes present in the red mud ponds
2. To characterize the physiological behavior of the isolated bacteria.
3. To reduce the alkalinity and revegetation of red mud ponds using microbes.

Chapter 2

Literature review

Red mud

Red mud is brick red in color and slimy in appearance, having an average particle size of <10 mm also known as red slime, red sludge or bauxite residue. It is comprised of oxides of iron, aluminum, sodium, calcium, titanium, silica and other trace elements part of the parent ore along with other minor constituents. Apart from alkalinity, red mud possesses high surface area (Paramguru *et al.*, 2005). The free moisture content of the red mud is highly variable (30-60%), depending upon the disposal method. It consists of dilute caustic solution with a pH of about 13. Furthermore, bauxite residues are characterized by the presence of Fe, Al, Si, Na, Ca, Ti and trace elements (Table 2.1).

Table 2.1: Composition of red mud of different locations (Thakur and Das, 1994)

Country	Fe ₂ O ₃	Al ₂ O ₃	TiO ₂	SiO ₂	Na ₂ O ₃
India					
Al. Corpn.	20.26	19.6	28.0	6.74	8.09
MALCO	45.17	27.0	5.12	5.7	3.64
HINDALCO	35.46	2.0	17.2	5.0	4.85
BALCO	33.8	15.58	22.5	6.84	5.2
NALCO	52.39	14.73	3.30	8.44	4.0
USA					
ALCOA Mobile	30.40	16.2	10.11	11.14	6.8
REYNOLD Arkansas	55.6	12.15	4.5	4.5	2.0
Sherwon	50.54	11.13	Traces	2.56	1.5-5.0
FRG Baudart	38.75	20.0	5.5	13	9.0
Hungarian	38.45	15.2	4.6	10.15	8.12
Jamaican	50.9	14.6	6.87	3.4	3.18
Surinam	24.81	19.0	12.15	11.9	9.29
Taiwan	41.3	20.21	2.9	17.93	8.16
Australia	40.5	27.7	3.5	19.9	3.8

Mud disposal

Mud disposal means its transporting and depositing in a tailing pond or lake or in a landfill, which covers the aspects of landscaping, rehabilitation, and the environmental impact. The important objectives remain the same, i.e., 1) reduced mud volume, 2) reduced caustic content, 3) reduced environment impact, 4) improved reclamation potential, and 5) improved rehabilitation possibilities. The usual earlier trend was to pump a slurry with 20-30% solids to the artificial ponds or lakes, requiring bottom sealing to avoid caustic leakage, high pumping energy, etc. (Colembra and Want, 1982). Improved dewatering techniques have facilitated semidry disposal methods by separating fine mud from the sand fractions and dewatering to a solid concentration of 50-60% before transporting by pipelines (Nguyen and Boger, 1998). At the disposal dam, the discharged mud is spread and allowed to consolidate and dry in layers following the method of thickened discharge and dry stacking (Chandler, 1987; Pohland and Tielens, 1987; Robinsky, 1987). This type of disposal strategy has many advantages such as reduced pumping costs, effective use of land, and minimum environmental impact (Cooling and Glenister, 1992). After sun drying, red mud desiccates in the field and is eventually hardened form cracks (Fig 2.1 a & b). The white soda flakes (Fig 2.1 a and b) also appear on the outer surface of the dried red mud, which is blown by the winds, and create environmental hazards.

Red mud vs. natural alkaline habitat

Soda lakes and deserts represent major types of most stable naturally occurring alkaline environments with pH values generally higher than 10 and occasionally reaching 12 (Jones *et al.*, 1994, 1998). The alkaline conditions in soda lakes were, for many years, known to be derived from the presence of unusually high levels of sodium carbonate. The formation of these environments is dependent on a group of conditions that include the topology and the climatic characteristics of a particular location as well as the geological composition of the terrain. The alkalinity is due to a shift in the $\text{CO}_2/\text{HCO}_3^-/\text{CO}_3^{2-}$ equilibrium towards a predominance of CO_3^{2-} that in the absence of Ca^{2+} or Mg^{2+} can remain in solution (in the presence of those ions, carbonate will precipitate as CaCO_3 or MgCO_3). Therefore, these lakes are characterized by high concentrations of sodium

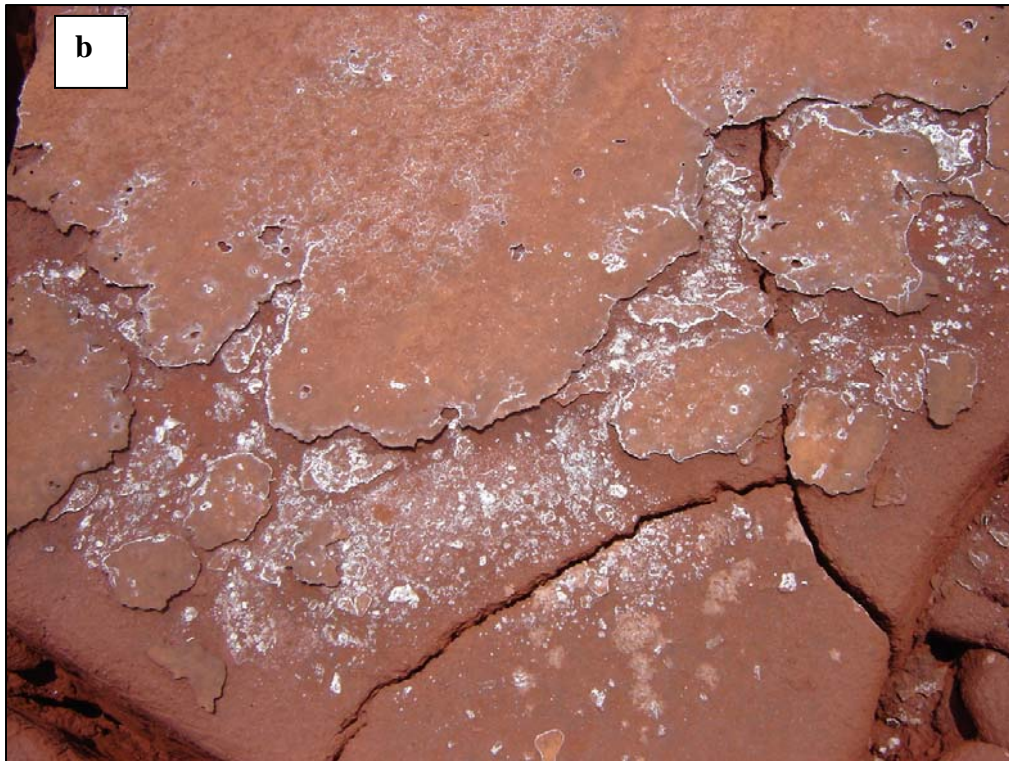


Fig 2.1 (a and b): Deposition of soda flakes, which appears white on the surface of dried red mud pond at NALCO, Damanjodi, Orissa (India)

carbonate and also represent saline, and sometimes hypersaline, environments due to the concomitant increase in the Cl⁻ concentration. The conditions necessary for the formation of a soda lake have much in common with those responsible for the generation of an athalassohaline (i.e., not derived from sea water) salt lake (Jones *et al.*, 1994, 1998; Tiago *et al.*, 2004). However, heavy metal analyses of the soda lakes are very few and the main focus remained to be the alkalinity of the system. Few reports pertaining to the heavy metal content of soda lake are as Mn 850, Pb 20, Cu 50, Zn 90, Ni 80, Cd 0.2, Cr 100, Fe 4.7(μg/g) (Krauskopf, 1979). Soda lakes situated at different locations have slightly differing concentration of metal. Iron concentrations were found to be highest in all soda lakes studied (Krauskopf, 1979).

Red mud differs from natural soda lakes in chemical, biological composition and origin. The chemistry of the red mud is basically dependent on types of ore used for the extraction of the alumina as well as the disposal method. The important parameters that have significance in its physicochemical properties are 1) moisture content, 2) rheology, 3) surface area, 4) particle size, 5) mineralogy, 6) contents of valuable metals, 7) presence of rare earth metals, and 8) presence of toxic substances, etc. (Paramguru *et al.*, 2005).

Bacterial diversity analysis

The historic way to characterize bacteria is to describe quantitatively as many phenotypic properties as possible, such as morphology, structure, cultivation, nutrition, biochemical metabolism, pathogenicity, antigenic properties, and ecology. Phenotypic similarities do not necessarily indicate phylogenetic relationships (relationship based on the ancestry of organisms) (Gillis and de Leg, 1992). In contrast to animals and plants, the morphology of microorganisms is in general too simple to serve as a basis for a sound classification and to allow for reliable identification. Thus, until very recently, microbial identification required the isolation of pure cultures (or defined cocultures) followed by testing for multiple physiological and biochemical traits (Amann *et al.*, 1995). Cultural methods will reveal only those physiological and nutritional types compatible with the cultural environment. The potential limitations of this approach are widely acknowledged and accepted (Torsvik *et al.*, 1996).

Molecular approach for diversity analysis

Staley and Konopka (1985) coined the term “great plate count anomaly” to describe this phenomenon, which has been known to microbiology for generations. By now, there is little doubt that in most cases, the majorities of microscopically visualized cells are viable but do not form visible colonies on plates. Two different types of cells contribute to this silent but active majority: 1) known species for which the applied cultivation conditions are just not suitable or which have entered a nonculturable state and 2) unknown species that have never been cultured before due to lack of suitable methods. It has been well documented for pathogens like *Salmonella enteritidis*, *Vibrio cholerae* and *V. vulnificus* that bacteria may quickly enter a nonculturable state upon exposure to salt, water, or low temperature (Amann *et al.*, 1995).

In order to sort out the problems associated due to cultivation based approach, genotypic methods are applied which basically depends upon the information derived from the nucleic acid (DNA and RNA). It is of primary interest to understand at which level various methods carry information and to realize the amount of time and work required. Obviously, genotypic methods presently dominate modern taxonomic studies.

Ribosomal RNA (rRNA)

Since protein synthesis is a very ancient process and present in all living cells, rRNA molecules are excellent targets to estimate evolutionary relationships. In 1965, Zuckerkandl and Pauling proposed that macromolecules such as nucleic acids and proteins could serve as evolutionary chronometers. The sequence of the units should contain information about the evolutionary distances among the organisms, including those of bacteria (Akkermans *et al.*, 1998). Analysis of 16S rRNA genes is now widely used for analysis of bacterial populations. The macromolecules that is most suitable for this would require the following prerequisites: 1) generally present, 2) functionally homologous in all organisms and 3) the sequence in the molecule should equally change as wide the evolutionary distance lower the mutation rate in the sequence.

Ribosomal RNA is proposed as one of the best candidate and it has been used by Woese *et al.*, (1990) for his studies on bacterial evolution. Major properties of rRNA are: 1) these are old molecules present in the ribosomes, 2) they are functionally constant, 3)

have a wide distribution, 4) are well conserved over large phylogenetic distances, 5) they occur in large number in cells (10^4 - 10^5 /cells), 6) in bacteria three types of rRNA molecules are present with different chain length and sedimentation rate(s): 5S rRNA (about 120 nucleotides), 16S rRNA (~ 1600 nucleotides) and 23S rRNA (~3000 nucleotides). The 5S molecule is too small and only suitable to distinguish major phylogenetic groups. 23S rRNA is excellent for phylogenetic studies, but so far few studies are available while 16S rRNA has been given most attention.

Microbial diversity of alkaline environments

The phylogenetic diversity of bacteria and archaea has been investigated, and variations in the bacterial community composition with the depth of a soda lake and other alkaline environments were analyzed (Duckworth *et al.*, 1996; Tiago *et al.*, 2004). But, it is the soda lakes, which has been studied more systematically to know the cultivable and uncultured bacteria. Microbial studies pertaining to the artificial alkaline environments such as bauxite residues are very scarce. Three novel alkaliphilic bacteria were isolated and characterized following enrichment at pH 10 using red mud tailing pond as source material (Agnew *et al.*, 1995). Low level of injured bacterial cells in the bauxite residue was isolated by Hamdy and Williams (2001), but they did not perform identification of these microbes. Except *Bacillus* sp., which was isolated by Agnew *et al.* (1995), no other species were reported for the bauxite residue sites. Bacterial diversity of red mud will give valuable information about the predominant bacteria, which could possibly be used to reduce the alkalinity and reclaim the red mud ponds and for other biotechnological applications.

Alkaliphiles are not confined exclusively to soda lakes but may be found in almost any environment, even in environments where the overall pH may not be particularly alkaline (Grant *et al.*, 1990). For instance, many alkaliphilic or alkali-tolerant members of the “Bacilli” are fairly ubiquitous. However, it seems probable that some organisms are unique to soda lakes, especially the haloalkaliphiles found in the hypersaline lakes such as Lake Magadi (Jones *et al.*, 1998). Although present-day soda lakes are geologically quite recent, they are supposed to be habitats of relict microbial

communities and are regarded as possible centers of the origin of microbial diversity (Zavarzin *et al.*, 1999).

Microbial communities in soda lakes have recently attracted attention as potential sources of industrially important enzymes (Horikoshi, 1996) and as a new perspective on microbial diversity (Duckworth *et al.*, 1996; Jones *et al.*, 1998; Zavarzin *et al.*, 1999). However, the diversity of haloalkaliphiles, particularly those inhabiting the hypersaline soda lakes, has not yet been adequately explored and most attention has been paid to a few separate groups of microorganisms. The major obstacle in understanding the soda lake microbial communities may be the ability to culture the organisms (Jones *et al.*, 1998). In this regard, the application of molecular techniques could provide new insights into the microbial composition of the soda lake environments, and the information revealed could serve as starting-point for the development of new cultivation techniques for yet uncultivated microorganisms (Jones *et al.*, 1998).

Tiago *et al.* (2004) estimated the bacterial diversity of non-saline alkaline environment. Heterotrophic populations were isolated and characterized from an alkaline groundwater environment generated by active serpentinization, which results in a Ca(OH)₂-enriched, extremely diluted groundwater with pH 11.4. One hundred eighty-five strains were isolated in different media at different pH values during two sampling periods. 38 different populations were identified and characterized. The majority of the isolates was gram positive with high G+C contents and was affiliated with three distinct groups; namely, strains closely related to the species *Dietzia natrolimnae* (32% of the isolates), to *Frigoribacterium/Clavibacter* lineages (29% of the isolates), and to the type strain of *Microbacterium kitamiense* (20% of the isolates). Other isolates were phylogenetically related to strains of the genera *Agrococcus*, *Leifsonia*, *Kytococcus*, *Janibacter*, *Kocuria*, *Rothia*, *Nesterenkonia*, *Citrococcus*, *Micrococcus*, *Actinomyces*, *Rhodococcus*, *Bacillus*, and *Staphylococcus*. Only five isolates were gram negative: one was related to the *Sphingobacteria* lineage and the other four were related to the *Proteobacteria* lineage. Despite the pH of the environment, the vast majority of the populations were alkali tolerant, and only two strains were able to grow at pH 11.

Remediation of bauxite residue

Bauxite residue is generated in very large quantities in Australia, producing up to 60 million tonnes of residue each year (Nguyen and Boger, 1998). The current method of residue management in Western Australia is known as the dry stacking disposal techniques. This technique first requires the finer fraction of residue (red mud) to be separated from its coarser fraction (red sand) (Nguyen and Boger, 1998). Due to its higher hydraulic conductivity (Wong and Ho, 1993) and thus ease of caustic removal by washing, red sand poses less of an environmental threat than red mud, and can be safely disposed. In contrast, red mud has a very low hydraulic conductivity and is saturated with caustic (Wong and Ho, 1994). After separation from red sand, the red mud is de-watered and dry stacked in red mud disposal areas (RDA) that are lined with impervious material (Hudson, 1987). Dry stacking involves discharging and spreading the red mud in layers and allowing each layer to dry before adding another layer (Chandler, 1987; Pohland and Tielens, 1987; Robinsky, 1987). This greatly reduces the volume of mud in the disposal space. In addition, gravity drain at the bottom of the RDAs enables continuous recovery of caustic, which in turn reduces the risk of caustic leakage and subsequent impacts on the environment.

At Alcoa World Alumina, Australia, filled RDAs are topped with red sand and amended with gypsum and sewage sludge (Glenister, 1992). This form of surface treatment has been shown to improve the structure of residue, and decrease its alkalinity and sodicity to levels that allow the establishment of alkaline tolerant plants (Fuller *et al.*, 1982; Ward 1986; Glenister 1987; Ho 1987; Wong 1990; Wong and Ho 1993). Although surface remediation helps to reduce erosion problem and may allow productive use of land, it does not alter the alkaline, saline and sodic nature of red mud deeper in profile. Continuous leaching of caustic and sodium from the red mud profile requires diligent maintenance of RDA linings to prevent ground water pollution. Refineries are thus faced with long term environmental responsibilities and liabilities that may extend beyond residue production (Valarie, 1999).

Paffenhoefer (1972) studied rehabilitation program of mined out bauxite areas and red mud pond surfaces at Gove, Northern Territory, Australia. The authors discussed the details of attempted vegetation on the pond surfaces. Attempts were made to have a self-

supporting vegetation cover that would survive on the surface of red mud ponds without any maintenance and under natural climactic conditions. Analysis of lateritic soil and red mud had shown nutrient deficiency particularly in nitrogen, phosphorus, potassium and zinc. It was concluded that the efforts succeeded in getting the desired species of grass/legume/tree to cover red mud ponds. A few species were marked for this purpose. These vegetation species were *Chloris gayana*, *Stylosanthes humilis*, *Cynodon dactylon*, *Acacia leptocarpa*, *Sporobolus virginis*, *Dolichos lab lab* and *Calapogonium mucunoides* which tolerated highly alkaline soil conditions and could grow into a stable and maintenance free vegetation over red mud ponds.

Several papers were published on reuse of the red mud. For example, red mud can be used as acidic amender or bottom sealant in disposal sites construction (Browner, 1995). It is used as catalyst in hydrodechlorination of tetrachloroethylene (Ordonez *et al.*, 2001). It is also used for iron and titanium oxides extraction (Parek and Goldberger, 1976), for the production of ceramic glazes (Wagh and Douse, 1991). It is also used for building material production as an additive to cement; for producing a coloring agent for paint works for ground floors of industrial and other buildings. It has use in producing of toned paper in the wood pulp and paper industry; producing iron ore sinter and pellets in the ferrous metallurgy; for improving the soil structure and as a micro fertilizer and a neutralizer of pesticides in agriculture; and extracting rare-earth metals and alumo-ferric coagulants as a technogenic raw material (Kovalenko, 1998).

Boriosco *et al.* (1982) found another way to cope up with pollution problems of red mud by taking seawater as a reactant. In the process counter current washing of sodium carbonate in red mud was carried out till its concentration fell down to 4-5 kg Na₂CO₃ per tonne of dry red mud. This red mud was mixed up with seawater to neutralize residual Na₂CO₃ by bringing pH to 9.5. This neutral material was disposed off to a large basin to settle down. Reaction of bauxite residue with seawater results in neutralization of alkalinity through precipitation of Mg-, Ca-, and Al-hydroxide and carbonate minerals. Reaction with seawater produced a residue pH of 8 to 8.5 (Menzies *et al.*, 2004).

Several workers used red mud as a soil amendment to increase the growth of plants. Summers *et al.* (1996) reported the phosphorus retention and leachates from

sandy soil amended with bauxite residue. Application of red mud to very sandy soil increased the yield and phosphorus response in subterranean clover (Summers *et al.*, 2001). Red mud applied at 80t/ha reduces by about 70% the amount of phosphorus lost in water drainage from sandy sites (Summers *et al.*, 1993). Ward and Summers (1993) modified the sandy soil with the fine residue from bauxite refining to retain phosphorus and increase plant yield. Eastham *et al.* (2006) reported a field evaluation of different forms of inorganic fertilizer, to assess their effectiveness as alternatives to poultry manure for supplying nutrients to dust control crops. They compared plant growth and nutrient uptake under different forms of nitrogen (N) and phosphorus (P) fertilizers with additional potassium (K) and trace elements on bauxite residue. They have also reported that diammonium phosphate (DAP) fertilizer blend could be used as an effective replacement for poultry manure for growing dust control crops on bauxite residue sand.

Past efforts to establish vegetation on red mud lakes have frequently involved chemical neutralization. Adequate growth of *Bermuda grass* is possible when sufficient gypsum was added to lower the pH to 8.3 for fine texture red mud. This amount was 34 tons/hectare, which is an expensive amelioration process. It has been found that fly ash and coarse texture red mud can be mixed for treatment in the ratio 20:80. After reducing the pH to 7-8 and after adding NPK fertilizer it becomes suitable for growth of Rhodes grass. It has been found that addition of organic amendments to red mud like paper pulp waste did not enhance the growth of Bermuda grass unless it is treated with gypsum. Attempts have also been made with glucose and potassium acid phosphate in red mud for the reduction of alkalinity (Regional Research Laboratory, Bhuvneshwar, 1996).

Courtney and Timpson (2005) investigated the use of mixing coarse fraction residue with fine fraction residue, at two different application rates (10% and 25%), with and without the use of gypsum as an ameliorant, for re-vegetation of the residue with *Trifolium pratense*. Optimum plant growth was observed in treatments that had also received gypsum amendment, with higher plant biomass, Mn nutrition and lower Al and Fe concentration. However, use of process sand at the higher application rate (25%) promoted lower levels of soluble Al and Fe and exchangeable Na in the substrate and, consequently, lower plant uptake of Na. Results indicate that co-disposal of the coarse fraction sand at 25% (w/w) with fine fraction residue improved the substrate and,

therefore, plant uptake and growth. They suggested that further monitoring is recommended to determine the effect of the absence of gypsum and other nutrient sources on plant growth.

Courtney and Timpson (2004) examined the chemical and physical amendment of fine fraction residue (red mud) at the Aughinish Alumina Ltd. Bayer Plant, Ireland followed by a two-year field investigation. Gypsum and sewage sludge were incorporated into the residue and amended mud was sown with *Lolium perenne* and *Holcus lanatus*. The amended substrate was low in manganese and magnesium. After first year, it contained adequate calcium levels, but there were deficiencies for nitrogen, manganese, potassium and magnesium. Sodium levels were not considered excessive and levels declined further in second year. Levels for nitrogen, calcium, manganese, magnesium, phosphorous and potassium were also reduced in the second year. As levels were already deficient in year one, the further decreases suggest severe nutrient shortage in the residue substrate. They have suggested long-term success of revegetation of bauxite residue, even after gypsum and organic amendment; the deficiencies of nutrients in the substrate must be overcome.

Restoring biological fertility

Attempts were made to rehabilitate the residue deposits and bauxite mines with and without neutralization by establishing vegetation cover using indigenous plants that are able to survive the adverse conditions (Wong and Ho, 1994). Extreme pH (10-12 initially) and salinity accompanied by poor structure and water holding capacity and low microbial activity also contribute to the sub-optimal nutrient status of bauxite residue. Bauxite residue is ill characterized substrate for plant and microbial growth and nutrient availability (Wong and Ho, 1993; 1994). The cycling of nutrients in soil and their acquisition by plants is mediated through the soil microbial population. Restoring this biological component of soil fertility should be an important aim of red mud rehabilitation. This has two aspects; firstly, managing top soils to optimize the survival of beneficial microorganisms, soil animals and seeds. Secondly, if topsoil is unavailable or severely degraded, then inoculation with key symbiotic microorganisms may be warranted (Wong and Ho, 1993; 1994).

Bioremediation offers a viable alternative to the regular use of physico-chemical methods of decontamination, which are not generally cost effective. Various factors such as specific microbial population, bioavailability of contaminants, soil type, temperature, pH, nutrients, and presence of oxygen or other electron acceptors influence the bioremediation process. Although bioremediation may not completely detoxify inorganic pollutant metals and radionuclides, yet it can alter the oxidation state, aiding in adsorption, uptake, accumulation, and concentration in micro- or macroorganisms. A good bioremediation approach will involve strategic use of all native microbes in an engineered way to achieve the best possible detoxification levels. In summary, bioremediation technologies have been successfully employed in the field and are gaining more and more importance with increased acceptance of ecofriendly remediation solutions.

Williams and Hamdy (1982) introduced biological activities in bauxite residue in order to neutralize its extra alkali. So in an envisaged programme, alfa hay was mixed with red mud, which stimulated bacterial growth resulting in production of organic acid within the system. It was observed that this acid neutralized excess alkali of red mud and made it amenable to earthworm and plant growth. Hamdy and Williams (2001) demonstrated that low levels of injured bacterial cells in the bauxite residue actively grew using various added nutrients and/or hay. The organisms grew from less than 10^2 to more than 10^9 cells g^{-1} bauxite residue and formed organic acids that lowered the pH from 13 to about 7.0. A total of 150 cultures were isolated from treated bauxite residue and included species of *Bacillus*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Flavobacterium* and *Enterobacter*. They have demonstrated by using scanning electron micrographs that untreated particles (control) of the bauxite residue were clumped together, and in treated bauxite residue these particles were highly dispersed with microcolonial structures. Furthermore, the treated bauxite residue supported the growth of several plants and earthworms that survived for over 300 days. In a test plot, bioremediation on a residue deposit at Alcoa Point Comfort, TX, the Bermuda grass hay used was effective mulch material and encouraged water filtration, leading to establishment and growth of salt –tolerant vegetative species.

Valerie (1999) investigated the microbiology of red mud and ascertained the possibility of using bacteria to reduce the alkalinity of red mud. To achieve this, bacteria were isolated from the red mud sample. Characteristics of these indigenous bacteria were investigated, including their ability to produce acidic substances in alkaline glucose medium. Finally, the ability of these acid producing bacteria to reduce the alkalinity of glucose amended red mud was determined using a series of pot trials. She showed that the properties of red mud had an influence on the types of bacteria present. Different types of bacteria were isolated from red mud. These bacteria have varying degrees of tolerance to high alkalinity and salinity. These were used as ameliorant in red mud in presence of added nutrients to encourage its growth. It was revealed that oxygen was necessary for pH reduction under the chosen conditions, and that the pH reduction was related to bacterial growth.

Rebuilding a quality soil is a challenge - equally important is proving that it has been achieved to the satisfaction of governments and the community. Consequently, developing and validating indicators of restoration success is currently of major interest (Tacey and Treloar, 1994). Ecosystem development is a long-term process; therefore early indicators of recovery, which can be used to predict long-term sustainability, are essential. For long-term success of revegetation of bauxite residue, even after gypsum and organic amendment, the deficiencies of nutrients in the substrate must be overcome (Courtney and Timpson, 2004).

Chapter 3

Materials and methods

Sample collection

Red mud samples were collected randomly from the red mud pond impoundment of National Aluminium Company Limited (NALCO), Damanjodi, Orissa (India) situated at 18°46'48"N and 82°53'36"E. NALCO is the largest alumina producers in Asia. The upper flakes of soda were removed and the samples were collected from 0-30 cm of depth by alcohol sterilized implements and sterile containers. The samples were stored at 4 °C until their use.

Isolation and cultivation of bacterial species

For isolation and enumeration of cultivable bacteria, red mud samples were diluted in saline (0.85%) and plated on alkaline nutrient agar, alkaline tryptic soya agar (Appendix I) and alkaliphilic Horikoshi agar medium and the plates were incubated at 37 °C. The grown bacterial colony from the plate was subcultured several times on the same medium from where it was picked.

Morphological and biochemical studies of red mud isolates

Gram staining

Bacterial smear from actively growing cells were spread on a glass slide and heat fixed. Filtered crystal violet was flooded for 10 sec. Briefly, washed in water to remove excess crystal violet. Gram's iodine was flooded for 10 sec and washed briefly in water. Further, it was decolourised with acetone until the moving dye front has passed the lower edge of the section and washed immediately in tap water. Safranin was used to counter stain for 15 sec and washed with water to remove the excessive stain. The slides were visualized under microscope at different magnifications.

Catalase test

Small amount of bacterial cells were placed onto a clean microscope slide. A few drops of H₂O₂ (3%) was added onto the smear. A positive result was the rapid evolution

of O₂ as evidenced by bubbling. A negative result was no bubbles or only a few scattered bubbles.

Oxidase test

Small amount of bacteria from an agar slant or plate was obtained with a sterile swab. One drop of N,N,N',N'-tetramethyl phenylenediamine dihydrochloride reagent was placed onto the culture on the swab. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

Nitrate reduction test

Nitrate media (Appendix I) was used to determine the ability of an organism to reduce nitrate (NO₃) to nitrite (NO₂) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate broth contained potassium nitrate as a source of nitrate. Bacteria were grown in nitrate agar plate. After sufficient growth of bacteria, 2-3 drops of sulfanilic acid and α -naphthylamine were added. If the organism had reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid was added; which reacted with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α -naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

Alkalinity, salinity and temperature test

To determine the growth of the bacterial cells in alkaline condition, different buffers were used to adjust the pH of media. All buffer solutions were prepared and sterilized separately and added to the media to a final concentration of 100 mM. Different buffers such as phosphate buffer, Tris buffer and carbonate-bicarbonate buffer (Appendix I) were used to adjust the pH at 7, 8 and 9 respectively. Carbonate-bicarbonate buffer was used to adjust the pH at 10.5 also, while sodium hydroxide alone was used to adjust the pH at 11.3.

Different sodium chloride concentrations (0, 2, 5, 7, 10, 12 and 15%) were amended in the alkaline nutrient broth (pH 10) to determine the survival of the bacterial isolates in saline conditions. Growth of bacterial isolates was recorded by measuring the absorbance at 600 nm of 24 hr grown cells. Four temperatures (25, 30, 37 and 45 °C)

were chosen to test the growth pattern of bacterial isolates. Bacterial isolates were grown in liquid and solid media and incubated at different temperatures as stated above.

Biolog test

The isolated strains were characterized by determining their substrate utilization pattern with Biolog GN/GP plates (Biolog Inc., USA). For determination, the bacterial cells were grown on alkaline nutrient agar media (pH 10) in four quadrants. Bacterial cells were harvested from fourth quadrant by using sterile swab and dissolved in inoculation fluid provided by the manufacturer. The Biolog GN/GP plates were inoculated by 150 μ l of the cell suspension that was adjusted to a density of $\sim 3.0 \times 10^6$ cells per mL by comparison with the turbidity standard supplied by the manufacturer. These plates were inoculated at 37 °C for 24 hr, omitting the 4 hr measurement. The color development in the micro plate wells was interpreted as positive, negative and borderline, in case it is not possible to differentiate positive from negative. The reading was also entered in the Biolog microlog GN/GP release 14.01/b databases to provide identification.

Fermentation of carbon substrates and biochemical tests of red mud isolates

A total of 35-carbohydrate fermentation tests were performed with isolated bacterial species according to the manufacturer's direction (Himedia Lab., Bombay, India). Inocula were prepared by growing the cells in alkaline nutrient broth (pH 10.5) at 37 °C in shaking condition until the inoculum turbidity was ≥ 0.5 OD at 620nm. Citrate utilization, lysine, ornithine and TDA tests were performed with all bacterial isolates of red mud by standard methods.

Sample preparation and organic acid estimation

One milliliter of the culture supernatant was pipetted into a screw-capped culture tube. The following reagents were added: 0.2 mL of 18 N H₂SO₄, 0.6 g of NaCl, 5 mL of diethyl ether, and 25 μ l of acetonitrile; the mixture was blended with a Vortex mixer for 1 min to mix the ether and aqueous phases. The tube was centrifuged at 2,500 rpm for 1 min to remove any dispersed aqueous droplets from the ether phase. The ether layer was transferred to another tube with a Pasteur pipette, and approximately 2 mm of ether was left at the solvent interface to ensure that none of the aqueous phase was transferred. To

the ether, 0.2 mL of 0.1 N NaOH was added, and the tube was gently shaken. With a Pasteur pipette, a small portion of the lower NaOH phase was removed and tested with pH paper. If necessary, 1 N NaOH was added, 1 drop at a time, to obtain a pH of 9.0 or greater. The tube was blended with a Vortex mixer for 1 min and centrifuged, and the ether phase was removed and discarded. Twenty-five microliters of acetonitrile was then added, and the tube was left uncapped for about 5 min to allow residual ether to evaporate. The tube was blended with a Vortex mixer to mix the contents, and 20 µl of sample was introduced into the chromatograph for analysis. Uninoculated medium was processed and analyzed in the same manner to determine the acids present. The concentration of acids produced by the bacteria was obtained by subtracting the concentration found in the uninoculated medium.

HPLC equipment for organic acid determination

The HPLC equipment was series 200 of Perkin Elmer, USA, equipped with Polypore-H column (Brownlee Column, Perkin Elmer, USA) and a Micro-Guard column (Perkin Elmer, USA). A 10µl bypass-sampling loop was used, and the diode array detector was adjusted to a wavelength of 210 nm and at a flow rate of 0.3 mL/min was maintained. Ambient temperature was used, and the flow rate was 0.3 mL/min, using column eluent, composed of 0.008 N sulphuric acid (HPLC grade). During chromatographic separations, the eluents were degassed with helium. Data were captured, amplified and analyzed using nuchrom software (Perkin Elmer, USA). A mixture of oxalic, citric, malic, fumaric, acetic and succinic acids (Bio-Rad Laboratories, USA) was used as a standard.

Antibiotic profiling of bacterial isolates

Bacterial isolates were grown in nutrient broth until the absorbance reached to 1.0. The grown bacterial cells were spread on nutrient agar and antibiotic discs were kept on it. These plates were incubated at 37 °C and the inhibition zone was noted. Ready precoated twenty antibiotic discs (Himedia Lab., India) were used to check the sensitivity of the bacterial isolates. These were: Norfloxacin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), Cefuroxime (30 µg), Ciprofloxacin (5 µg), Cefaperazone (75 µg), Ceftazidime (30 µg), Roxithromycin (30 µg), Calaritomycin (15 µg), Co-Trimoxazole (25 µg), Netillin (30 µg), Cefaclor (30 µg), Cephotaxime (30 µg),

Cephadroxil (30 µg), Azithromycin (15 µg), Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units), Amikacin (30 µg), Sparfloxacin (5 µg) and Ampicillin/sublactam (10/10 µg).

Molecular methods

Extraction of DNA

Several protocols have been developed and described for the preparation of genomic DNA from microorganisms. Most based upon the methods of Kirby (1957) and Marmur (1961), which generally comprise:

- a) Chemical cell disruption by enzymic digestion and detergent lysis;
- b) Extractions with organic solvents and
- c) Selective recovery of the DNA.

The method was optimized specifically for the application of isolated DNA in the polymerase chain reaction, to amplify 16S rRNA genes.

Isolation of genomic DNA from red mud isolates

A single colony of red mud bacterial isolate was picked from a freshly grown plate and transferred it into 20 mL of alkaline Nutrient Broth in a 250 mL of flask. The culture was incubated for 16-24 hr at 37 °C with vigorous shaking (120 cycles/minute in a rotary shaker). Cells were harvested in 2.0 mL sterile microfuge tube. Media was decanted from the microfuge tubes and were kept in an inverted position for 1 min to allow the last traces of media to drain away (The cell pellet may be washed here using 10 mM Tris-HCl pH 8.0). Each cell pellet was resuspended in 0.8 mL saline-EDTA buffer thoroughly. Freshly prepared lysozyme solution (50 µl of 10 mg/ml) was added, mixed well and incubated at 37 °C for 20 min. SDS (0.2 mL of 10%) was added, mixed well by inversion, and incubated in the water bath at 60 °C for 15 min. The above mixture was extracted with phenol: chloroform: isoamyl alcohol (25:24:1). Upper aqueous phase was transferred to the sterile microfuge tube. Equal volume of isopropanol was added to precipitate DNA. After centrifugation, the supernatant was discarded and the pellet was air dried. DNA was dissolved in 50 µl TE buffer. DNase free RNase solution was added to a final concentration of 40 µg/mL and incubated at 37 °C for 30 min, with occasional shaking. This was extracted with equal volume of phenol: chloroform: isoamyl alcohol

(25:24:1). DNA was precipitated from the upper aqueous layer with 2 volumes of ethanol. It was centrifuged and the pellet was redissolved in 50 μ l Tris-EDTA (pH 8.0) and stored at 4 °C for further use.

Electrophoresis of DNA on agarose gels

DNA was loaded on agarose gels prepared in 0.5x TBE buffer, pH 8.0 (appendix I) using a 6x loading dye (Appendix I). Agarose concentration was 0.7% for genomic DNA (w/v). Ethidium bromide (0.5 μ g/mL) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 50 volts for 45-60 min and visualized on a U.V. transilluminator.

DNA Purification

The DNA was purified by elution through the Wizard DNA Clean up system (Promega Inc., USA) according to manufacturer's instructions in order to remove contaminants, which can hamper in manipulation of DNA.

Spectrophotometric quantification of DNA

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260} . For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 μ g/mL. The quality of the DNA was evaluated by measurement of the A_{260} and A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0. Ratios less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

Ethidium bromide fluorescent DNA quantification

DNA was checked by agarose gel containing ethidium bromide (0.5 μ g/mL) electrophoresis. The quantity of DNA was visually determined with reference to a known DNA concentration of lambda phage (MBI Fermentas, USA) by comparing the intensity of fluorescence. The molecule is linear double stranded undigested DNA.

REP PCR based DNA fingerprinting

The reaction mixtures for the REP PCR contained 1x PCR buffer (Invitrogen, USA), each deoxynucleotide triphosphate at a concentration of 200 μ M, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 μ M and 2.5U of Taq DNA polymerase in a final volume of 100 μ l. DNA amplification was performed with Genamp PCR system

(Applied Biosystem, USA) by using the following program; initial denaturation 95 °C for 2 min, 35 cycles of 92 °C for 30 sec, 38 °C for 80 sec and 60 °C for 200 sec, final extension at 68 °C for 8 min and final soak at 4 °C. The REP PCR primers were REP F 5' - III ICG ICG ICA TCI GGC -3' and REP R 5'-ICG ICT TAT CIG GCC TAC-3'.

Amplification of 16S rDNA and Purification of PCR products

The forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTTC-3' (Weisberg *et al.*, 1991) were used for the amplification of 1.5 kb gene of 16S rRNA. Variable region 3 was amplified using forward primer 5'-GACTCCTACGGGAGGCAGCAG-3' and reverse primer 5'-ATTACCGCGGCTGCTGG-3' for amplification of 250 bp.

DNA amplification was performed with the Genamp PCR system (Applied Biosystem, USA). The reaction mixture for the PCR contained 1x PCR buffer, each dNTPs at a concentration of 200 µM, 1.5 mM MgCl₂, each primer at a concentration of 0.1 µM and 2.5U of Taq DNA polymerase in a final volume of 100 µl. PCR conditions used were same for amplification of 1.5 kb and 250 bp fragment of 16S rDNA which was as follows: Preheating at 92 °C for 2 min, 36 cycles of 92 °C for 1 min, 48 °C for 30 sec and 72 °C for 2 min and final extension of 72 °C for 6 min 10 sec. Amplified DNA was verified by electrophoresis of aliquots of PCR products (5 µl) on a 1.0% agarose gel in 0.5% TBE buffer. 16S rDNA amplicon was gel eluted using QIAquick columns (Qiagen Inc., USA).

PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning. The DNA fragment was excised from the gel, using the QIAquick gel extraction kit (Qiagen Inc., USA) as per manufacturer's direction. Purified PCR products were eluted with 40 µl TE buffer (pH 8.0).

Ligation in T-vectors

The 16S rDNA amplicon was ligated into pTZ57R/T (Fermentas Inc., USA) or pGEM-Teasy (Promega Inc., USA) vector. For ligation of amplicon in pTZ57R/T vector, the final reaction volume was kept 30µl (Appendix I) and incubated at 22 °C for 12 hr. The final reaction volume for pGEM-Teasy vector was kept at 10µl (Appendix I) and incubated at 4 °C for 12 hr for ligation. The reaction mixture was kept overnight and analyzed on 0.7% agarose gel.

Genetic Transformation of *E. coli*

A single colony of *E. coli* DH5 α was picked from a freshly grown plate and transferred into 20 mL of LB broth in a 250 mL flask. The culture was incubated for 16-20 hr at 37 °C with vigorous shaking (200-250 cycles/min in a rotary shaker). 200 μ l of the above culture was aseptically transferred into 20 mL of fresh LB broth in a 250 mL flask. The culture was incubated with vigorous shaking at 37 °C for 2-3 hr. To monitor the growth of the culture, determined the OD₅₉₀ every one-hour (The OD₅₉₀ should be ~ 0.5). The above culture was transferred to sterile, disposable, ice-cold 50 mL polypropylene tubes. The culture was incubated on ice for 10 min. The cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. The tubes were kept in an inverted position for 1 min to allow the last traces of media to drain away. The pellet was resuspended in 10 mL of ice-cold 0.1 M CaCl₂ and stored on ice for 10-15 min. Cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. The cell pellet was resuspended in 1 mL of ice-cold 0.1 M CaCl₂. CaCl₂ treatment for 2 hr induces considerably a transient state of “competence” in the *E. coli* cells. A 100 μ l of the suspension of competent cells was transferred to a sterile and prechilled microfuge tube (1.5 mL capacity). Plasmid DNA sample (~100 ng in a volume of 5 μ l or less) was added to each tube and mixed the content of the tubes gently. The tubes were incubated on ice for 30 min. Then with the help of a floater, tubes were incubated in a circulating water bath that was preheated to 42 °C for exactly 2 min. The tubes were transferred immediately on an ice bath and chilled the cells for 1-2 min. LB broth (1 mL) was added to each tube and the cultures were incubated for 45-60 min at 37 °C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The cells (100 μ l) were plated on each 90 mm LB-antibiotic-X-GAL and IPTG plates. The plates were inverted and incubated at 37 °C overnight. Transformed colonies were appeared in 12-16 hr. Transformants were selected based on blue and white colonies.

Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5 α (LacZ-) bacterial host cells were plated on Luria Agar (Appendix I) medium containing 50 μ g/mL ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for

colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T or pGEM-T easy vector is located in the multiple cloning site (MCS) of the plasmid's *lacZα* gene; if insert was present, non-functional β-galactosidase is produced, and the transformed bacterial colony is white. White colonies were picked and grown in 2 mL LB containing ampicillin (50 μg/mL) and simultaneously patching of these cultures were done on LA containing ampicillin. Plasmid DNA was isolated (described in the proceeding section) and re-amplification of the insert was done using vector's promoter specific sequences.

Isolation and purification of plasmid DNA from bacteria by alkaline lysis method

A single transformed *E. coli* colony was transferred into 2 mL of LB medium containing appropriate antibiotic (ampicillin used in a final concentration of 50 μg/mL) in a capped 15-mL tube. The culture was incubated overnight at 37 °C with vigorous shaking. 1.5-2.0 mL of the above culture were poured into a microfuge tube. Centrifuged at 8000 rpm for 5 min. The medium was removed, leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 200 μL of ice-cold Solution I (Appendix 1) and mixed well by to ensure that the bacterial pellet was completely dispersed in this solution. 200 μL of freshly prepared Solution II (Appendix 1) was added and the tube was closed tightly and mixed the contents by gentle inversion of the tubes five to ten times. The tubes were incubated on ice for few min. A 300 μL of ice-cold Solution III (Appendix I) was added and mixed by inversion to disperse Solution III through the viscous bacterial lysate. The tube was stored on ice for 5-10 min and centrifuged at 12,000 rpm for 10 min at 4 °C in a microfuge. The supernatant was carefully transferred to a fresh tube. DNA was precipitated with equal volume of isopropanol. The mixture was allowed to stand at room temperature for 5-10 min and centrifuged at 10,000 rpm for 10 min at 4 °C in a microfuge. The pellet was air-dried and dissolved in 500 μL of TE buffer (pH 8.0). DNase free RNase solution was added to a final concentration of 20 μg/mL and incubate at 37 °C for 30 min, with occasional shaking. The solution was extracted once with equal volume of phenol: chloroform:isoamylalcohol (25:24:1). The upper aqueous layer was transferred to a fresh microfuge tube, and then added one-tenth volume of 3.0 M sodium acetate (pH 4.5) and 2 volumes of ethanol, mixed well and kept it at -20 °C for 1 hr. The tubes were centrifuged at 10,000 rpm for 20 min at 4 °C in a microfuge tube. The pellet was rinsed

with 1 mL of 70% ethanol at 4 °C. The pellet was allowed to dry in air for 5-10 min. The pellet was redissolved containing plasmid DNA in 50 µl of TE (pH 8.0) and stored at – 20 °C for further use.

Restriction analysis of DNA samples by agarose gel electrophoresis

DNA 17 µl (500 ng) was added in a sterile microfuge tube. The appropriate 10x restriction enzyme assay buffer was added and mixed thoroughly by tapping the tube. 1 µl (2-5 units) of the restriction enzyme was added, mixed by tapping the tube. The mixture was incubated at the 37 °C for 1-2 hr. To stop the reaction, 4-5 µl gel-loading buffer was added, mixed by vortexing briefly (as the DNA samples need to be analyzed directly on agarose gel).

Sequencing

The 16S rDNA inserts were sequenced for both strands using T7 and SP6 for pGEM-T easy vector and M13 forward and reverse for pTZ57R/T vectors. The sequence was generated by chain termination method using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

Extraction of DNA from red mud

Total DNA was extracted from red mud samples, according to the direct DNA extraction method described by Mau (1997). This protocol represents a modified version of the protocol of Wilson (1987), optimized for the extraction of genomic DNA from sediments. Apart from it, several other methods were also used for extraction of genomic DNA.

Method 1

One gram of red mud was added in eight, sterile, 2 mL eppendorf tubes. Firstly, the red mud aliquots were washed with 500 µl TE buffer (pH 8.0). These aliquots were resuspended with 560 µl TE buffer (pH 8.0) and approximately 10 µg crystalline lysozyme in each tube. The compounds were mixed thoroughly by inverting the eppendorf tubes several times during incubation, at 37 °C for approximately 60 min. The mixtures were incubated again, at 37 °C for approximately 60 min after addition of 6 µl Proteinase-K (10 mg/mL), and 30 µl SDS (10%), Subsequently, 100 µl NaCl (5M) were added, and the tubes were incubated for 2 min at 65 °C. After addition of 80 µl preheated

CTAB-solution (10% CTAB in 0.7 M NaCl), the tubes were incubated for another 10 min at 65 °C. Freeze thawing or bead beating method was used prior to solvent extraction as described under the heading “Common steps for extraction of DNA from red mud”.

Method 2

In a screw-capped microcentrifuge tubes, 0.5 g sieved red mud was added. To this, 0.5 mL of modified CTAB extraction buffer (equal volumes of 10% CTAB in 0.7 M NaCl with 240 mM potassium phosphate buffer (pH 8.0) was added and the tubes were vortexed briefly. Tubes were then incubated at 70 °C for 10 min in a water bath. Freeze thawing or bead-beating method was used prior to solvent extraction as described under the heading “Common steps for extraction of DNA from red mud”.

Method 3

Red mud samples (1 g) were mixed with 2 mL of 120 mM sodium phosphate buffer (pH 8.0) by shaking at 150 rpm for 15 min. The slurry was pelleted by centrifugation at 6,000 x g for 10 min. The pellet was washed again with phosphate buffer, resuspended in 2 mL of lysis solution (0.15 M NaCl, 0.1 M Na₂EDTA [pH 8.0]) containing 15 mg of lysozyme/mL, and incubated at 37 °C water bath for 2 hr with an agitation at 20 to 30 min intervals. Then 2 mL of 0.1 M NaCl 0.5M, Tris-HCl (pH 8.0), and 10% sodium dodecyl sulfate was added. Freeze thawing or bead-beating method was used prior to solvent extraction as described under the heading “Common steps for extraction of DNA from red mud”.

Method 4

Red mud sample (0.25 g) was ground with liquid nitrogen by using a mortar and pestle for about 5 min or until a fine powder obtained. The powdered soil was suspended in 0.5 mL of skim milk powder solution (0.1 g of milk powder in 25 mL of H₂O) by vigorous vortexing. The soil and debris are removed by centrifugation (12,000 rpm) at 4 °C for 10 min, and the supernatant was mixed with 2 mL of SDS extraction buffer (0.3% SDS in 0.14 M NaCl, 50 mM sodium acetate of pH 5.1) by vortexing. Freeze thawing or bead beating method was used prior to solvent extraction as described under the heading “Common steps for extraction of DNA from red mud”.

Method 5

DNA was extracted from MoBio soil extraction Kit as per manufacturer's direction (MoBio, USA). PowerMax™ Bead Solution tube was filled with 10 g of red mud sample and vortexed vigorously for 1 min. PowerMax™ Bead Solution tubes were placed in a shaking water bath set at 65 °C and shook at maximum speed for 30 min. Tubes were centrifuged at 2500 x g for 3 min at room temperature. Supernatant was transferred to a clean Collection Tube. 5 mL of solution C2 (provided by the manufacturer) were added and inverted twice to mix and incubated at 4°C for 10 min. It was centrifuged at 2500 x g for 4 min at room temperature. Avoiding pellet, supernatant was transferred to a clean Collection Tube (provided). 4 mL of Solution C3 (provided by the manufacturer) was added and inverted twice to mix to incubate at 4 °C for 10 min. The contents were centrifuged at 2500 x g for 4 min at room temperature and supernatant was transferred to a clean collection tube (provided). 30 mL of Solution C4 (provided by the manufacturer) was added to supernatant and invert twice. First, Spin Filter was filled with solution from C4. Centrifuged at 2500 x g for 2 min at room temperature. Flow through was discarded and added second volume of supernatant to same Spin Filter and centrifuged at 2500 x g for 2 min at room temperature. Flow through was discarded and repeated until entire volume was processed. Solution C5 10 (mL) (provided by the manufacturer) was added to spin filter and centrifuged at 2500 x g for 3 min at room temperature. Spin Filter was placed in a new Collection Tube (provided). A 5 mL of sterile Solution C6 was added to the center of spin filter membrane and centrifuge at 2500 x g for 3 min at room temperature. Spin Filter was discarded and DNA was collected.

Common steps for extraction of DNA from red mud

[Freeze-thaw cycles: Samples were frozen for 15 min at -80 °C and then immediately placed in a water bath (70 °C) to rapidly thaw the sample. This process was repeated three times.

Bead Beating: After incubation, 0.5 g each of 0.1 mm glass and 0.5 mm zircona/silica beads were added. Tubes were then shaken in the Fast Prep instrument (Qbiogene) at 2500 rpm for 30 sec for three times with intermittent cooling in ice.]

The upper aqueous phases were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and, finally, with an equal volume of

chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 0.3 volumes of ammonium acetate and 0.7 volumes of isopropanol to the aqueous phase, followed by 30 min centrifugation at 12,000 rpm. DNA pellets were resuspended in TE buffer and combined in 100 µl. DNA was precipitated again by adding 0.1 volumes 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol (absolute). The pellet was washed with 500 µl of 70% ethanol, and centrifuged another 10 min. After removing the ethanol, the pellet was dried briefly for 2-3 min and resuspended in a final volume of 40 µl 10 mM Tris buffer (pH 8.0).

Quality check and purification of the extracted DNA was performed as described in section 3.2.1.3 and 3.2.1.4. Amplification, ligation and transformation were performed for clone library preparation as described before.

Clone library preparation and screening

The DNA was purified with a Wizard column (Promega Inc., USA) and subjected to whole genome amplification (GenomiPhi DNA Amplification Kit, GE Healthcare, USA) as per manufacturer's direction. Amplification and cloning of 16S rDNA was done as described earlier. Screening of recombinant clones in clone library was done using M13F and M13R primers (Appendix I). The amplicon (1.5 kb) was digested with *RsaI*. Clones having different restriction patterns were selected for partial sequencing (as described above). The 16S rDNA gene sequences of clone library were checked in chimera check program of Ribosomal Database Project-II (RDP-II).

Analysis of sequence data

Sequences were analyzed by using CHECK-CHIMERA program of the RDP II (Maidak *et al.*, 2001), in order to detect the presence of possible chimeric artefacts generated by PCR. Similarities were calculated for nearly complete 16S rDNA sequences using only unambiguously determined nucleotide positions. The 16S rDNA gene sequences of red mud isolates and clone library were compared with those available in GenBank/ EMBL databases using BLAST program (Altschul *et al.*, 1997) and at RDP-II (Cole *et al.*, 2003). The sequences of closely related strains and uncultured bacteria were retrieved from RDP-II and aligned using multiple alignments CLUSTALW program (Thompson *et al.*, 1997). The evolutionary distance was calculated by Kimura 2 parameter and phylogenetic dendograms were constructed by neighbor-joining using

MEGA 4 software (Tamura *et al.*, 2007). Bootstrap values of 1500 replicates were performed to assess the statistical support for the tree.

Measurement of buffering capacities

The buffering capacity of the bacteria was measured by an acid pulse technique. Experiments were conducted in 10 mL glass vials with 7 mL samples of cell suspensions which were stirred magnetically at room temperature. The pH values of these suspensions were 7.0 and 10.0. Valinomycin (final concentration, 10 mM) was then added from small volumes of concentrated stock solutions in acetone; the final acetone concentrations did not exceed 0.2%. The cells were allowed to equilibrate for about 2 hr with intermittent mixing. Immediately before the assay, 0.23 mL of freshly prepared carbonic anhydrase (20 mg/mL in 300 mM KCl) was added. Vigorous mixing with a small magnetic flea was performed after insertion of the pH electrode. After 5 to 10 min, an acid pulse was added, usually as a 50 to 150 μ L portion of 100 mM HCl in 300 mM KCl, and changes in external pH were recorded for 3 min. When assays were performed, the pH was initially adjusted in the pre-incubation period of 2 hr. In these cases, 50 μ L aliquots of 100 mM HCl or 100 mM KOH (in 300 mM KCl) were added at intervals of about 20 min until the desired pH was attained. Because of the mixing artifact, pH changes during the first 15 sec were not used in these plots.

Measurement of glycolysis activity and zeta potential

To evaluate the pH dependency of red mud isolates, glycolysis activity of two red mud isolates, namely RM9P and RM8, were measured in the presence and absence of valinomycin at various pHs between 7.0 and 10.0. These two isolates were considered as representative isolates because of their growth and survival at pH 7 to 11.3. The cells were cultured until the exponential phase ($OD_{600} = 1.0$) in nutrient broth medium where pH was set at 7, 8, 9 and 10 with buffers at 100 mM concentration. The cells were harvested, and washed with the same buffer in which these were previously grown. The washed cell pellet was re-suspended in the same buffer and incubated for 2 hr at the same temperatures in which the cells were cultured to deplete their metabolic energy. The cells were washed once again with the same buffer and re-suspended in the same buffer. The

basal composition of the reaction mixture for the glycolysis reaction contained 150 mM potassium phosphate buffer, 1 mM MgSO₄·7H₂O, and one of the following buffers at 100 mM with the pH adjusted to the desired values, using KOH: MES (7.0), MOPS (pH 8.0), TAPS (pH 9.0), or TABS (pH 10.0) (Appendix I). Subsequently, the energy-depleted cells were added at an OD₆₀₀ of about 4.0, and then valinomycin in ethanol solution was added at a final concentration of 7 μM. The control series received 0.1% (vol/vol) ethanol. The reaction was started by the addition of glucose at a final concentration of 10 mM. The mixtures were incubated at 37 °C, for appropriate time periods. After being incubated, the mixtures were centrifuged at 12000 rpm for 5 min at 4 °C, and the supernatant was filtered through a 0.22 μm pore size membrane. The glucose concentrations of the filtered supernatants were measured, using the DNS method.

For zeta potential analysis, cells were resuspended in 1 mM KNO₃ at concentrations of about 5.3 x10⁸ per mL for RM9P and RM8. The pH was adjusted with KOH or HNO₃, with a new suspension made for each determination. The solutions were placed in the zeta potential analyzer Muteck SZP06 (BTG Mutek GmbH, Germany) and the streaming potential of the bacterial cells were determined.

After incubation in respective buffer, the supernatants were collected and 10 μl (filtered from 0.22 μm filter paper) of volume were injected in the HPLC and analyzed for organic acid exudation (as described in the previous section).

In vitro* studies of *Aspergillus tubingensis

Aspergillus tubingensis a good phosphate solubilizer was isolated from the *Eucalyptus* plantation (Reddy *et al.*, 2002) and tested for its ability to grow at different pHs, different concentrations of red mud amended media and tolerance to Na, Al and Fe.

pH tolerance of *A. tubingensis*

An actively growing mycelial disc (0.5 mm) of *A. tubingensis* was cut from the edge of the colony and was aseptically inoculated to Czapek-Dox modified liquid media (Appendix 1) having different pHs 2.5 to 12 in a 250 mL Erlenmeyer flasks. The flasks were incubated at 28 °C for 3 days. After 3 days of incubation, the mycelium was filtered and dried for 48 hr at 70 °C. The dry biomass and the pH of the culture filtrate were measured.

Metal tolerance of *A. tubingensis*

Aluminium (0, 100 and 200 µg/mL), Na (0, 100 and 200 µg/mL) and Fe (0, 100, 200, 300, 400, 500 and 600 µg/mL) in the form of $\text{AlSO}_4 \cdot 16\text{H}_2\text{O}$, Na_2SO_4 and FeCl_3 respectively were amended in the Czapek-Dox modified liquid media separately. The fungus was inoculated and incubated 28 °C under shaking conditions. After one week of growth, the mycelium was harvested and dried. The dried mycelia were digested with HNO_3 and perchloric acid (3:1) for analyzing the Al, Na and Fe present in the mycelium using inductively coupled argon plasma emission spectroscopy (ICP).

Effect of red mud amended media on *A. tubingensis*

Different concentrations of red mud (0, 1, 2, 5 and 10%) were amended in Czapek-Dox liquid media and the pH was recorded. The contents were autoclaved and the mycelial disc of (0.5 mm diam.) *A. tubingensis* was inoculated and incubated at 28 °C under shaking condition. After 3 days of incubation the contents were filtered and the pH of the culture filtrate was recorded. The mycelium was further washed to remove soil particles adhered to the mycelia and dried for 48 hr at 70 °C. The dried mycelia were digested with concentrated HNO_3 and perchloric acid (1:3). Different elements such as Al, Na and Fe present in the mycelium were measured by ICP.

Nursery trials for amelioration of bauxite residue

Different substrates such as sludge, top soil, fly ash, and gypsum were added to red mud for reduction of pH. Five to 25% of these substrates were amended with red mud and the pH change was noted. Nursery trials were carried out at TIFAC-CORE of Thapar University, Patiala. The red mud and fly ash were brought from National Aluminum Company, Damanjodi, Orissa. Local campus sludge and topsoil were used for the amendment of the red mud. Sludge, top soil and fly ash were added at 20% concentration and gypsum at 10% concentration to the red mud. The bacterial isolates were grown in modified nutrient broth (Appendix 1) at 37 °C with constant shaking at 120 rpm unless the bacterial absorbance (A_{600}) reached at 1.0. *Aspergillus tubingensis* was grown on pikovaskaya agar (Appendix 1) at 28 °C for 3 days. The spore inoculation of *A. tubingensis* (~360 Log cfu/plot) and the indigenous bacterial consortium (~350 Log cfu/plot) mixed in a sterile soil rite was inoculated to each plot. The Bermuda grass,

which was a native of Damanjodi, Orissa, was planted uniformly in each block of nursery trials.

The experiments were designed in such a way that every chemical amendment had three treatments such as red mud amended with gypsum contained control (without any microbial inoculation), bacterial and *Aspergillus tubingensis* inoculation. The same pattern was followed in sewage sludge, topsoil, fly ash and without any chemical amendment of red mud. After six months of nursery experiment, physico-chemical characteristics such as pH, Electrical conductivity, organic carbon, available phosphorus, total nitrogen; and metal concentrations of treatments were measured and analyzed. The plants and soil samples were harvested after six months. The pH and electrical conductivity (EC) of the soil was checked by the method of International Society of Soil Science (1930). The total organic carbon (Walkley and Black, 1934), total nitrogen (by Total Organic Carbon Analyzer), and available phosphorus (Bray and Kurtz, 1945) was estimated. The mineral nutrition (Fe, Ca, Mg, Na and K) and aluminum content was estimated by Page *et al.* (1982). Enzymatic assays and microbial load were analyzed and all these methods are mentioned in the proceeding section. The plants were analyzed for their growth parameters and also for the different mineral nutrients. One gram of oven dried ground leaves/shoots and roots were digested (Page *et al.*, 1982) and the concentrations of calcium, magnesium, potassium, iron and aluminium were measured using an inductively coupled plasma emission spectrophotometer.

Determination of red mud pH and electrical conductivity (EC)

Air-dried red mud (20 g) was taken in 100 mL beaker and 50 mL of distilled water was added to it and thoroughly stirred for 2-3 min using a glass rod. Further, it was kept in shaking condition (120 rpm) for 3 hr. Suspension was allowed to settle for 30 min. The instrument was checked with two buffer solutions of known pH viz. one acidic and other alkaline. The electrode was rinsed with distilled water and carefully wiped with filter paper. The pH of the samples were measured of sample by immersing the electrode in supernatant solution. The pH value was recorded when the reading was stabilized (usually after 1 min).

Red mud mixed with water and stirred as described previously. KCl solution (0.01 M; EC=1.413 dS m⁻¹) was used to calibrate the meter. The electrode was dipped in the supernatant solution and recorded the EC.

Total Organic Carbon in soil (Walkley-Black, 1934)

Dried red mud (1 g) was transferred into a 150 mL conical flask and 10 mL of 1N K₂Cr₂O₇ was added and mixed well. The blank was prepared in which all reagents except soil were added. The conical flask was kept on teflon /asbestos sheet and 20 mL of concentrated H₂SO₄ from the sides of the flask was added. The flask was swirled during addition. The flask was allowed to stand for 30 min and there after 70 mL of water was added. These flasks were swirled thoroughly and allowed the soil particles to settle overnight. The supernatant was decanted and read the color intensity using red filter at 660 nm. For standard, 0, 10, 20, 30, 40 and 50 mg of anhydrous sucrose crystals was weighed into a 100 mL volumetric flask and repeated the same procedure.

Calculation

The quantities of sucrose (0, 10, 20, 30, 40 and 50 mg) were multiplied by 0.4207 and found the quantities (%) of carbon present. Because 10 mg sucrose contained 4.207 mg C and if suppose 4.207 mg C was found in 1000 mg of soil, it meant 100 mg soil contained = (4.207 x 100)/ 1000 mg carbon; Organic carbon (%) = Colorimetric reading x 0.0042. Because organic matter contains 58% carbon so, Organic matter = Organic carbon (%) x 100/ 58 or organic matter = org. C (%) x 1.724 (Van Bemmelen factor).

Available Phosphorus in red mud (Bray and Kurtz, 1945)

Red mud sample (2.5 g) was weighed in a 150 mL conical flask and 50 mL of Bray's P-1 extractant was added and kept on shaker for 5 min (soil- to- solution ratio 1:20). It was filtered through Whatman No. 1 filter paper quickly so as to collect the filtrate within 10 min. 5 mL of filtered aliquot was transferred into 50 mL volumetric flask and diluted to about 20 mL. 8 mL of ascorbic acid (0.042 g) solution was added and the volume was made to 50 mL. After 10 min, the color intensity nm was measured at 882. Blank was prepared with the extracting solution also (without soil). For standard, 0, 2, 5, 10, 15 and 20 mL were pipetted out of working phosphorus (KH₂PO₄) solution (1mg/L), in 6

different volumetric flasks and added 5 mL of extractant. 8 mL of ascorbic acid solution was added and finally made the volume to 50 mL with distilled water. The phosphorus concentration of these solutions was 0.04, 0.1, 0.2, 0.3 and 0.4 µg/mL respectively.

Calculation

$$\text{Available phosphorus (mg/kg)} = (Q \times V) / (A \times S)$$

Q = quantity of phosphorus in µg read on X- axis against a sample reading; V = volume of extracting reagent (mL); A = volume of aliquot used (mL); S = weight of soil sample taken (g)

Determination of Mineral Elements by inductively coupled plasma emission spectrophotometer (Page *et al.* 1982)

Red mud (1 g) was taken in a 100 mL conical flask. 15 mL of concentrated nitric acid was added and kept for 1 hour and then added 5 mL of perchloric acid. The flask was kept on a hot plate in acid-proof digestion chamber having fume exhaust system and heated at about 100 °C for first one hour and then raised the temperature to about 200 °C. The digestion was continued until the contents become colorless and only white dense fumes appeared. Reduced the acid content to about 2-3 mL by continuous heating at the same temperature. It was removed from the hot plate, cooled and diluted HCl was added. The solution was filtered through Whatman No. 42 filter paper and the final volume was made 20 mL with diluted HCl. The concentration was measured for mineral elements in the filtrate using inductively coupled plasma emission spectrophotometer. The calibration curve was prepared for each element by recording absorbance of a series of standard solutions of increasing concentrations.

Calculation

$$\text{Mineral ion (mg/g)} = C \times 20/W$$

C = concentration in the sample obtained on X-axis against the reading

W = weight of the mycelium taken

Determination of soil metals by SEM-EDX

The red mud samples were dried, sieved (<200 mesh), and ground. Finely crushed soil samples were vacuum air-dried and analyzed qualitatively and quantitatively using

an energy dispersive X-ray (EDX) Phoenix Microanalyzer, which was equipped with a Hitachi Scanning Electron Microscopy (SEM). The microanalyzer had qualitative, quantitative, imaging, and element mapping capabilities. This EDX system could detect light elements with 0.1% detectability limit and heavy elements with better than 0.1% detectability limit. The software used for analysis is SEM QUANT version 3.0 from EDAX Inc. The EDX scan time was set for 200 seconds.

Anion analysis of the red mud

Red mud was mixed with water in 1:5 ratio. The water-soluble extract was subjected to anion analysis with different anion selective probe potentiometer. The work was carried out by TCIRD (Thapar Corporate Industrial Research and Development, Patiala) center.

Soil microbial analysis

Soil samples were collected from different treatments in sterile containers and samples (1 g dry wt.) were suspended in 10 mL of single distilled water and disrupted by vortexing for 1–2 min. The resulting suspensions were serially (1/10) diluted in a sterile saline solution (NaCl 0.9%). Total heterotrophic neutrophilic, alkaliphilic and actinomycetes cultivable bacteria were estimated from colony forming units (CFU) growing on a non-selective nutrient media, nutrient media having pH 10.5 and actinomycetes agar (Appendix I). Plates were incubated for 2 days at 37 °C. Three replicates per dilution were maintained.

Statistical analysis

Three replicates were used for each treatment in nursery experiment. The data was completely randomized in this experiment and were subjected to one-way analysis of variance (ANOVA). The significant differences among the means were compared with Tukey's test at $P < 0.05$ using Graphpad Prism version 4.0 software.

Chapter 4

RESULTS

Microbial diversity analysis of red mud

Isolation and characterization of bacteria

Red mud samples were collected from different locations of red mud pond of National Aluminum Company, Damanjodi, Orissa, India in different seasons and the bacteria were isolated. Bacterial isolation was performed at three different times with different samples collected at three different time intervals from the red mud pond. The bacteria isolated from these samples varied between 5 to 100 cfu per gram of red mud samples at pH 10.5 irrespective of the complex media used for the isolation. Most of the bacterial colonies were appeared at 10^{-3} dilution. No growth was observed at 10^{-6} or 10^{-7} dilution. Based on their morphology and growth, 10 different bacteria were considered for further studies. These isolates were designated as RM9P, RM10E, RM10D, RM11R, RM12W, RM13Y, RM1, RM1A, RM6 and RM8.

Sub-optimal level of nutrients along with high alkalinity, salinity, sodicity and metal concentrations did not support the growth of bacteria and plants. Hence, methylene blue hanging drop experiment was carried out to know whether the isolated bacteria were metabolically injured or not. All isolated bacteria exhibited the light blue color of methylene blue in hanging drop experiment, which was an indication for the level of active dehydrogenases and the metabolic status of the cells.

The bacterial isolates had diverse morphotypes. The diversity found either in cell or in colony morphology and pigmentation was rather discrete. RM9P, RM11R and RM1 had pink pigmentation. RM10D and RM8 were yellowish in color and rest other isolates were whitish to cream whitish in color. RM9P, RM10D, RM10E, RM11R, RM1A, RM6 and RM8 showed gram positive reaction while two isolates namely RM13Y and RM1 showed variable gram reaction and only one isolate, RM12W showed gram negative reaction (Table 4.1). All isolates were catalase positive except RM11R, which was weak positive for catalase test (Table 4.1). RM10E, RM11R, RM1A and RM6 were oxidase positive and RM9P, RM10D, RM12W, RM13Y, RM1 and RM8 were oxidase negative (Table 4.1). The ability to reduce nitrate was a positive character of all isolates. Only one

isolate RM10E was found to be positive for utilization of citrate. RM8 and RM10D were positive for urease production and RM13Y and RM1A showed small production of extracellular urease (Table 4.1). Extracellular acid phosphatase was positive for RM10E, RM11R, RM12W, RM1, RM1A, RM6 and RM8 (Table 4.1). RM1 and RM8 were found to hydrolyze xylan and carboxy methyl cellulose (CMC) extracellularly and RM11R and RM6 were able to produce small amount of xylanase and cellulase in presence of substrates xylan and CMC respectively (Table 4.1). For all other biochemical tests such as lysine, ornithine, ONPG, TDA hydrolysis and H₂S production, these isolates were found to be negative.

Organic acid exudation was estimated in presence of sugar (sucrose) amended in the red mud extract media, so that original conditions of this extreme site can be mimicked. Bauxite residue was extracted with the aqueous solution and amended with carbon source (sucrose). All but three red mud bacterial isolates (RM11R, RM13Y and RM1) were found to produce acetic acid (Table 4.1). RM6 produced succinic acid along with acetic acid and only trace amount of oxalic acid were found in RM13Y and RM1 as shown in Table 4.1.

Growth studies

When subjected to salinity and temperature tests, RM9P was able to grow in 15% NaCl amended media, while other isolates were grown either at 3% and 6% of NaCl amended media (Table 4.2). Four temperatures (25, 30, 37 and 45 °C) were chosen to test the growth pattern of bacterial isolates. All bacterial isolates were grown at 25 to 37 °C and four bacterial isolates (RM9P, RM10E, RM12W and RM8) showed growth at 45 °C (Table 4.2).

All bacterial isolates were grown in nutrient media with different pH in buffered media. The growth of red mud isolates was determined in liquid and solid media amended with different buffers. RM9P, RM11R, RM12W and RM8 isolates were able to grow at pH 10.5, and two isolates (RM9P and RM8) showed growth at pH 11.3 and other isolates were grown in buffered media of pH 8.0 (Table 4.2). All isolates showed growth in unbuffered media when sodium hydroxide was used to adjust the pH at 10.5.

Table 4.1: Biochemical characterization of the bacterial isolates from the red mud pond

Bacterial Isolates	Gram Staining	Catalase	Oxidase	Nitrate Reduction	Citrate Utilization	DNase	Urease	Xylanase/CMCase	Acid phosphatase	Organic Acid Production*
RM9P	+	+	-	+	-	-	-	-	-	AA
RM10E	+	+	+	+	+	+	-	-	+	AA
RM10D	+	+	-	+	-	-	+	-	-	AA
RM11R	+	±	+	+	-	-	-	±	+	-
RM12W	-	+	-	+	-	-	-	-	+	AA
RM13Y	Variable	+	-	+	-	-	±	-	-	-
RM1	Variable	+	-	+	-	-	-	+	+	-
RM1A	+	+	+	+	-	-	±	-	+	AA
RM6	+	+	+	+	-	-	-	±	+	AA, SA
RM8	+	+	-	+	-	+	+	+	+	AA

+: positive reaction; -: negative reaction; ±: weak positive;

* -: no acid production; AA: acetic acid; SA: succinic acid; Acid production was studied in soil extract media (Appendix I) amended with sucrose.

Table 4.2: Effect of sodium chloride concentration, temperature and pH on the growth of bacterial isolates of red mud

Bacterial isolate	NaCl (%)	Temperature (°C)	Growth in buffered alkaline condition					
			7	8	9	10.5	11.3	12
RM9P	0-15	25 – 45	+++	+++	+++	+++	+++	-
RM10E	0-7	25 – 45	+++	+++	-	-	-	-
RM10D	0-5	25 – 37	+++	+++	-	-	-	-
RM11R	0-5	25 – 37	-	+++	+++	+++	-	-
RM12W	0-5	25 – 45	+++	+++	+++	+++	-	-
RM13Y	0-10	25 – 37	-	+++	++	-	-	-
RM1	0-5	25 – 37	+++	+++	++	++	-	-
RM1A	0-5	25 – 37	+++	+++	-	-	-	-
RM6	0-3	25 – 37	-	+++	++	-	-	-
RM8	0-7	25 – 45	+	+++	+++	+++	+++	-

-: no growth; +: poor growth; ++: good growth; +++: luxurious growth

Carbohydrate fermentation and antibiotic profiling

The ability to ferment various carbohydrates by these bacteria was determined. Majority of the isolates were able to ferment different carbon substrates (Table 4.3). RM9P and RM13Y were able to ferment two carbon sources, RM10E and RM1 fermented three carbon sources, RM11R and RM12W fermented four carbon sources and other isolates were able to ferment more than five carbon sources (Table 4.3).

Bacterial isolates were checked for their sensitivity to different antibiotics. Three red mud isolates were found resistant to some antibiotics, but rest all other isolates were sensitive. RM10E was resistant to 3 antibiotics, RM1A was resistant to 6 antibiotics and RM8 was resistant to 1 antibiotic (Table 4.3).

Biolog test

Different carbon substrate utilization patterns of red mud isolates were observed and matched with the BIOLOG database. Red mud isolates were able to utilize number of varied carbon substrates, which ranged from 19 to 78 (Table 4.4). The maximum numbers of 78-carbon substrates were utilized by RM1A and only 19 were utilized by RM9P (Table 4.4). Different carbon substrates (49) were utilized by two isolates RM11R and RM6. Forty-seven, 56, 59 and 66 different carbon substrates were utilized by RM10D, RM13Y, RM8 and RM1 respectively (Table 4.4). Homology searching of the Biolog database revealed that only RM1A had 30% similarity with *Bacillus sp.* In other red mud isolates similarities varied from 0 to 1% with no probability with the existing database of the Biolog (Table 4.4). The similarity percentage of the red mud isolates was less than 35% in all cases, clearly indicating that these bacterial isolates were different from the reported bacteria.

The metabolic diversities of the red mud isolates were shown by the ability of isolates to metabolize any substrate, including amino acids and carboxylic acids on the basis of color development in different wells on Biolog plates. RM10E and RM12W utilized the maximum number of carbon substrates along with amino acids and carboxylic acids, while RM9P utilized few carbohydrates, 8 carboxylic acids, one fatty acid methyl ester and no amino acid.

Table 4.3: Fermentation of carbon substrates and antibiotic profiling of the red mud bacterial isolates

Bacterial isolate	Carbon substrate fermentation[#]	Antibiotic Resistance*
RM9P	Glucosamine, Ribose	-
RM10E	Dextrose, Trehalose, Maltose,	Cj, Cu
RM10D	Fructose, Dextrose, Mannose, Salicin, Glucosamine, Mannitol, Ribose, Rhamnose, Cellobiose, Melezitose, D-Arabinose	-
RM11R	Lactose, Glucosamine, Mannitol, Ribose	-
RM12W	Ribose, Glucosamine, Mannitol, Lactose,	-
RM13Y	Glucosamine, Ribose	-
RM1	Maltose, Dextrose, Ribose	-
RM1A	Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose,	Nt, Nx, Cj, Ak, Ca, C
RM6	Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Mellibiose, L-Arabinose, Mannose, Inulin, Salicin, Inositol, Mannitol, Ribose, Rhamnose, Melezitose, D-Arabinose	-
RM8	Maltose, Fructose, Dextrose, Trehalose, Glycerol, Salicin, Mannitol, Cellobiose, D-Arabinose	Cj

[#] Total of 35-carbohydrate fermentation tests was performed with isolated bacterial species using HiCarbo Kit (Himedia Lab., Bombay, India).

* Nt: Netillin (30 µg), Nx: Norfloxacin (10 µg), Cj: Cefaclor (30 µg), Ak: Amikacin (30 µg), Ca: Cefotaxime (30 µg), C: Chloramphenicol (30 µg), Cu: Cefuroxime (30 µg); -: no antibiotic resistance observed, out of twenty antibiotics of the current study

Table 4.4: Carbon substrate utilization of red mud isolates on Biolog GP plates and their similarities with the Biolog database.

Bacterial isolate	No. of Carbon substrate utilized	% Similarity with the Biolog database
RM9P	19	<5
RM10D	47	<5
RM10E	20	<5
RM11R	49	<5
RM12W	20	<5
RM13Y	56	<5
RM1	66	<5
RM1A	78	30
RM6	49	<5
RM8	59	<5

REP PCR based fingerprinting

After physiological studies, genetic diversity of red mud isolates were studied by PCR based molecular typing. Amplification of the regions between adjacent repetitive extragenic elements (REP PCR) of isolate-specific DNA fingerprints was generated. Rep PCR for all isolates yielded a complex genomic fingerprint consisting of ~12 Kbp to 100 bp amplicons of varying intensity (Fig 4.1). The maximum number of amplicons was generated by RM12W and minimum of three amplicons was found in case of RM8. Visual observation of the DNA fingerprints clearly showed that all isolates were genetically distinct. These results showed complex banding patterns, which reflected a high degree of inter or intra specific genetic unrelatedness among isolates (Fig 4.1).

Identification of the bacterial isolates

All bacterial isolates were subjected to 16S rDNA amplification using universal primers, and about 1.5 kb amplicon was observed in all isolates (Fig 4.2). 16S rDNA PCR products were cloned into pGEM-T Easy Vector (Promega Inc., USA). The plasmid DNA was extracted from different clones and amplified with T7 and SP6 primers. The 1.5 kb amplified products of different clones were subjected for restriction enzyme analysis (*RsaI*, *AluI*, *HinfI*, *TaqI* and *NdeII*) analysis to see the variation in the 16S rDNA region. The 16S rDNA products from three selected clones were then sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with the primers T7 and SP6. The 16S rDNA sequences of red mud isolates ranged from 1486 to 1576 bp. The sequences were analyzed by multiple sequence alignment to check the similarities among the isolates. The homologies among the sequences were from 77 to 97% between isolates. Minimum of 77% similarity was found in RM12W with RM10D, RM1 and RM8 and maximum 97% similarity was found between RM13Y and RM1 (Table 4.5). None of these isolates had shown 99% or more similarity among themselves (Table 4.5). RM10E and RM6 had shown 94% similarities.

Sequences were compared for the similarity in the GenBank DNA database using BlastN (NCBI) (Altschul *et al.*, 1997). Pairwise alignment revealed that 16S rDNA of bacterial isolates had 96% to 99% similarity with the sequences of NCBI database.

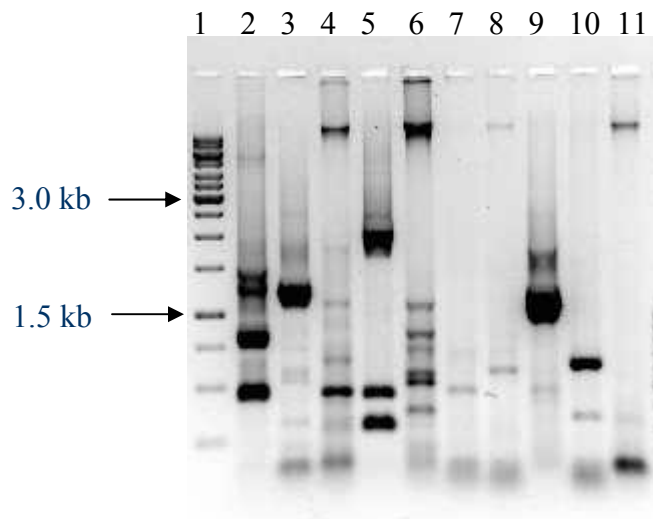


Fig 4.1: Comparison of purified genomic-DNA-based fingerprints generated by REP-PCR for different red mud isolates. Lane 2- RM9P; Lane 3-RM10E, Lane 4-RM10D, Lane 5- RM11R; Lane 6- RM12W; Lane 7-RM13Y; Lane 8- RM1; Lane 9- RM1A; Lane 10- RM6; Lane 11- RM8, Lane 1: 1 kb (Fermentas) size marker.

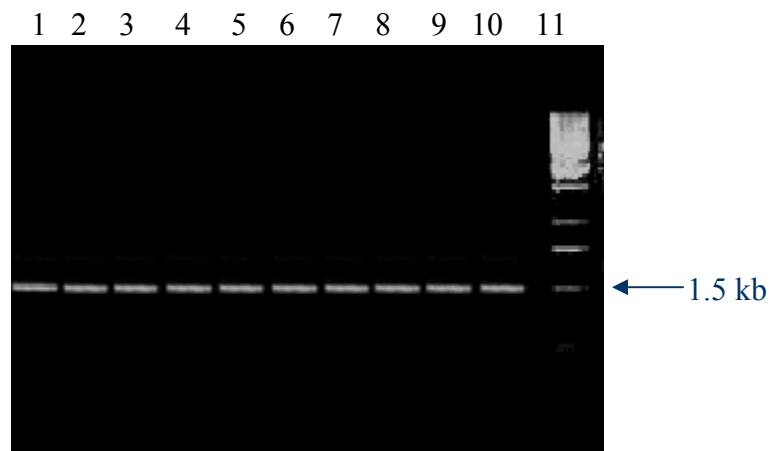


Fig 4.2: 16S rDNA amplification of red mud isolates. Lane 1-10: RM9P, RM10E, RM10D, RM11R, RM12W, RM13Y, RM1, RM1A, RM6 and RM8 and Lane11: 1 kb marker (Fermentas)

RM9P had shown 98% similarity with *Planococcus maitriensis*, *Planococcus maritimus* and *Planococcus sp.* *P. maritimus* had isolated from alkaline Lonar lake, India. RM10E had 99% similarity with the *Bacillus cereus*, which was reported to degrade pesticide. RM10D had shown 98% similarity with *Microbacterium hominis*, which was isolated from phenol degrading environment. RM11R had 99% similarity with *Salinicoccus roseus*, which inhabited in Eastern Mediterranean Sea. RM12W had shown 99% similarity with the *Pseudomonas pseudoalcaligenes*, *Pseudomonas sp.* IC017 and Uncultured bacterial clone 2As4. These isolates and clone were either isolated form contaminated site or from marine sediments. RM13Y had similarity with *Kocuria palustris*, *Kocuria sp.* CNJ787 PL04 and *Kocuria flavus* CT9 with 96 to 99% similarity. These bacteria were also isolated from marine sediment (Gulf of Mannar). RM1 showed 98% similarity with *Kocuria polaris*, *Kocuria sp.* and *Kocuria rosea*. These isolates were also reported form marine environments. RM1A showed only 95% similarity with the uncultured bacterium and *Bacillus sp.* ge15. RM6 and RM8 showed 99% with *Bacillus sp.* and *Agromyces sp.* respectively. These isolates were also reported either from marine environment or from contaminated sites.

The 16S rDNA gene sequences of red mud isolates determined in this study were deposited in the GenBank of NCBI data library under accession numbers EF675619 to EF675628 (Appendix II). The related sequences showing similarity in BLAST were retrieved from GenBank and RDP-II and aligned using the program CLUSTALW (Thompson *et al.*, 1997). The resulting multiple alignments were optimized visually and the evolutionary distance was calculated by Kimura 2 parameter. Phylogenetic dendograms were constructed by neighbor-joining method using MEGA 4 software (Tamura *et al.*, 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses.

Phylogenetic analysis revealed that most of the bacteria were related to the phylum Firmicutes and Actinobacteria and one isolate was related to gram negative bacteria. All the sequences in the dendogram were divided into three groups (Group A, B and C) with high bootstrap values. There were five isolates in 'group A' which were related to Phylum Firmicutes and associated with the genera *Bacillus*, *Planococcus* and

Table 4.5: Percentage similarity of 16S rDNA sequences of bacterial isolates using multiple sequence alignment (ClustalW).

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	RM9P	1511	2	RM10E	1514	92
1	RM9P	1511	3	RM10D	1486	80
1	RM9P	1511	4	RM11R	1512	90
1	RM9P	1511	5	RM12W	1499	80
1	RM9P	1511	6	RM13Y	1573	80
1	RM9P	1511	7	RM1	1487	81
1	RM9P	1511	8	RM1A	1515	94
1	RM9P	1511	9	RM6	1511	92
1	RM9P	1511	10	RM8	1489	81
2	RM10E	1514	3	RM10D	1486	81
2	RM10E	1514	4	RM11R	1512	91
2	RM10E	1514	5	RM12W	1499	80
2	RM10E	1514	6	RM13Y	1573	80
2	RM10E	1514	7	RM1	1487	82
2	RM10E	1514	8	RM1A	1515	93
2	RM10E	1514	9	RM6	1511	94
2	RM10E	1514	10	RM8	1489	81
3	RM10D	1486	4	RM11R	1512	81
3	RM10D	1486	5	RM12W	1499	77
3	RM10D	1486	6	RM13Y	1573	92
3	RM10D	1486	7	RM1	1487	92
3	RM10D	1486	8	RM1A	1515	81
3	RM10D	1486	9	RM6	1511	80
3	RM10D	1486	10	RM8	1489	91
4	RM11R	1512	5	RM12W	1499	80
4	RM11R	1512	6	RM13Y	1573	81
4	RM11R	1512	7	RM1	1487	82
4	RM11R	1512	8	RM1A	1515	90
4	RM11R	1512	9	RM6	1511	89
4	RM11R	1512	10	RM8	1489	81
5	RM12W	1499	6	RM13Y	1573	76
5	RM12W	1499	7	RM1	1487	77
5	RM12W	1499	8	RM1A	1515	80
5	RM12W	1499	9	RM6	1511	80
5	RM12W	1499	10	RM8	1489	77
6	RM13Y	1573	7	RM1	1487	97
6	RM13Y	1573	8	RM1A	1515	81
6	RM13Y	1573	9	RM6	1511	80
6	RM13Y	1573	10	RM8	1489	90
7	RM1	1487	8	RM1A	1515	82
7	RM1	1487	9	RM6	1511	81
7	RM1	1487	10	RM8	1489	91
8	RM1A	1515	9	RM6	1511	92
8	RM1A	1515	10	RM8	1489	81
9	RM6	1511	10	RM8	1489	80

Table 4.6: Red mud isolates and their closest relative species inferred from 16S rRNA gene sequences of existing database

Red Mud Isolate	Nearest match	Phylum	Accession no.	% Similarity of 16S rDNA
RM9P	<i>Planococcus maitriensis</i> (AJ544622)	Firmicutes	EF675619	98
RM10E	<i>Bacillus cereus</i> (AJ577274)	Firmicutes	EF675620	99
RM10D	<i>Microbacterium hominis</i> (AM181504)	Actinobacteria	EF675621	99
RM11R	<i>Salinicoccus roseus</i> (X94559)	Firmicutes	EF675622	99
RM12W	<i>Pseudomonas pseudoalcaligenes</i> (Z76675)	Proteobacteria	EF675623	98
RM13Y	<i>Kocuria palustris</i> (Y16263)	Actinobacteria	EF675624	96
RM1	<i>Kocuria polaris</i> (Y16263)	Actinobacteria	EF675625	98
RM1A	<i>Bacillus sp.</i> (AJ276809) /uncultured bacterium	Firmicutes	EF675626	92/95
RM6	<i>Bacillus litoralis</i> (EF371376)	Firmicutes	EF675627	98
RM8	<i>Agromyces mediolanus</i> (D45052)	Actinobacteria	EF675628	97

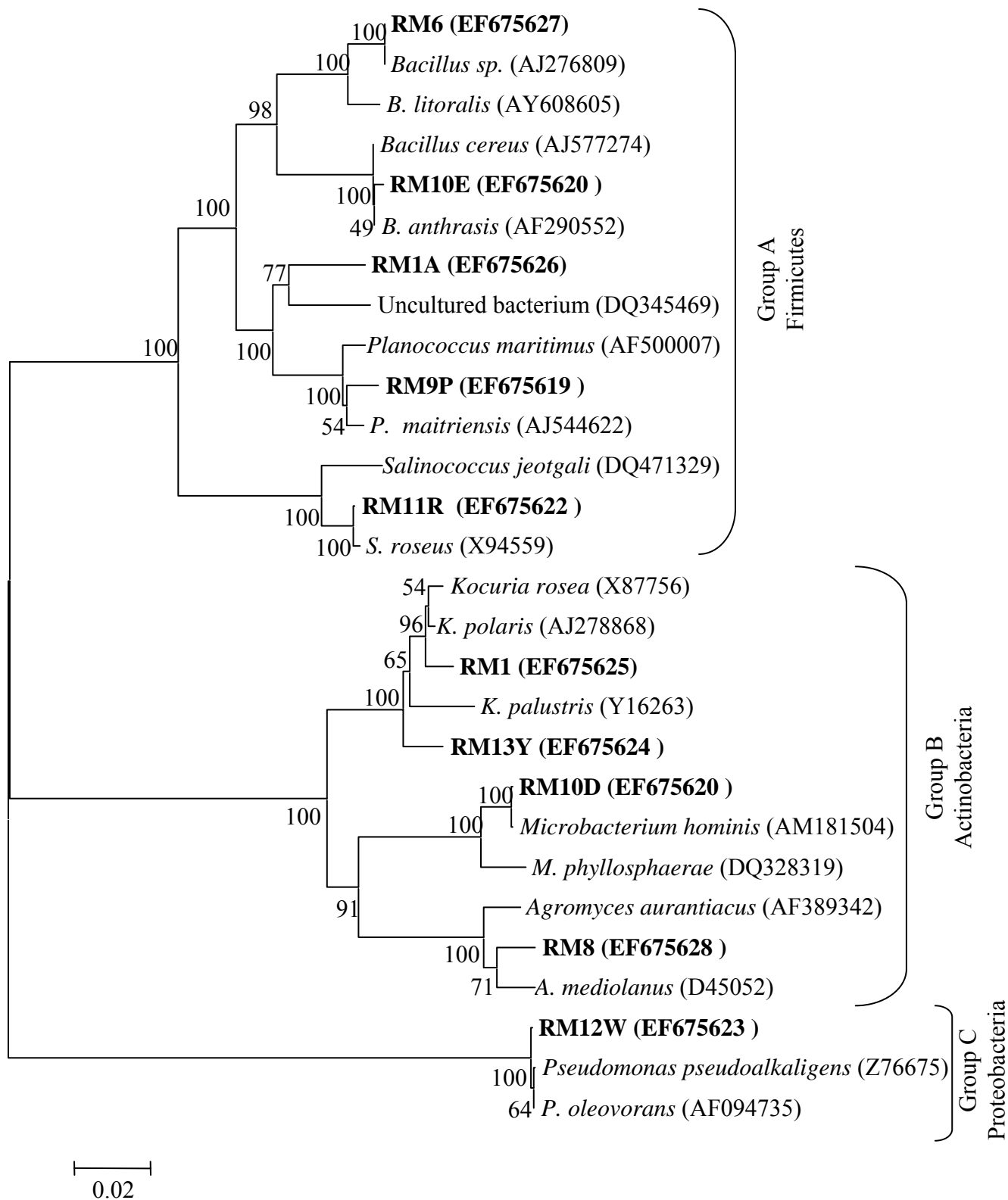


Fig 4.3: Neighbor-joining tree based on bacterial 16S rDNA sequence data from different isolates of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1500 replicates.

Salinococcus. The isolate RM6 was phylogenetically related to *Bacillus sp.*, RM10E with *Bacillus sp.*, and RM1A with an uncultured bacterium. RM9P was related with *Planococcus maitriensis*, RM11R with *Salinococcus roseus*.

‘Group B’ consisted of 4 isolates belong to phylum Actinobacteria related to genus *Microbacterium*, *Kocuria* and *Agromyces*. RM1 showed 98% similarity with *Kocuria polaris* and RM13Y showed 96% similarity with *Kocuria palustris*. RM10D isolate was phylogenetically related to *Microbacterium hominis* and RM8 with *Agromyces mediolanus* (97%). Group C consisted of only one isolate, which showed 98% similarity with *Pseudomonas pseudoalkaligenes* (Fig 4.3, Table 4.6).

DNA extraction from red mud

Crude DNA was extracted from red mud using various methods as described in materials and methods (Section 3.3). Different extraction procedures were checked with various combinations of lytic agents along with garden soil as a control to extract genomic DNA. The physical and chemical properties of red mud and garden soil were completely different. In terms of bacterial counts, garden soil had $\sim 10^8$ cfu/gm as compared to 5-100 cfu/gm of red mud. The garden soil had high C, N, and available P content and slightly alkaline pH (~ 7.6) whereas red mud showed trace amount of C, N, and available P content and high alkalinity (pH 10-13). Different extraction procedures (method 1 to 4) were applied using freeze thawing and bead beating and extracted DNA was checked for concentration and quality. Apparently, only one method for DNA extraction worked for red mud was method 1 by both the freeze-thawing and bead beating. Freeze-thawing had yielded very low amount of DNA than the bead beating method. Other methods including the commercial kit from Mobio did not yielded good amount of DNA and loading of this DNA in agarose gel was not visualized by ethidium bromide fluorescent staining. Crude DNA extracted from red mud by hot SDS, CTAB and proteinase K and mean yields ranged from 2.37 to 9.97 $\mu\text{g/gm}$ of dry wt. of red mud (Table 4.7). The purity of red mud DNA was low as compared to DNA of garden soil. Agarose gel electrophoresis of these DNA had revealed that it was extracted along with contaminant. It was very viscous and could not move from the well (Fig 4.4). For control (garden soil), every method had

Table 4.7: Purity ratio, DNA yield and 16S rDNA amplification from red mud and garden soil sample

Treatment	DNA yield ($\mu\text{g/g}$ of dry wt. of cells)		A_{260}/A_{280} ratio		16S rDNA PCR amplification	
	<i>Red mud</i>	<i>Garden Soil</i>	<i>Red mud</i>	<i>Garden Soil</i>	<i>Red mud</i>	<i>Garden Soil</i>
Freeze-thawing method						
Method 1	2.37 \pm 0.32	16.9 \pm 1.3	1.47 \pm 0.09	1.67 \pm .002	+	+
Method 2	0.09 \pm 0.01	15.3 \pm 1.7	1.62 \pm 0.05	1.52 \pm 0.03	-	+
Method 3	0.10 \pm 0.01	15.1 \pm 1.5	1.46 \pm 0.05	1.21 \pm 0.03	-	+
Method 4	0.09 \pm 0.03	8.96 \pm 0.96	1.66 \pm 0.03	1.23 \pm 0.02	-	+
Bead-beating method						
Method 1	9.97 \pm 0.91	20.3 \pm 1.1	1.12 \pm 0.06	1.72 \pm 0.09	+	+
Method 2	0.46 \pm 0.06	17.5 \pm 0.9	1.36 \pm 0.03	1.47 \pm 0.05	-	+
Method 3	0.37 \pm 0.05	13.5 \pm 0.87	1.31 \pm 0.02	1.34 \pm 0.03	-	+
Method 4	0.23 \pm 0.02	7.93 \pm 0.81	1.48 \pm 0.02	1.41 \pm 0.03	-	+
Method 5	0.32 \pm 0.08	19.7 \pm 1.2	1.41 \pm 0.03	1.52 \pm 0.04	-	+

-: no amplification with crude DNA; +: PCR amplification with crude DNA

yielded high concentration of DNA as compared to red mud. DNA extraction by Method 1 with freeze-thawing had yielded 7 times more DNA concentration in garden soil than the red mud and Method 1 with bead beating yielded two times more DNA concentration as compared to red mud. Other methods had also yielded high concentration of DNA in garden soil, which ranged from 8.96 to 19.6 $\mu\text{g/g}$ as compared to insignificant amount of DNA in red mud (Table 4.7). Direct amplification of V3 region of 16S rDNA was performed from each DNA extracted from various methods. DNA of garden soil extracted by different methods resulted in 250 bp amplification of 16S rDNA, but DNA extracted from the red mud by method 1 along with freeze-thawing showed very light amplification (Fig 4.6).

Clone library screening

16S rDNA was amplified using the red mud high molecular wt. DNA as template. No or very small amplification was obtained with crude and purified red mud DNA (Fig 4.6), because of co-extraction of the contaminants which were not removed after purification steps. In order to overcome the inhibition of PCR reactions and to obtain successful results on difficult to amplify samples like red mud, a pre-PCR was applied which is consisting of whole-genome amplification (WGA) by multiple displacement amplification (MDA) using $\phi 29$ DNA polymerase and random hexamer primers in an isothermal reaction to increase copy number and simultaneously diluting inhibitory substances. 300 bp to ~ 50 kb DNA smear was resolved on agarose gel of the isothermal amplification (WGA) of red mud samples (Fig 4.5).

After WGA amplification, 16S rDNA amplification by PCR was employed (Gonzalez *et al.*, 2005) (Fig 4.6). 16S rDNA was successfully amplified with the WGA amplified red mud DNA as template, but not with the crude or purified DNA of red mud as described previously. 16S rDNA of 1.5 kb amplicon was further purified, ligated in pTZ57R/T vector and transformed in *E. coli* DH5 α . Amplicons of different red mud samples collected at different time intervals were pooled together and used for clone library construction. Clone library was screened visually by selecting the white transformed colonies and then subjected to colony PCR using M13F and M13R primers. The amplified 1.5 kb region of 16S rDNA was further selected for the restriction

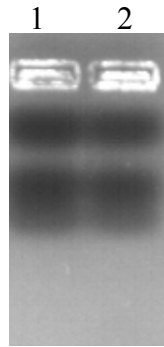


Fig 4.4: High molecular wt. DNA isolation from red mud. DNA is seen in the well due to presence of co-extraction of contaminant.

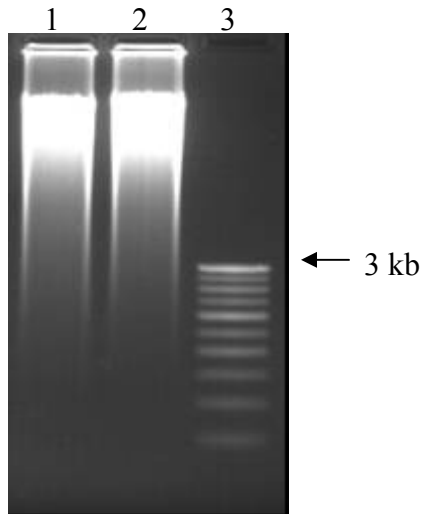


Fig 4.5: Whole genome amplification of high molecular wt. DNA from red mud. Lane 1 and 2 contain samples of amplified DNA of red mud samples; and Lane 3: 100 bp DNA molecular wt. marker (Fermentas).

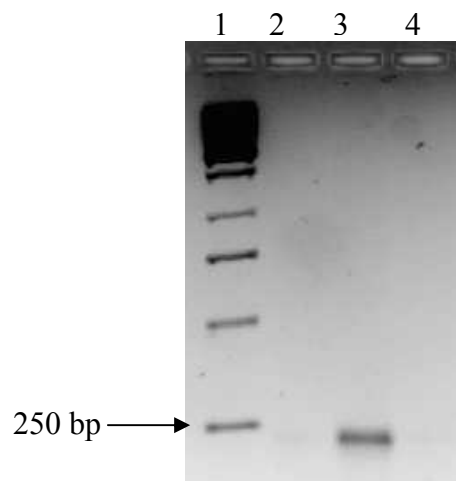


Fig 4.6: Amplification of V3 region of 16S rDNA from crude DNA directly isolated from red mud, which is hardly visible in agarose gel because of PCR inhibition (Lane 2), from whole genome amplified DNA of red mud sample (Lane 3) and negative control (Lane 4). Lane 1: 1 kb DNA molecular wt. marker (Fermentas)

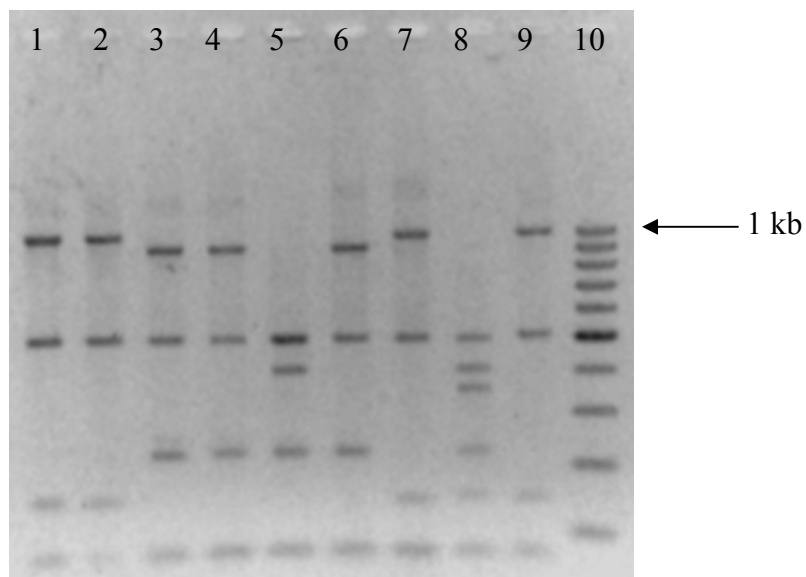


Fig 4.7: Restriction analysis by *RsaI* of 16S rDNA amplicon (1.5 kb) of clone library. Lane 1,2,7 and 9; Lane 3,4 and 6; Lane 5 and Lane 8 having different restriction pattern constitute different operation taxonomic unit (OTU). One clone from each OTU had been selected for the partial sequencing. Lane 10: 100 bp DNA molecular wt. marker (Fermentas).

digestion with *RsaI* to differentiate the clone based on their restriction pattern (Fig 4.7). About 250 clones were screened. Clones having similar restriction pattern were grouped together and one clone was selected from each group for sequencing using M13F and M13R primers. 41 clones were sequenced partially. Clone library sequences were submitted under accession numbers *EU665639 - EU665677* (Appendix II). All sequences were checked by Chimera Check program of RDPII database. Two apparent chimeric sequences were detected and omitted in further studies. The 16S rRNA gene sequences of the 38 clones with an average length of 700 nucleotides (minimum 387 nucleotides; maximum, 742 nucleotides) were compared to the 16S rDNA databases (RDP II and NCBI), and closest matches were identified. The similarities between red mud environmental clone sequences and that of a described species or of uncultured clone were depicted in table 4.8. Most bacterial clones were affiliated to Beta- (34%) and Gamma- (56%) proteobacteria while 4.8% clones represented Bacteroidetes as shown in the table 4.8. Out of 14 clones, which belong to beta-proteobacter class, 7 clones had shown high similarity (99%) with uncultured Burkholderiaceae bacterium or *Ralstonia sp.* K101 (Table 4.8).

Beta-Proteobacter

RMCL-1, 7 and 18 were affiliated to the clones isolated from recent Hawaiian volcanic deposits or with bacteria isolated from soil in Japan having 2,4-Dichlorophenoxyacetic acid-degrading genes. All these clones had 99% similarity with each other. One clone RMCL-58 had shown 97-98% similarity with uncultured bacterium PT06 or *Cupriavidus basilensis* strain SE15. RMCL-28, 68, 112 showed 98-99% and RMCL-62, 105 showed 100% similarity with *Cupriavidus basilensis* N-Ams; AM048887 or uncultured bacterium 005C-G01 that were reported from the mars exploration rovers assemble and diesel-contaminated soil of Baekun Mountain. RMCL-28 had shown similarity with the clone isolated from groundwater contaminated with high levels of nitric acid-bearing uranium waste at the NABIR-FRC.

Gamma-Proteobacter

23 clones which belong to gamma-proteobacter, 6 clones (RMCL-14, 19, 23, 90, 102, 125) had 99% while one clone (RMCL-142) had 100% similarity with the uncultured bacterium 1s8; *Serratia marcescens* strain RJT which is reported from the

swine effluent impacted environments. RMCL-107, 143, 63 clones had shown 99% similarity with Uncultured bacterium s1s12a; f2h21 from clone library of microbiota of the ant lion (Table 4.8). These had considerably good similarity with the *Serratia sp.* also which was reported for mineralization of chlorpyrifos.

RMCL-144 had 100% similarity with the *Stenotrophomonas maltophilia*, uncultured bacterium rRNA402, *Xanthomonas sp.* BBCT38, uncultured bacterium S23639. *Stenotrophomonas maltophilia* had also been isolated from alkaline environment such as the inner Mongolia Baer soda lakes (Ma *et al.*, 2004). These clones are reported from various other sources such as an anaerobic psychrophilic enrichment cultures obtained from a greenland glacier ice core, or marine microorganisms of Coco's Island.

RMCL-25 had 97% similarity with the ant-degrading strains *Stenotrophomonas sp.* or extracellular medium-chain-length poly (3- hydroxyalkanoate) depolymerase secreting *Stenotrophomonas sp.* (Table 4.8).

RMCL-66, 3, 16 and 115 had shown 99% similarity with different uncultured bacterium from rhizosphere and phyllosphere bacteria and *Serratia marcescens* strain RJT having fipronil degrading capacity. RMCL-4, 93, 95, 103 had shown 99% similarity with the uncultured bacterium clone PDC-OTU10 (Table 4.8).

Bacterioidetes

RMCL-202 and RMCL-2 showed 99 and 100% similarity with the uncultured bacterium 654966 of Bacterioidetes subdivision respectively. RMCL-88 had shown 99% similarity with the Iron-reducing enrichment clone Cl-A7. RMCL-89, RMCL-61 and 121 had shown homology with *Serratia marcescens* strain P1, capable of converting DL-lactate to pyruvate isolated from the deep-sea sediment.

4.8: Diversity of non-cultivable bacteria of red mud sites and their affiliation to the 16S rDNA sequences of existing database

Class	Red mud clones	Total numbers of clones	Closest relative in database	% identity to closest related
Beta proteobacterium	RMCL-1, 7, 18, 31, 92, 98, 114	7	Burkholderiaceae bacterium KVD-1700-16; DQ490285/ Ralstonia sp. K101; AB212229	99%
	RMCL-58	1	Bacterium PT06; DQ136051/Cupriavidus basilensis strain SE15; EU447184	97-98%
	RMCL-28, 68, 112	3	Cupriavidus basilensis N-Ams; AM048887 / uncultured bacterium 005C-G01; AY662034	98-99%
	RMCL-62, 105	2	Cupriavidus basilensis N-Ams; AM048887	100%
	RMCL-88	1	Iron-reducing enrichment clone CI-A7 clone CI-A10; DQ677002/ Burkholderiaceae bacterium KVD-1700-16; DQ490285	99%
Gamma proteobacterium	RMCL-107, 143, 63	3	Uncultured bacterium; s1s12a; DQ068886/ uncultured bacterium; f2h21; DQ068786	99%
	RMCL- 89	1	Serratia marcescens strain P1; EU031439	99%
	RMCL-61, 121	2	Serratia marcescens strain P1; EU031439	100%
	RMCL-14, 19, 23, 90, 102, 125	6	Serratia marcescens strain RJT ;EU233275/ uncultured bacterium; 1s8; DQ068829	99%
	RMCL-142	1	Serratia marcescens strain RJT ;EU233275/ uncultured bacterium; 1s8; DQ068829	100%
	RMCL-66	1	Uncultured bacterium clone spb28b5; DQ321561	100%
	RMCL-144	1	Stenotrophomonas maltophilia; 15; AY169434/uncultured bacterium; rRNA402; AY959175/Xanthomonas sp. BBCT38; EF471219/uncultured bacterium; S23_639; EF572540	100%
	RMCL-4, 93, 95, 103	4	Uncultured bacterium clone PDC-OTU10; AY700617/ Serratia sp. EP28; AM403719	99%
	RMCL-25	1	Gamma proteobacterium PI_GH4.1.G2; AY162052/ Stenotrophomonas maltophilia; M5-1; AY880274	97%
	RMCL- 3	1	Uncultured bacterium; 1s8; DQ068829/ Serratia sp. BBTR23; DQ337601	99%
Bacteriodetes	RMCL-16, 115	2	Uncultured bacterium, clone C 4-81; AM947381	99%
	RMCL-202	1	Uncultured bacterium; 654966; DQ404637	99%
	RMCL-2	1	Uncultured bacterium; 654966; DQ404637	100%

Salient features

- The bacterial load of red mud varied from 5 to 100 cfu per gram of red mud. Ten bacterial isolates which were further studied, had different Rep-PCR profile and 16S rDNA sequences. 16S rDNA sequences showed that these isolated closely related to *Planococcus sp.*, *Bacillus sp.*, *Pseudomonas sp.*, *Kocuria sp.*, *Micrococcus sp.*, *Agromyces sp.* and *Salinococcus sp.* These isolates mainly belong to Phylum-Firmicutes, Actinobacteria and Proteobacteria. Sequence alignment of closely related bacteria and red mud bacterial isolates showed that similarity varied from 97 to 99%, but none of the isolates had shown 100% sequence similarity with the bacteria of the existing database. Biolog test was performed to study the metabolic diversity of these isolates. None of the isolates had shown homology with the Biolog database.
- Screening of the clone library resulted in distinct 16S rDNA sequences as compared to isolated cultures. These were mainly belonging to Proteobacteria and Bacteroidetes. These bacterial clones were affiliated to Beta- (34%) and Gamma- (56%) Proteobacteria while 4.8% clones represent Bacteroidetes. Interestingly, all sequences of clone library had 99% or more similarity with the uncultured and cultivable isolates. But these clones had shown no homology with the cultivable isolates of red mud. The present study gives the first report on bacterial diversity analysis of the red mud.

Alkaline tolerance by red mud isolates

Reduction of media pH by red mud isolates

Red mud isolates were grown in media adjusted to two different pHs *i.e.* 7.0 and 10.0 to test their potential to decrease the media pH. Their growth was relatively same on both the pHs in unbuffered media. But there was little variation of growth among the isolates which ranges from 9.0 to 9.31 log cfu (Fig 5.1). The maximum growth was observed in case of RM10E, RM10D and RM12W (9.3 log cfu). Three isolates, RM10D, RM1 and RM1A were able to reduce the initial pH of 10.0 by six units in 36 hr. RM1A was able to reduce it further from 3.80 at 36 hr to 3.76 at 168 hr. RM11R had shown the pH change from 7.6 (36 hr) to 6.35 (168 hr) when grown at pH 10.0. All other isolates were able to change the medium pH 4 to 5 units. RM10E and RM12W had a maximum pH change at 36 hr. At pH 7.0, RM10D and RM1A had reduced the medium pH by 3 units at 36 hr, and decreased further at 168 hr. Interestingly, RM9P showed initial rise of 1.29 units of medium pH at 36 hr, which reached to pH of 5.1 at 168 hr when grown at pH 7.0. The rest of the all isolates had shown minor reductions in the medium pH (Fig 5.1).

Buffering capacity of red mud isolates RM9P and RM8

A diverse group of bacteria thrive in highly alkaline environments (Krulwich and Guffanti, 1983). Alkaliphilic bacteria develop low proton and high sodium concentrations, and are thus different from neutrophilic bacteria in energy metabolism (Pitryuk, 2001). Considerable efforts had been made to understand how cellular metabolism reacts to variations in pH (Ingram and Marr, 1996). The function and structure of the cell wall of a living cell may change during shift in alkalinity, due to passive and active (energy consuming) processes. In the present study, the effect of

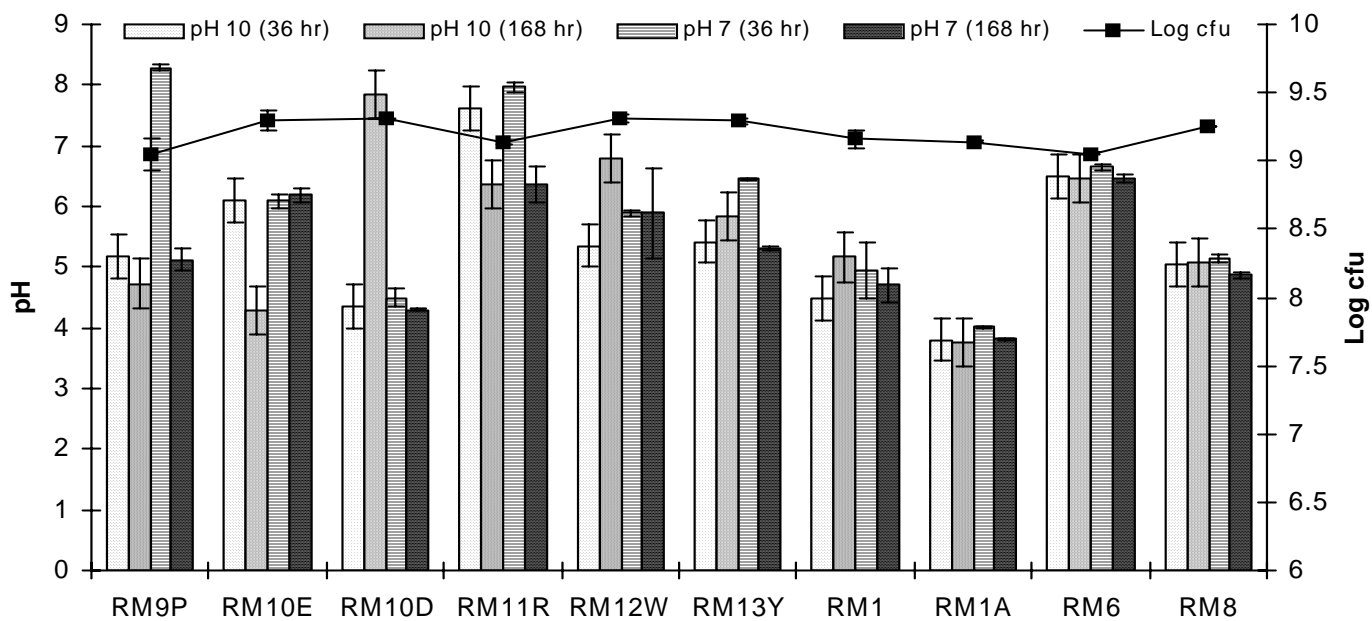


Fig 5.1: Reduction of nutrient media (pH 7.0) and its variant (pH 10.0) by red mud isolates at different time intervals. Values are mean \pm SD (n = 3)

different pH was studied on two selected bacterial red mud isolates. Out of ten isolates, RM9P and RM8 were found to grow vigorously at neutral (pH 7.0) and alkaline (pH 11.0) buffered condition. Hence, these two isolates were taken as representative isolates of red mud to study the behavior at different pHs and also to study the alkaline tolerance mechanism.

The buffering capacities of red mud isolates RM9P and RM8 were measured at pH 7.0 and 10.0. These isolates were grown in modified nutrient media having pH 7.0 and 10.0 using 50 mM of phosphate and carbonate buffer, respectively, in aerobic condition. pH ranges used for the estimation of buffering capacities were 6.5 to 9.5 for RM9P grown at pH 7.0, but pH ranges of 7.0 to 10.0 were used for the RM9P grown at pH 10.0. The buffering capacity of RM8 grown at pH 7.0 was observed in pH ranges 6.5 to 9.5 and 6.5 to 10.5 pH ranges were used for RM8 grown at pH 10.0 for buffering capacity estimation. Outer buffering capacity (Bo) and total buffering capacity (Bt) were estimated as a function of pH. There was considerable change in the buffering capacities of both isolates at various pHs. RM9P and RM8 when grown at pH 10.0 showed maximum total and outer buffering capacities when compared to bacterial growth at pH 7.0. RM9P grown in neutral conditions had shown maximum total and outer buffering capacities 5.0 and 1.6 (mM H⁺/pH unit/mg of protein) at pH 8.67 (Fig 5.2 a and b). But in alkaliphilic conditions, RM9P had exhibited 11.7 and 7.5 (mM H⁺/pH unit/mg of protein) of total and outer buffering capacities at pH 9.68. RM8 grown in neutral condition showed maximum 1.97 and 1.2 (mM H⁺/pH unit/mg of protein) of total and outer buffering capacities at pH 9.11 while at alkaline condition it showed 3.2 and 2.9 (mM H⁺/pH unit/mg of protein) of total and outer buffering capacities at pH 10.15 (Fig 7.3 a and b). Both bacterial isolates showed maximum buffering capacities in alkaline conditions irrespective of their growth conditions. Both the bacterial isolates showed lower buffering capacities when grown at neutral conditions as compared to the alkaline conditions. RM9P had more buffering capacity than the RM8 at both pH 7.0 and 10.0 (Fig 5.2 and 5.3). The total buffering capacity of RM9P in alkaline condition was 4 fold higher than the RM8 in same condition and 6 fold higher than the RM8 grown in neutral condition.

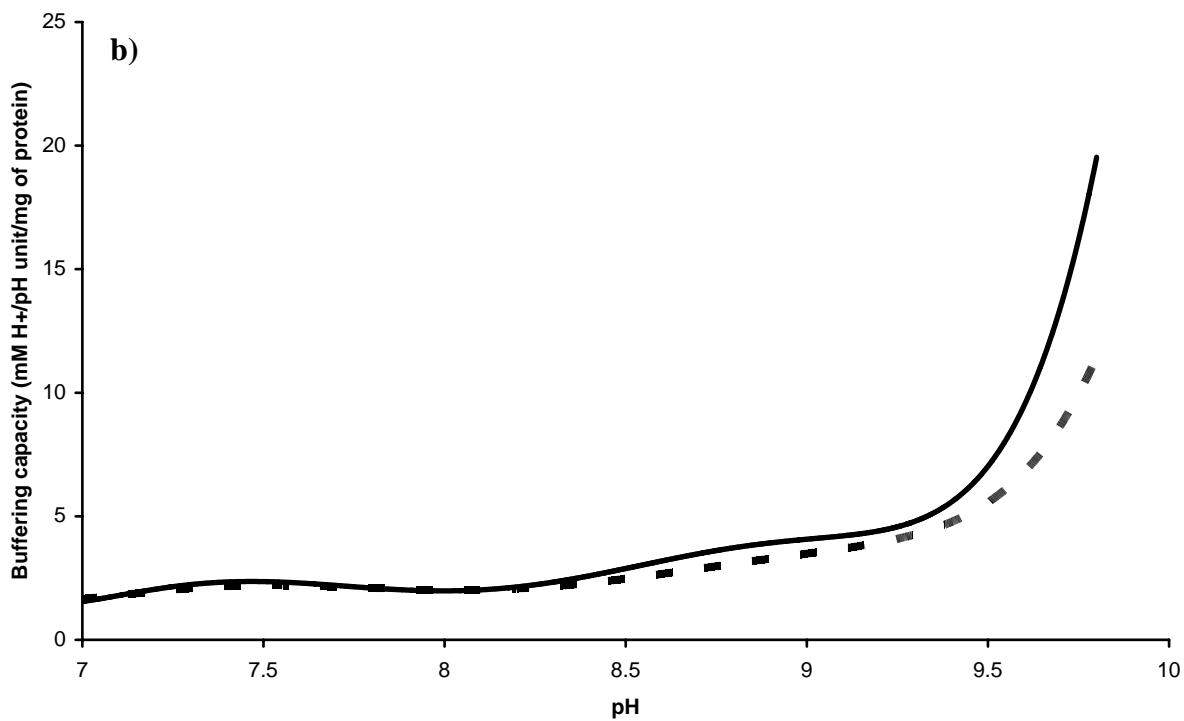
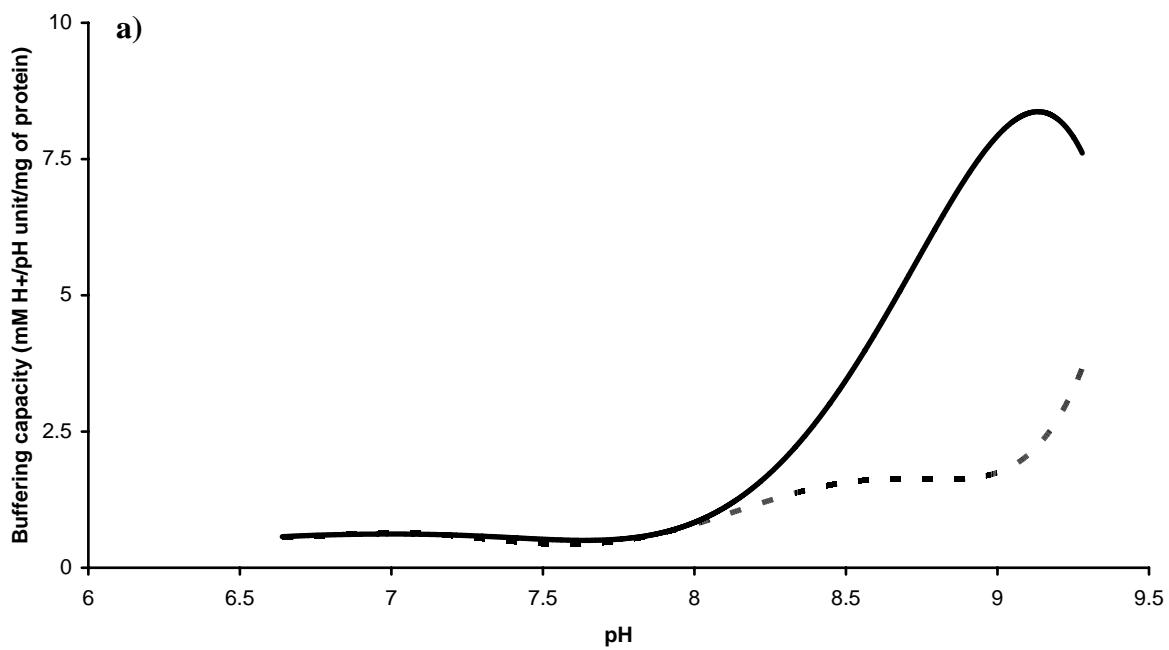


Fig 5.2: Total (firm line) and Outer (dotted line) buffering capacities of RM9P at a) pH 7.0 and b) pH 10.0

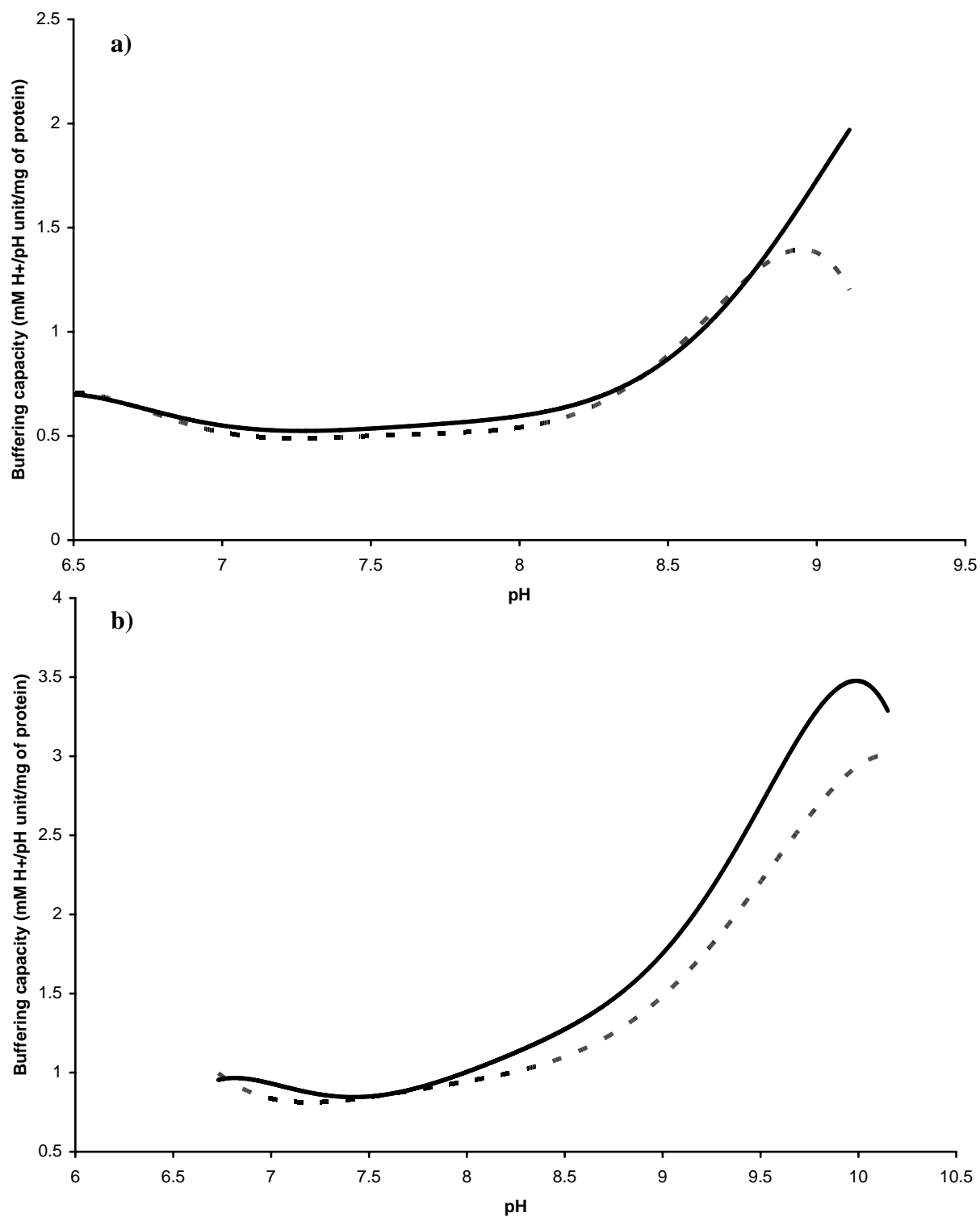


Fig 5.3: Total (firm line) and Outer (dotted line) buffering capacities of RM8 at a) pH 7.0 and b) pH 10.0

Effect of alkalinity on glycolysis of the red mud isolates RM9P and RM8

Whether rise in external pH can affect the glycolysis or energy building process intracellularly was checked by growing the bacterial isolates RM9P and RM8 at four different pHs i.e. 7, 8, 9 and 10. Maximum glycolytic activity of RM9P was observed at pH 7.0. It decreased with the increase of pH (Fig 5.4 a). At pH 7.0, unused glucose was 3.9 mM and it had increased to 9.0 mM at pH 10.0. Glycolytic activity was checked in presence of valinomycin, which dissipates Δ pH, using both the red mud isolates at various pHs (7, 8, 9 and 10) (Fig 5.4 a and b). Glycolytic activity of RM9P was rapidly decreased in presence of ionophore valinomycin at pH 7.0. It was 6.17 mM of unspent glucose at pH 7.0, which was 58% increase from the corresponding non-valinomycin treated glycolytic activity of RM9P (Fig 5.4 a). The unused glucose was increased from pH 7.0 to 10. The abrupt rise in unused glucose was found from pH 7.0 to 8.0 (3.9 to 8.9 mM of glucose) in non-valinomycin treated RM9P bacterial strain, which was not evident in case of valinomycin treated cells. In valinomycin treated cells (RM9P) the 6.17 and 6.79 mM of unspent glucose was estimated at pH 7.0 and 8.0 respectively which showed slight decrease in glycolytic activity.

The maximum glycolytic activity of RM8 was observed at pH 7.0, which was decreased with the increase in external pHs (Fig 5.4 b). The trend of glycolytic activity of RM8 was similar as compared with RM9P. RM8 at pH 7.0 had 6.7 mM of unused glucose concentration, which was higher than the unused glucose concentration of 3.9 mM at pH 7.0 of RM9P. But, similar unused glucose concentration was found at pH 8.0 with valinomycin treated RM9P cells. In valinomycin treated and untreated cells of RM8, as the pH increased the glucose consumption was decreased. Unused glucose of 7.0 and 6.7 mM concentration was estimated at pH 7.0 in valinomycin treated and untreated cells which reached at 8.7 and 8.5 mM of unused glucose at pH 10.0 (Fig 5.4). At pH 8.0 and 9.0, relatively constant values of 7.6 and 7.7 were observed. As compared with the RM9P (untreated cells), RM8 did not show decrease in glycolytic activity with increasing pH.

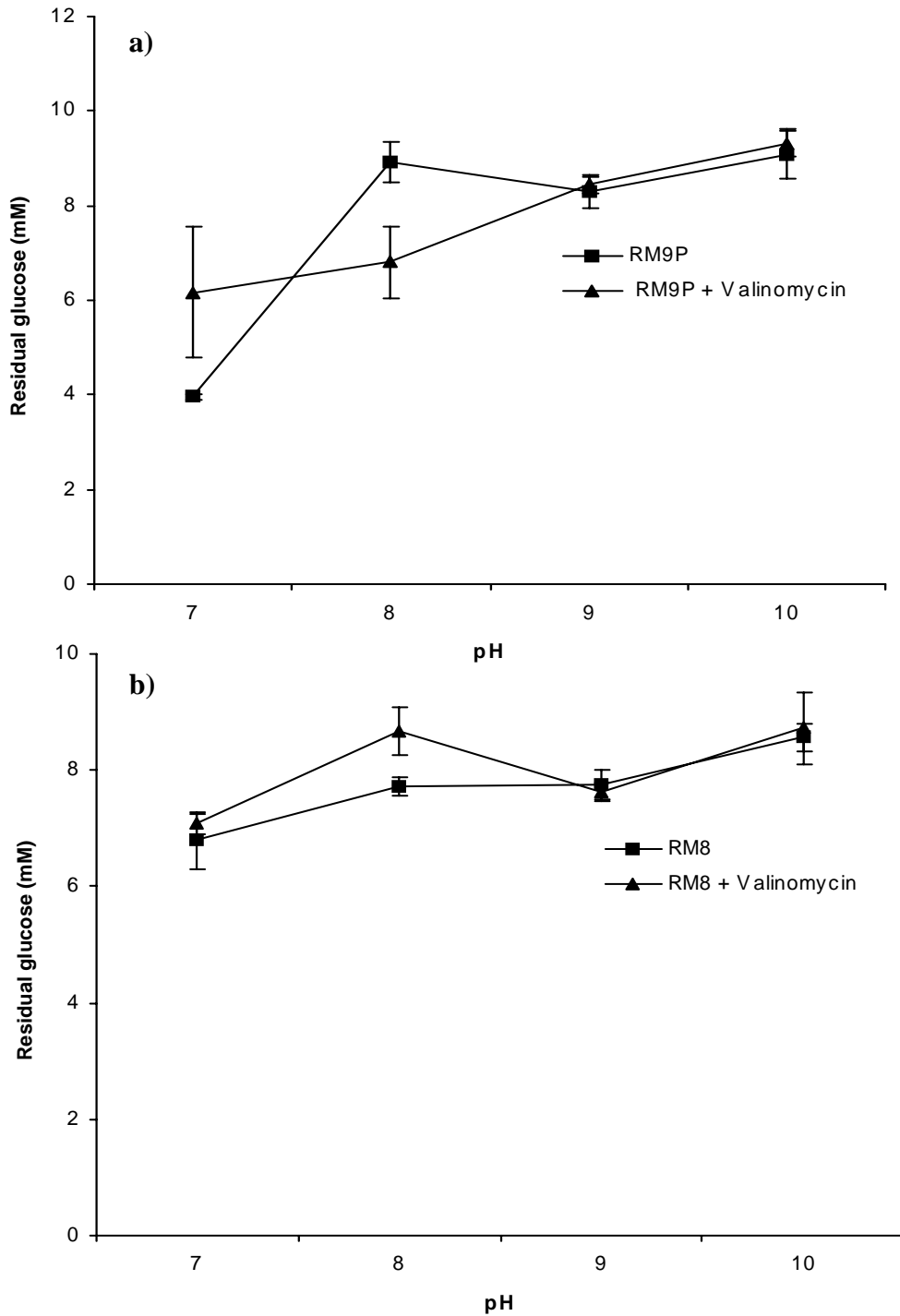


Fig 5.4: Glycolysis activities of red mud isolate a) RM9P and b) RM8. The experiments were carried out in the presence (triangle symbols) or absence (square symbols) of valinomycin at different pHs. Data are mean \pm SD (n=3)

Effect of alkalinity on the zeta potential of the red mud isolates RM9P and RM8

Surface charge determination was done using zeta potential analyzer. Alkaline condition might alter the physiology and cell membrane structure of the cell. Zeta potential of RM9P and RM8 were estimated at various pHs (7, 8, 9 and 10), where the cells were grown. The maximum zeta potentials, -28.5 and -24.6 mV, were observed at pH 7.0 in RM9P and RM8 respectively. With the increase in alkalinity the charge was increased. Zeta potentials of -22.3 and -18.3 mV were observed at pH 10.0 in RM9P and RM8, respectively. Higher zeta potential was observed in case of RM8 where the negativity of the surface was less (Fig 5.5).

Organic acid production

At pH 7.0, 8.0, 9.0 and 10.0, organic acid exudation by RM9P and RM8 were checked in presence and absence of valinomycin. Under oxic condition; oxalic, succinic, trace amount of acetic and two other low molecular wt. organic acids were detected. In comparison to RM9P, the exudation of organic acid was low in RM8. Organic acids were detected more in normal cells at various pHs than the valinomycin-treated cells in both the isolates. However, at pH 8.0, RM9P showed more acid production in case of valinomycin treated cells than the normal cells. The production of organic acids was increased with the increase in alkalinity (pH) i.e. from pH 7.0 to 9.0 and then decreased at pH 10.0. The maximum organic acid recorded in RM9P isolates were 290 and 234 nMoles/10 μ l at pH 9; and 78 and 38 nMoles/10 μ l were recorded in RM8 isolate in valinomycin untreated and treated cells, respectively (Fig 5.6).

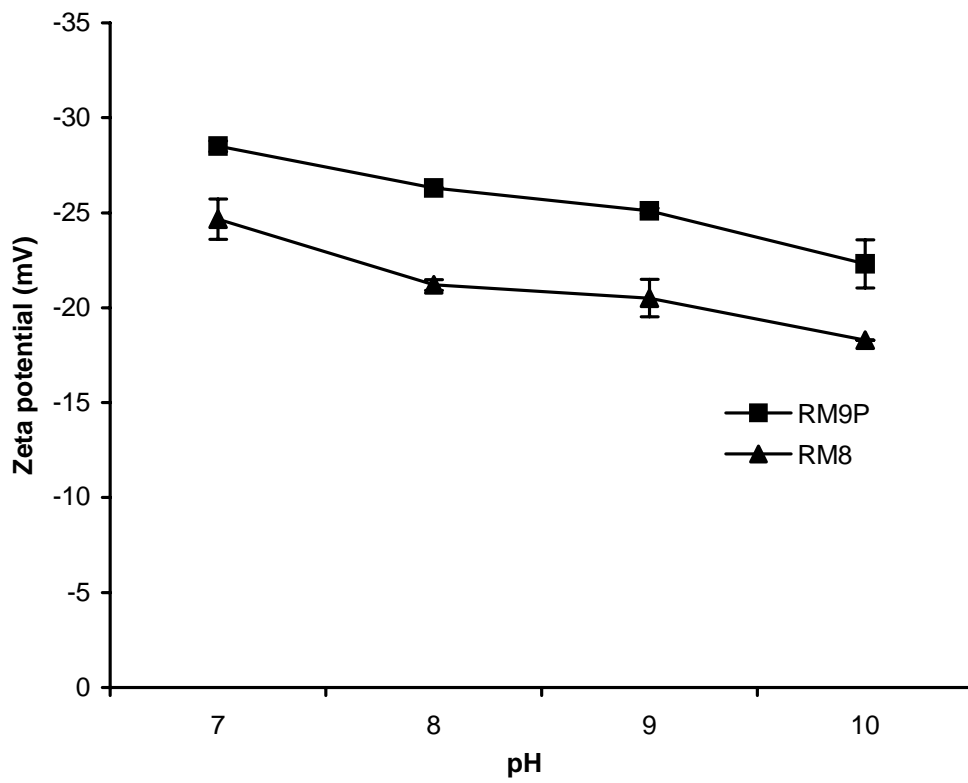


Fig 5.5: Zeta potential of red mud isolate RM9P (square symbols) and RM8 (triangle symbols) at different pHs. Values are mean \pm SD (n=3)

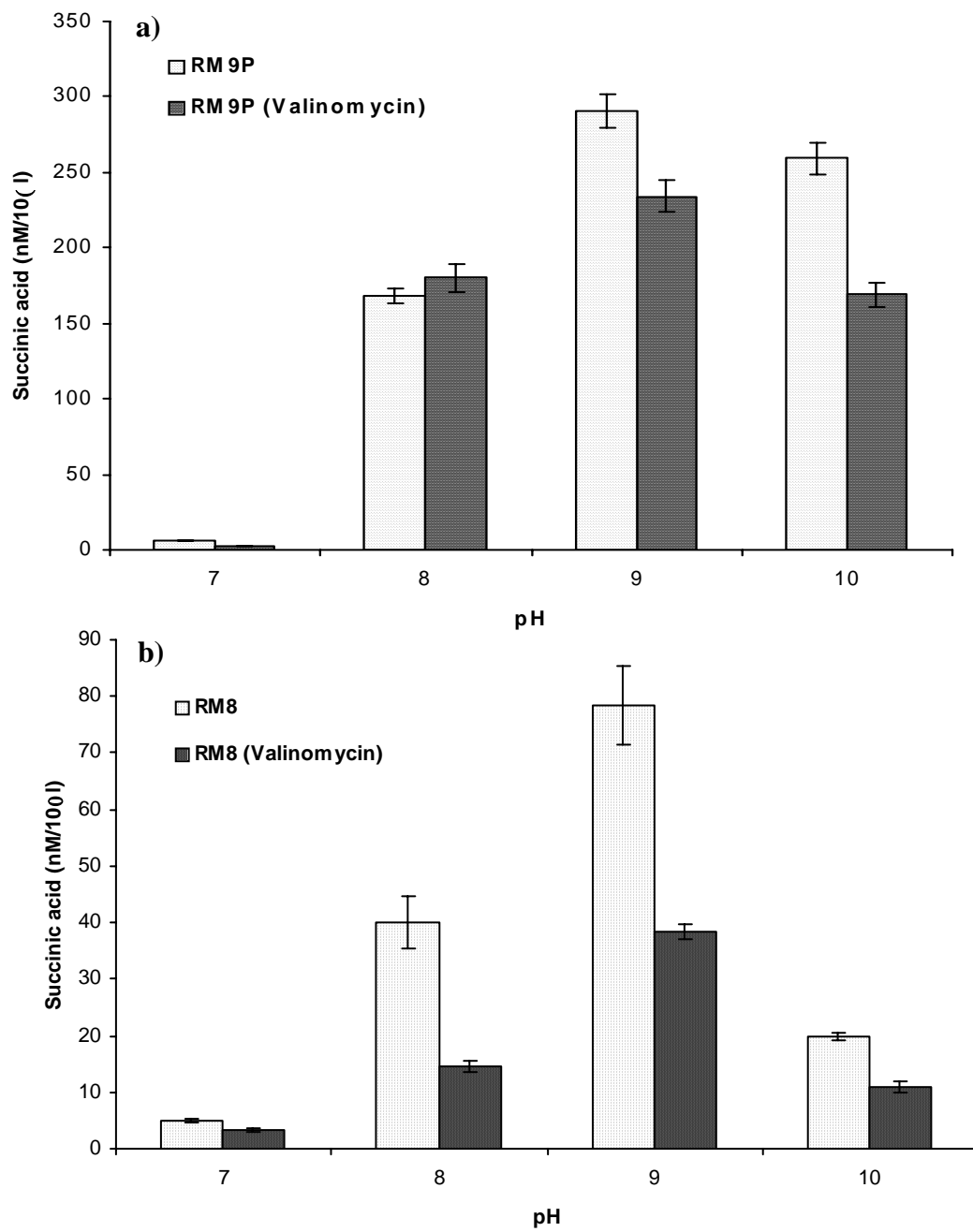


Fig 5.6 :Succinic acid exudation at various pHs in presence and absence of valinomycin in red mud isolates a)RM9P and b) RM8. Mean \pm SD (n=3)

Salient features

- All bacterial isolates were grown in media where pH was set at 7.0 and 10.0. These isolates had shown pH reduction of the media either at 36 or 168 hr. The maximum reduction of the pH was observed in case of RM1A isolate.
- Two isolates RM9P and RM8, were studied for alkaline tolerance mechanism because of their growth at neutral and alkaline pH. RM8 showed more buffering capacity at both the neutral (pH 7.0) and alkaline (pH 10.0) range as compared to RM9P.
- Glucose consumption was more at neutral pH in both RM9P and RM8 isolates. It had decreased with the increase of alkalinity. RM9P had shown more glucose consumption than the RM8 at pH 7.0.
- Zeta potential of both the isolates was increased with the increase of alkalinity. RM9P had shown more negativity in zeta potential at all pH tested as compared to RM8.
- Organic acid exudation was higher in isolate RM9P than the RM8. Succinic acid exudation was increased up to pH 9.0 in both the isolates RM9P and RM8.

***In vitro* studies of *Aspergillus tubingensis* in red mud amended media**

Aspergillus species are widely known for their organic acid producing capacity and mineralization of phosphate. The possibility of using exogenous *Aspergillus* sp., which are efficient organic acid producers, to reduce the alkalinity of the red mud, was explored in this study.

The ability of *A. tubingensis* to reduce the medium pH was tested in this study so as to check whether this organism could reduce the pH of the red mud amended soil. *A. tubingensis* was grown in different pHs and it had been observed that *A. tubingensis* was able to grow from pH 2.5 to 12.0. The maximum growth was observed at the pH 3.5 (Fig 6.1). Growth in neutral to alkaline (pH 7 and 12) condition was less as compared to growth at acidic pH. Profound growth (>1.0 g/L) was observed at acidic pH (3.5 to 6.5). The final pH of the media was below 3, except that of *A. tubingensis* grew at pH 11 and 12 where the final pH of the medium was reduced to 4.8 and 4.9 respectively (Fig 6.1).

Red mud contains high level of aluminum, iron and sodium. Hence, *A. tubingensis* was grown in different concentrations of aluminum, iron and sodium to study the tolerance. Three concentrations of Al (0, 100 and 200 µg/ml) were used to know the effect on *A. tubingensis*. 100 µg/ml of Al concentration had effect on growth and the growth was increased by 50% from the control while at 200 µg/ml of Al it decreased by 57% compared to control (Fig 6.2). Aluminum significantly inhibited the growth of *A. tubingensis* at 200 µg/ml (Fig 6.2). To determine the maximum concentration of NaCl tolerance by *A. tubingensis*, different concentrations of NaCl were amended in the media. The results showed that up to 100 µg/ml of Na, the growth of *A. tubingensis* was not affected, but at higher concentrations (150 and 200 µg/ml), the growth was inhibited. The level of Na increased in the mycelium as the concentration of Na increases in the growth medium (Fig 6.3).

The ability of *A. tubingensis* to grow in presence of different Fe concentrations was tested and the results indicated that the growth of *A. tubingensis* increased up to 600 µg/ml of Fe tested in this study when compared to control. The maximum growth of *A. tubingensis* was recorded at 400 µg/ml of Fe. Growth was increased in all iron-

amended media as compared to control. The level of Fe accumulation was also significantly increased as the concentration of Fe increased in the medium ((Fig 6.4).

When *A. tubingensis* was grown in presence of different concentrations of red mud, the mycelial growth increased as the concentration of the red mud amended in the medium was increased. The maximum growth was observed when the *A. tubingensis* was grown at 10% red mud amended medium. The pH of the medium was drastically reduced to below 3.0 in all the treatments after the growth of the *A. tubingensis* (Fig 6.5). The mycelium grown in presence of red mud was digested and analyzed for different element such as Al, Na, and Fe as these are normally present in red mud at higher concentration. The Al level increased in the mycelia with the increase in the concentration of the red mud. The maximum uptake of the Al was observed in presence of 10% of red mud (Fig 6.6). The level of Na decreased as the concentration of red mud increased in the growth medium. The content of Fe increased in the mycelium as the concentration of red mud increased in the medium (Fig 6.6). From these results, it was clear that *A. tubingensis* could tolerate high levels of Na, Fe, Al and high pH when inoculated into the red mud soil.

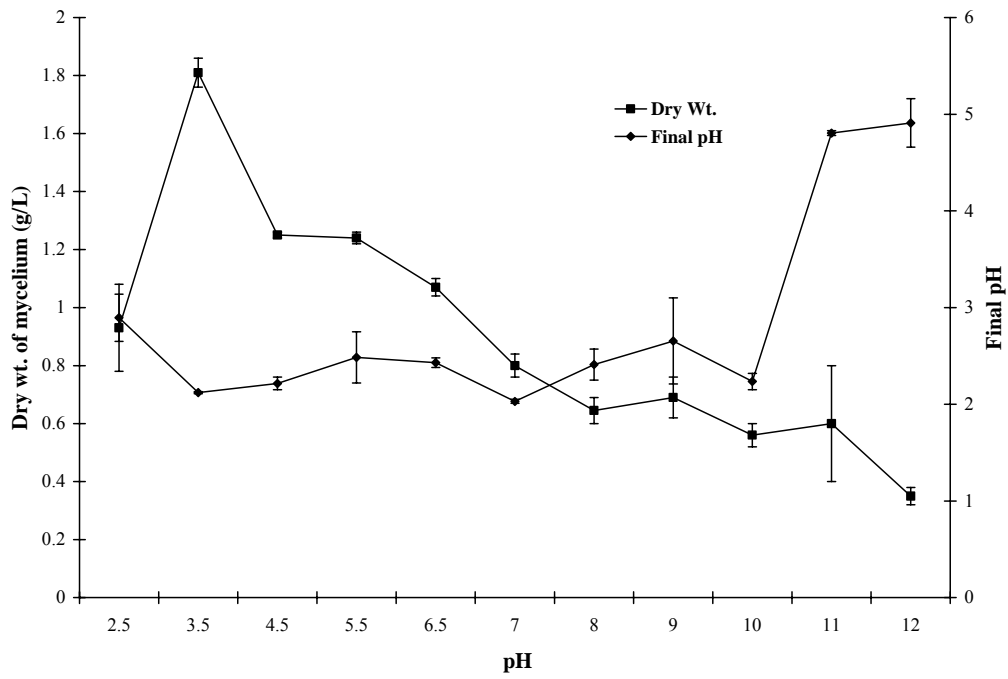


Fig. 6.1. Effect of pH on the mycelial growth of *A. tubingensis* and the final pH of the culture filtrate. Data are mean \pm SEM.

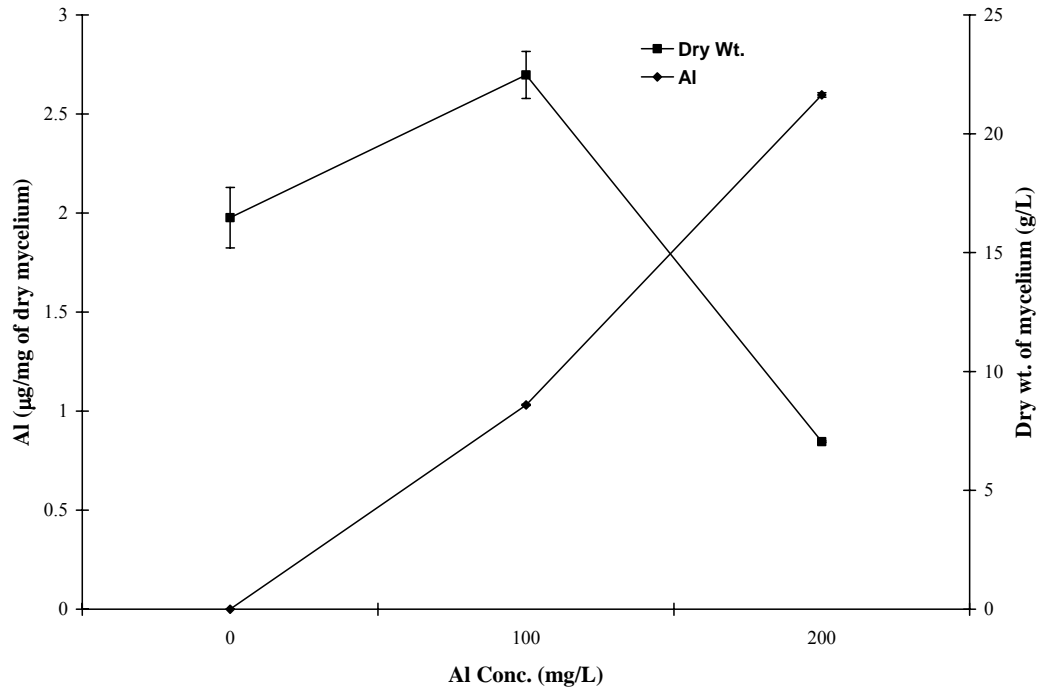


Fig 6.2: Influence of Al on the growth of *A. tubingensis* and their accumulation in the mycelium. Data are mean \pm SEM.

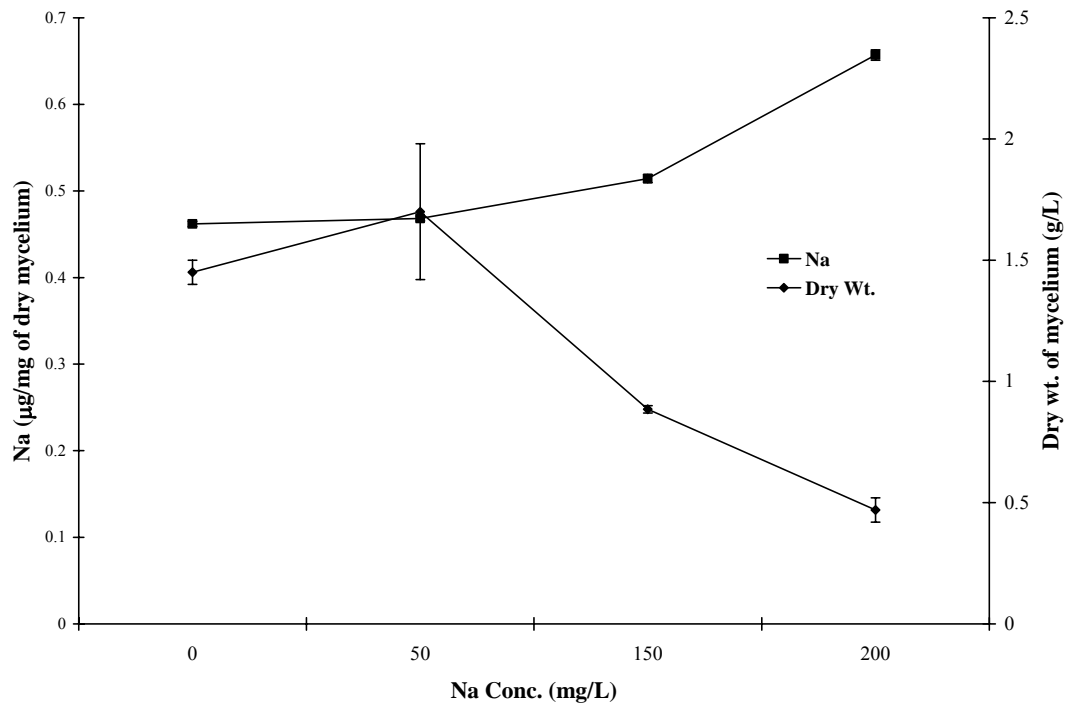


Fig 6.3: Influence of Na on the growth of *A. tubingensis* and their accumulation in the mycelium. Data are mean \pm SEM.

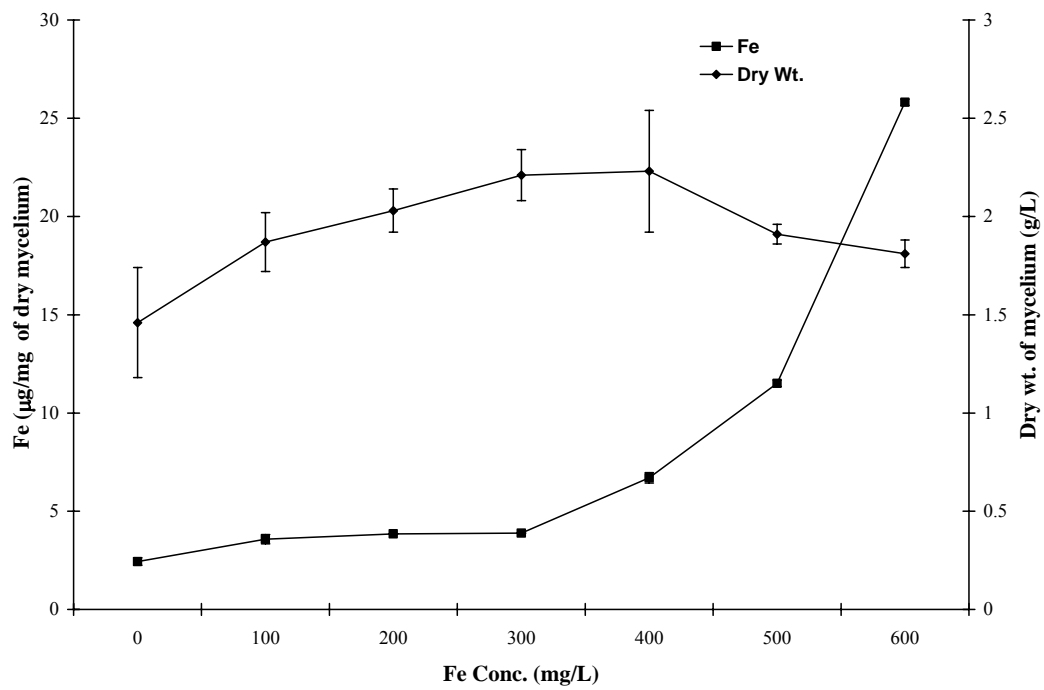


Fig. 6.4: Influence of Fe on the growth of *A. tubingensis* and their accumulation in the mycelium. Data are mean \pm SEM.

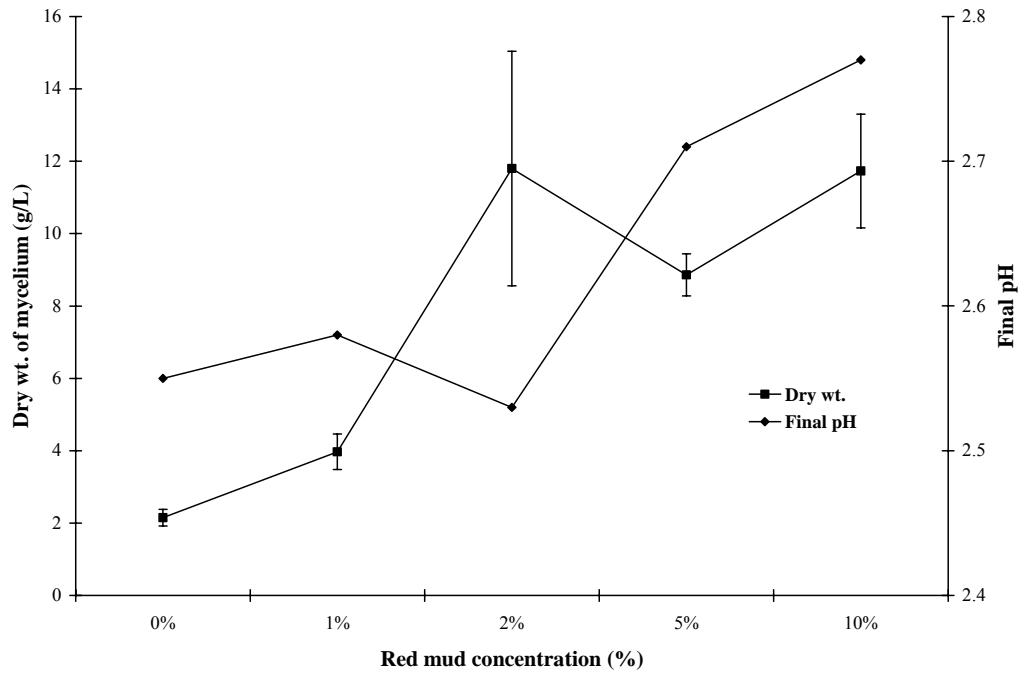


Fig 6.5: Mycelial growth of *A. tubingensis* and the final pH of the culture filtrate when grown in presence of different red mud concentrations. Data are mean \pm SEM.

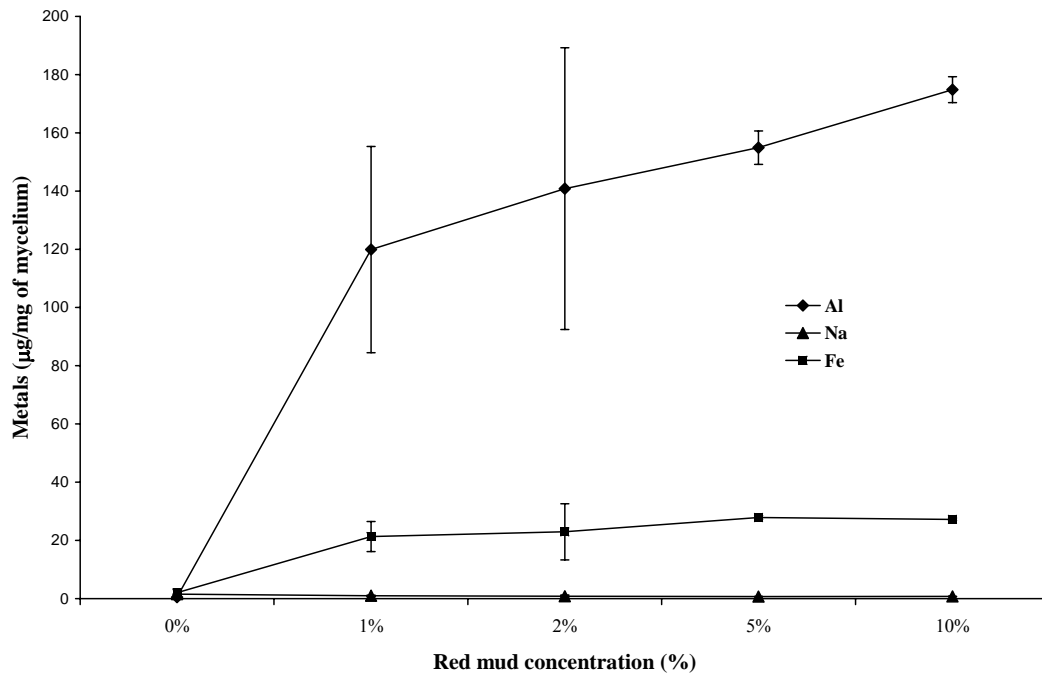


Fig. 6.6: Accumulation of different metals (Al, Na and Fe) by *A. tubingensis* in presence of red mud. Data are mean \pm SEM.

Salient features

- *Aspergillus tubingensis* had shown growth in Czapek-Dox medium in the pH range 2.5 to 12.0. It was able to grow at high concentration of aluminum, iron and sodium when grown in metal amended Czapek-Dox medium.
- When grown in red mud (0-10%) amended media, the pH was reduced to less than 3.0. Aluminum and iron content was increased in *A. tubingensis* with the increase of red mud concentration, but sodium content was decreased with the increase of red mud concentration.

Amelioration of red mud in nursery studies

Nursery trial

Sub optimal level of nutrients combined with high sodium, pH and other heavy metals are considered to be the major constraints of red mud revegetation and bioremediation. An attempt was made to alleviate the toxicity of the red mud by using different chemical treatments like gypsum, top soil, sewage sludge and fly ash; and consortium of bacteria (isolated from the red mud) and *Aspergillus tubingensis*, were used as biological treatments.

The physicochemical characteristics of the red mud samples were analyzed which were collected over the span of three years in different seasons from National Aluminum Company (NALCO), Damanjodi, Orissa, India. It had very stable chemical composition, characterized by the dominance of iron, aluminum and sodium. The pH of the bauxite residue was very high and never recorded below 10.3. The organic carbon, available phosphorus and total nitrogen was very low. Predominantly, iron and aluminium are two major constituents of red mud (Table 7.1). Anion analysis of red mud revealed the presence of different anions such as - chloride 17 ± 5.9 (g/L); sulphate 1082 ± 302.5 (g/L); carbonate 4.766 ± 1.6 (g/L); nitrate 4.35 ± 0.4 (g/L); fluoride 13.1 ± 3.8 (g/L); silicate 4.65 ± 0.71 (g/L) and sulphide was not detectable.

Application of different ameliorants such as gypsum, topsoil, fly ash and sludge with red mud was studied to remediate the red mud. The addition of 5–25% w/w gypsum to red mud reduced the pH to 10.5–7.5 as compared with pH 11.9 of red mud (Fig. 7.1, Table 7.1.1). Higher reduction of pH was evident in gypsum than the other ameliorants. The reduction of pH by gypsum is mainly attributed to the acidity of gypsum. Addition of sludge at different concentration showed ~2 unit of pH decrease, while other ameliorant decreased about ~1 unit of pH (Fig. 7.1, Table 7.1.1).

Top soil, fly ash and sludge (20%) and gypsum (10%) were amended with red mud and the physico-chemical characteristics of these mixtures were studied. Maximum organic carbon and organic matter were recorded in sludge amended red mud, followed by fly ash and topsoil as compared to red mud (Table 7.1). Available phosphorus content was higher in sludge and fly ash amended red mud as compared to other amendments (Table 7.1). Total nitrogen was highest in sludge followed by topsoil and fly ash amended

red mud. But no nitrogen was detected by TOC (total organic carbon) analyzer in red mud and gypsum amended red mud (Table 7.1). Alkaline phosphatase, invertase and urease were recorded highest in sludge with red mud. Acid phosphatase and cellulase enzymes were recorded highest in fly ash with red mud. Enzymatic activities were very low in case of control red mud (Table 7.1). Neutrophilic bacteria and actinomycetes were present in more numbers in sludge amendment. Alkaliphilic bacteria were recorded highest in topsoil amendment followed by fly ash, sludge and gypsum when compared to control red mud (Table 7.1). Metal analysis of these amendments showed that iron content was highest in red mud followed by sludge amendment and lowest content was recorded in fly ash. Aluminum was recorded highest in fly ash amendment and lowest in sludge amendment. Titanium, sodium, calcium, potassium and magnesium were also analyzed (Table 7.1).

An alkali tolerant, Bermuda grass which is native of aluminum refinery area in Damanjodi (from where the sample was collected) was planted in each treatment to see the performance and survival of this grass (Fig. 7.1.1; Table 7.1.1). Data were collected after completion of the six months of the experiments. A total of twenty different treatments which were used in the present study are as follows: Red Mud; red mud amended with bacteria (RMB); red mud with *A. tubingensis* (RMA); red mud with sludge (RMSC); red mud with sludge and bacteria (RMSB); red mud with sludge and *A. tubingensis* (RMSA); red mud with fly ash (RMFC); red mud with fly ash and bacteria (RMFB); red mud with fly ash and *A. tubingensis* (RMFA); red mud with gypsum and bacteria (RMGB); red mud with gypsum and *A. tubingensis* (RMGA); red mud with top soil (RMTSC); red mud with top soil and bacteria (RMTSB) and red mud with top soil and *A. tubingensis* (RMTSA) (Fig 7.1.1; Table 7.1.1).

Table 7.1: Chemical and microbiological characteristics of red mud amended with different treatments

Chemical parameters	Red mud	Red mud with gypsum	Red mud with sludge	Red mud with topsoil	Red mud with fly ash
% C	0.3±0.05	0.4±0.01	1.18±0.01	0.55±0.02	0.66±0.04
pH	11.90±0.10	9.73±0.25	11.15±0.1	11.34±0.20	11.50±0.1
Organic matter	0.6±0.17	0.8±0.33	2.04±0.8	0.95±0.37	1.16±0.43
Avail. P (mg/Kg)	0.2±0.1	0.3±0.02	1.81±0.2	0.38±0.1	1.68±0.32
Nitrogen (mg/Kg)	0	0	6 ±0.2	4.9± 0.23	4.9±0.26
Alkaline phosphatase (µM/g/hr)	3±10	790±2.01	900±21	600±2	135.17±2
Acid phosphatase (µM/g/hr)	99±10	340±9.8	300±9	270±5	108±15
Cellulase (µM/g/24hr)	100±9.9	700±10	590±10	330±10	497±85
Invertase (µM/g/3hr)	150±10	199±10	1500±10	470±9	669±10
Urease (µM/g/hr)	2±1	21±1.07	23.26±1	16.51±2	22.44±1.4
Neutrophilic bacteria (cfu x10⁴/g)	17±1	439±14	1211±80	937±73	982±57
Alkaliphilic bacteria (cfu x10⁴/g)	9.5±4.3	92.5±13	199±22	230±79	217±15
Actinomycetes (cfu x10⁴/g)	6.5±2	58.5±2	146±11	83±7	32±5
Iron (%)	47.3±1	37.6±1.1	43.6±4.4	37.4±1	31.5±1.1
Titanium (%)	2.2±0.4	1.9±0.14	2.1±0.55	1.9±0.50	1.7±0.5
Calcium (%)	1.2±0.09	0.8±0.05	0.8±0.09	1.02±0.1	0.55±0.05
Potassium (%)	0.2±0.07	0.05±0.01	0.03	0.11±0.07	0.12±0.02
Magnesium (%)	0.1±0.01	0.1±0.01	0.13±0.01	0.12±0.03	0.02±0.01
Sodium (%)	5.7±0.1	5.3±0.05	4.8±0.04	5.5±0.04	3.2±0.05
Aluminium (%)	8±0.50	7.2±0.7	6.6±0.04	7.2±0.03	11.8±0.3

Values are mean ± SD (n=3)

Table 7.1.1: Reduction of pH of red mud with addition of different concentrations of amendments

Substrate (%)	Gypsum	Sludge	Fly ash	Top soil
0	11.9±0.2	11.9±0.1	11.9±0.1	11.9±0.2
5	10.5±0.2	11.63±0.1	11.7±0.2	11.57±0.2
10	9.7±0.5	11.15±0.2	11.5±0.3	11.3±0.2
15	9.5±0.14	11.03±0.2	11.3±0.1	11.21±0.1
20	9.0±0.3	10.63±0.3	11.3±0.2	11.17±0.2
25	7.5±0.3	10.01±0.2	11.21±0.3	11.1±0.2

Values are mean ± SD (n =3)

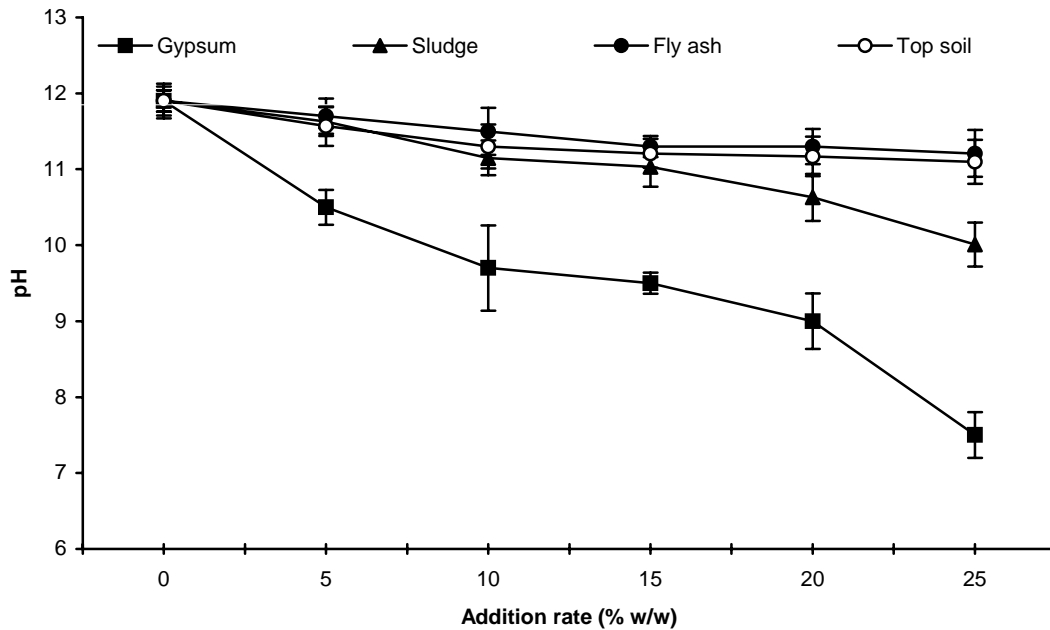


Fig 7.1: Reduction of pH of red mud with addition of different substrates. Values are mean ± SD (n = 3)

Table 7.1.2: Schematic representation of different treatments of nursery trial

Red mud with fly ash	Red mud with gypsum	Red mud with sludge	Red mud with topsoil	Red mud
Red mud with fly ash and <i>A. tubingensis</i>	Red mud with gypsum and <i>A. tubingensis</i>	Red mud with sludge and <i>A. tubingensis</i>	Red mud with topsoil and <i>A. tubingensis</i>	Red mud with <i>A. tubingensis</i>
Red mud with fly ash and bacteria	Red mud with gypsum and bacteria	Red mud with sludge and bacteria	Red mud with topsoil and bacteria	Red mud with bacteria



Fig 7.1.1: Effect of indigenous red mud bacterial consortia and *Aspergillus tubingensis* for the ameloration of the bauxite residue amended with different substrates in nursery trail after six months. Column 1, 2, 3, 4 and 5 are red mud amended with fly ash, gypsum, sludge, topsoil and red mud alone respectively. Row a, b and c is without microbes, with *A. tubingensis* and with indigenous bacterial consortium respectively.

Chemical analysis of soil

Reduction of pH is a primary aspect of bioremediation of bauxite residue. The pH of the red mud was ~12.0 at the beginning of the nursery experiment, which was significantly decreased in all the treatments (Table 7.1.3). In the red mud control treatment, there was a reduction of pH of one unit after six months as compared to initial pH, which was further decreased to 0.5 unit in both bacteria and *A. tubingensis* inoculated red mud treatments (Table 7.1.3). In the sludge amended red mud, initial pH of 11.15 decreased to 10.27 after six months, in the control treatment. *A. tubingensis* treatment had significantly decreased the pH to 9.86 as compared to bacterial treatment (10.04) and control (10.27). The control treatment of fly ash amended red mud had one unit of pH decrease after six months as compared to initial sample. In this treatment also, there was no significant difference observed between bacterial and *A. tubingensis* treatments. However, these two treatments had significantly decreased pH with respect to control (Table 7.1.3). The maximum decrease of pH was observed in gypsum amended red mud. Initial pH of 9.73 was decreased to 8.05 in control treatment after six months. However, no significant decrease was observed in control, bacterial and *A. tubingensis* treatments (Table 7.1.3). The initial pH of topsoil amended red mud was 11.34, which decreased significantly to 9.85 in control treatment after six month. The bacterial and control (six month) treatments showed no significant difference in pH reduction. But, *A. tubingensis* showed more significant reduction compared to other treatments of sludge amendment (Table 7.1.3). Overall, a maximum reduction of 4 units of pH was observed in gypsum amendment followed by top soil (Table 7.1.3).

Electrical conductivity (EC)

Soil electrical conductivity (EC) is a property of soil that is determined by standardized measures of soil conductance (resistance⁻¹). Soil electrical conductivity is related to factors that affect soil productivity, use, and management. Red mud had high EC of 3.84 (mS/cm), which was significantly decreased to 2.89 mS/cm after six months time in control treatment. The bacterial treatment had shown maximum significant reduction of EC (0.74 mS/cm) as compared with *A. tubingensis* (1.47 mS/cm) and control

red mud treatment (Table 7.1.3). The EC of sludge-amended red mud was reduced to 0.64 from 3.5 mS/cm in six months compared to control treatment. A slight reduction was observed in bacterial and *A. tubingenis* treatments as compared to control treatment of sludge amendment (Table 7.1.3). Fly ash-amended red mud had significantly decreased EC from 3.97 mS/cm to 0.53 mS/cm in six months in control treatment. No significant difference was observed in control (six month), bacteria and *A. tubingenis* treatments (Table 7.1.3). The least reduction of EC was observed in case of gypsum amended red mud after six months time. The initial EC of 3.63 was decreased to 1.8 mS/cm in six month in control treatment and no significant difference was observed in control, bacterial and *A. tubingenis* treatments (Table 7.1.3). The top soil-amended red mud had initial EC of 3.2 mS/cm, which was decreased to 0.4 mS/cm in six months in control treatment. No significant difference was observed in control and bacterial treatments. Furthermore, slight reduction was observed in *A. tubingenis* as compared to control and bacteria (Table 7.1.3).

The maximum reduction of EC was observed in red mud with topsoil and *A. tubingenis*, which was 0.3 when compared to 3.8 mS/cm as that of control red mud (Table 7.1.3). The EC was significantly higher in gypsum treatments when compared to all other treatments but lower than that of control red mud. This was in contrast to the pH, as maximum pH reduction was noted in gypsum-amended red mud treatment (Table 7.1.3).

Table 7.1.3: Effect of bacterial consortia and *A. tubingensis* on the reduction of pH and electrical conductivity of the different amendments of red mud

Treatments	pH	EC (mS/cm)
Red mud		
Before	11.90±0.10a	3.84±0.17a
Control	11.08±0.01c	2.89±0.04e
Bacteria	10.48±0.03de	0.74±0.01h
<i>A. tubingensis</i>	10.49±0.02de	1.47±0.02g
Red mud with sludge		
Before	11.15±0.1c	3.5±0.1c
Control	10.27±0.02f	0.64±0.01hi
Bacteria	10.04±0.02g	0.50±0.01ij
<i>A. tubingensis</i>	9.86±0.04h	0.46±0.005j
Red mud with fly ash		
Before	11.50±0.1b	3.97±0.07a
Control	10.55±0.08d	0.53±0.01ij
Bacteria	10.34±0.07ef	0.5±0.01ij
<i>A. tubingensis</i>	10.35±0.08ef	0.53±0.04ij
Red mud with gypsum		
Before	9.73±0.25hi	3.63±0.25b
Control	8.05±0.03j	1.8±0.04f
Bacteria	7.94±0.09j	1.80±0.02f
<i>A. tubingensis</i>	7.96±0.05j	1.71±0.01f
Red mud with top soil		
Before	11.34±0.20b	3.2±0.1d
Control	9.85±0.02h	0.4±0.01jk
Bacteria	9.81±0.07h	0.41±0.005jk
<i>A. tubingensis</i>	9.64±0.04i	0.30±0.01k

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are mean \pm SD (n = 3)

Organic carbon and organic matter

Organic carbon and organic matter were significantly increased in bacterial and *A. tubingen* inoculated red mud when compared to control red mud treatment. Organic carbon and organic matter increased 160% and 125% in bacterial and *A. tubingen* treatments respectively as compared to control red mud (Table 7.1.4). In gypsum amended red mud, no significant difference in organic carbon and organic matter content was observed in control and *A. tubingen* treatments. A 50% increase of organic carbon and organic matter was observed in bacterial treatment when compared to control and *A. tubingen* treatment (Table 7.1.4). In sludge amended red mud, the bacterial treatment had higher content of organic carbon and organic matter as compared to control and *A. tubingen* treatment. *A. tubingen* treatment showed 109% higher content of organic carbon and organic matter when compared to control treatment (Table 7.1.4). Topsoil amended red mud had no significant difference in organic carbon and organic matter in control and *A. tubingen* treatments. The bacterial treatment had shown 84% increase in organic carbon and organic matter when compared to control *A. tubingen* treatment (Table 7.1.4). Red mud amended with fly ash had also shown increased organic carbon and organic matter in all treatments. A small increase was observed in *A. tubingen* treatment as compared to control. Bacterial treatment showed significantly more organic carbon and organic matter as compared to control and *A. tubingen* treatments (Table 7.1.4). Organic carbon and organic matter was increased in all treatments (Table 7.1.4). These were recorded highest in sludge, followed by fly ash, topsoil, gypsum and without any chemical amendment of red mud respectively. In sludge amended red mud, organic carbon (OC) and organic matter (OM) were highest in bacteria followed by fungal amendment. Also, in all other treatments, OC and OM were recorded higher in the case of bacteria than the *A. tubingen* treatment (Table 7.1.4).

Available Phosphorus

Available phosphorus was similar in the red mud control after six months when compared to *A. tubingen* inoculated red mud. The bacterial treatment had significantly increased available P compared to control and *A. tubingen* treatment of red mud (Table

7.1.4). In gypsum amended red mud, control and bacterial treatment showed similar content of available P, while *A. tubingensis* treatment had significantly increased available P (Table 7.1.4). Bacterial inoculation in sludge amended red mud had increased available P as compared to control and *A. tubingensis* treatments. *A. tubingensis* treatment had shown 123% increase of available P as compared to control (Table 7.1.4).

In topsoil amended red mud, bacterial and control treatments had no significant difference in content of available P, but *A. tubingensis* had shown slight increase of available P compared with control and bacterial treatments (Table 7.1.4). The bacterial treatment of fly ash amended red mud, had significantly increased available P as compared to control treatment. Slightly decreased available P was observed in *A. tubingensis* treatment when compared with bacterial treatment (Table 7.1.4) of fly ash amended red mud. The available P was estimated highest in the same treatment where organic carbon was highest i.e. red mud amended with sludge and bacterial (Table 7.1.4). In three treatments red mud amended with sludge, fly ash and red mud alone; bacterial treatments had more available phosphorus than the *A. tubingensis* amended treatment. This trend was not observed in case of topsoil and gypsum amended red mud treatments. A more than 200% increase of available P was estimated in red mud with sludge and bacteria as compared to control (Table 7.1.4).

Nitrogen

Total nitrogen was increased significantly in bacteria inoculated red mud as compared with control red mud. A more than 400% increment was observed in bacterial treatment of red mud as compared to control. But, no nitrogen was estimated in *A. tubingensis* treatment of red mud (Table 7.1.4). Nitrogen was not detected in control and *A. tubingensis* treatment of gypsum amended red mud by total organic carbon (TOC) analyzer and kjeldahl method. An abrupt increase of 388 mg/Kg of nitrogen was recorded in bacteria inoculated, gypsum amended red mud when compared to control treatment, which was devoid of nitrogen (Table 7.1.4).

In sludge amended red mud, significant increase in total nitrogen was observed in bacterial and *A. tubingensis* treatments as compared to control. Bacterial treatments had significantly higher nitrogen than *A. tubingensis* and control treatments. Decreased total

nitrogen was observed in bacteria and *A. tubingensis* treatments of topsoil amended red mud when compared to the control. Control treatments had increased total nitrogen than the other two treatments. No significant difference was observed in bacteria and *A. tubingensis* treatment of topsoil amended red mud in relation to nitrogen (Table 7.1.4). In fly ash amended red mud, no difference was observed in all three treatments *i.e.* control, bacteria and *A. tubingensis* in relation to the nitrogen level (Table 7.1.4). Nitrogen was not detectable in red mud by kjeldahl method and total organic carbon (TOC) analyzer. The enormous increase was observed in gypsum with bacterial treatment and it was the highest total nitrogen among all treatments. Bacterial inoculation in all treatments showed increased nitrogen as compared to control and *A. tubingensis* treatments (Table 7.1.4).

Table 7.1.4: Effect of bacterial consortia and *A. tubingensis* on the chemical structure of different red mud amended treatments

Treatments	Organic Carbon (%)	Organic matter (%)	Avail. P (mg/Kg)	Nitrogen (mg/Kg)
Red mud				
Control	0.43±0.01j	0.75±0.30j	1.40±0.2fgh	5.68±0.2de
Bacteria	1.13±0.07ghi	2.03±0.75ghi	2.14±0.15de	29.3±0.7c
<i>A. tubingensis</i>	0.97±0.02hi	1.62±0.66hi	1.43±0.2fgh	0e
Red mud amended with gypsum				
Control	0.93±0.03i	1.69±0.63i	0.38±0.1i	0e
Bacteria	1.39±0.36efg	2.05±0.72efg	0.36±0.1i	388.1±11.5a
<i>A. tubingensis</i>	0.93±0.002i	1.61±0.65i	1.7±0.2efg	26.78±1.55c
Red mud amended with sludge				
Control	1.62±0.02e	2.79±1.13e	1.92±0.2ef	15.6±4.2d
Bacteria	3.82±0.11a	6.58±2.62a	5.90±0.2a	40.4±4.4b
<i>A. tubingensis</i>	3.40±0.09b	5.86±2.33b	4.30±0.2b	0e
Red mud amended with top soil				
Control	1.27±0.02fgh	2.20±0.88fgh	1.04±0.2h	10.8±2.9d
Bacteria	2.34±0.05d	4.04±1.62d	1.14±0.17h	5.5±1.1de
<i>A. tubingensis</i>	1.28±0.03fgh	2.21±0.88fgh	1.32±0.2gh	5.8±0.1de
Red mud amended with fly ash				
Control	1.45±0.04efg	2.50±0.99efg	1.71±0.1efg	5.9±0.2de
Bacteria	2.71±0.03c	4.68±1.89c	2.92±0.2c	6.4±0.6de
<i>A. tubingensis</i>	1.58±0.03ef	2.74±1.09ef	2.61±0.1cd	7.5±0.7de

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are mean \pm SD (n = 3)

Microbial count and their survival in different amendments of red mud

The total number of neutrophilic, alkaliphilic and actinomycetes were determined in each treatment.

Neutrophilic bacterial count

The neutrophilic bacterial count was significantly decreased in *A. tubingensis* inoculated red mud as compared to control red mud. Bacterial inoculation in red mud showed significantly higher number of (1449×10^4 cfu/g) of neutrophilic bacteria than of the control (197×10^4 cfu/g). In sludge-amended red mud, *A. tubingensis* treatment significantly increased in neutrophilic bacterial count compared to the control. The bacterial treatment had significantly decreased neutrophilic count than the control (Table 7.1.6). Bacterial and *A. tubingensis* treatments significantly increased the neutrophilic bacterial count in topsoil-amended red mud when compared with the control. *A. tubingensis* treatment significantly increased the numbers of neutrophilic bacterial count than the bacterial and control treatments (Table 7.1.6). In fly ash-amended red mud, bacteria and *A. tubingensis* had shown significant enhancement of the neutrophilic bacterial count as compared with control treatment. But, no significant difference was observed in bacterial and *A. tubingensis* treatments. In gypsum-amended red mud, bacterial and *A. tubingensis* treatments had significantly increased the neutrophilic bacterial count than the control red mud. Slight increase was observed in bacterial treatment as compared to *A. tubingensis* treatment (Table 7.1.6).

The highest number of neutrophilic bacteria (2840×10^4 cfu/g) were counted in topsoil with *A. tubingensis* amended red mud, which was two-fold increase from its control treatment (without any microbial amendment) and 180% higher than the control red mud (Table 7.1.6). This was followed by four treatments, namely red mud with topsoil and bacteria; fly ash with bacteria and *A. tubingensis*; and gypsum with bacteria, where there was no significant difference was observed. The number of neutrophilic bacteria ranged from 1900-2250 ($\times 10^4$ cfu/g) in these treatments (Table 7.1.6). The maximum rise of neutrophilic bacteria was observed in red mud with bacteria where it was $1449 (\times 10^4$ cfu/g) as compared to $197 (\times 10^4$ cfu/g) of control red mud.

Alkalitolerant bacterial count

The significant rise of alkalitolerant bacteria was observed in bacterial and *A. tubingenis* treatments of red mud as compared to the control. However, bacterial treatment showed significantly increased number of alkalitolerant bacteria as compared to the *A. tubingenis* treatment (Table 7.1.5). In sludge-amended red mud, the bacterial treatment had significantly increased number of alkalitolerant bacteria as compared to *A. tubingenis* and control treatments. *A. tubingenis* treatment significantly increased the number of alkalitolerant bacteria than the control treatment (Table 7.1.5). In topsoil-amended red mud and fly ash-amended red mud, the increase of alkalitolerant bacteria was relatively same. In these treatments, *A. tubingenis* and bacteria showed a increased number of alkalitolerant bacteria as compared to its respective control. *A. tubingenis* treatments showed a significantly increased number of alkalitolerant bacteria as compared with bacterial treatments (Table 7.1.5). In gypsum-amended red mud, bacterial treatment had shown significant increase of 1926 ($\times 10^4$ cfu/g) than control treatment (129 $\times 10^4$ cfu/g). *A. tubingenis* showed significant increase of alkalitolerant bacteria when compared with control but was less in comparison with bacterial treatment (Table 7.1.5).

The alkalitolerant bacterial population was recorded highest in red mud amended with topsoil and *A. tubingenis*; and red mud-amended with gypsum and bacteria. In these treatments, no significant difference was found. Bacterial treatments had higher amounts of alkalitolerant bacteria as compared to their respective controls. But, in red mud with fly ash amendment, no significant differences were found in bacterial treatment as compared to the control. *A. tubingenis* inoculants showed the increase of alkalitolerant bacteria in different treatments as compared to its control, except in the sludge amendment where it had decreased significantly than the control.

Actinomycetes count

No significant differences were observed in the actinomycetes count of control, bacterial and *A. tubingenis* treatments of in red mud; red mud amended with sludge; and red mud amended with fly ash treatments. No significant differences were observed in bacterial and control treatments of top soil amended red mud. *A. tubingenis* treatment had significantly increased actinomycetes than the control and bacterial treatments (Table 7.1.5). A significant increase of actinomycetes count was observed in bacteria and *A.*

tubingensis treatments of gypsum amended with red mud. But, no significant difference was observed in case of bacterial and *A. tubingensis* treatments. Actinomycetes counts were relatively less as compared to neutrophilic and alkalitolerant bacteria. Top soil with *A. tubingensis*; gypsum with *A. tubingensis* and bacteria had highest actinomycetes count in the range of 1075 to 1125 ($\times 10^4$ cfu/g) which were not significantly different (Table 7.1.5).

Table 7.1.5: Effect of bacterial consortia and *A. tubingensis* on the microbial count (neutrophilic, alkaliphilic and actinomycetes) of different red mud amended treatments of nursery experiment.

Treatments	Neutrophilic bacteria (x10 ⁴ cfu /g)	Alkaliphilic bacteria (x10 ⁴ cfu /g)	Actinomycetes (x10 ⁴ cfu /g)
Red mud with sludge			
Control	1184±79bcdef	1536±22ef	146±11.3b
Bacteria	462±70def	2570±342bc	229.5±10.6b
<i>A. tubingensis</i>	1880±73abc	1188±33fg	206±5.6b
Red mud with top soil			
Control	1340±73bcde	620±79gh	105.5±17.6b
Bacteria	1900±446ab	2700.5±351bc	158.5±3.5b
<i>A. tubingensis</i>	2840±158a	3654±3a	1125±91.9a
Red mud with fly ash			
Control	1782±579abc	2236±158cd	69±65b
Bacteria	2250±70ab	2196±5cd	217±12.7b
<i>A. tubingensis</i>	2381.5±75ab	2934±36b	387.5±41.5b
Red mud with gypsum			
Control	666±149cdef	129.5±13h	87.5±2.12b
Bacteria	1926.5±325ab	3630±19a	1225±35.3a
<i>A. tubingensis</i>	1888.5±334abc	2141±140cde	1075±106a
Red mud			
Control	197±70ef	209.5±43h	16.5±4.9b
Bacteria	1449±182bcd	1771.5±231def	58±5.6b
<i>A. tubingensis</i>	16±2f	1218±229fg	7±0.4b

Values sharing a common letter within the column are not significant at P<0.05. Values are mean ± SD (n = 3)

Metal analysis

The sources of metals in soil are diverse, including mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge amendments, the use of pigments and batteries. Metals in soil are present as free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides or they may form part of the structure of silicate minerals. The toxicity of metals in soil depends on their bioavailability. Red mud had high metal (Fe, Al and Na) content and appears red due to the presence of high content of iron oxide. Here, soil metals such as iron and aluminum along with some micronutrients were analyzed.

Soil iron content

Red mud control treatment had 42% of iron content after six months. It was significantly decreased in bacterial and *A. tubingensis* treatments of red mud. The bacterial treatment had shown significant reduction of iron content when compared to *A. tubingensis* and control treatments (Table 7.1.6). In gypsum amended with red mud, *A. tubingensis* treatment had more iron content as compared to control, but slight reduction of iron content was observed in the bacterial treatment as compared with control treatment (Table 7.1.6). In sludge amended red mud, control and *A. tubingensis* treatments, there was significant difference in the iron content was observed. But reduction was observed in the bacterial treatment as compared to other two treatments (Table 7.1.6). The bacterial and *A. tubingensis* treatments had shown significant reduction in soil iron content as compared to the control in topsoil amended red mud. But no significant difference was observed between the bacterial and *A. tubingensis* treatments (Table 7.1.6). The bacterial and *A. tubingensis* treatments had shown significant reduction in soil iron content as compared to control in fly ash-amended red mud. The bacterial treatment decreased the content of iron as compared to *A. tubingensis* treatment (Table 7.1.6). Red mud amended with sludge (control and *A. tubingensis*) had maximum iron content (Table 7.1.6). Red mud amended topsoil with bacteria and *A. tubingensis*; and red mud amended fly ash and bacteria had shown maximum reduction in iron content and these three treatments were significantly similar to each other. In the gypsum amendment, bacteria had shown significant reduction with control and *A.*

tubingensis treatment. Little reduction of iron content was observed in red mud and red mud amended with sludge. Significant reduction was found in bacterial inoculation of all treatments when compared to the control and *A. tubingensis* treatments (Table 7.1.6).

Soil calcium content

Calcium contents were similar in control and bacterial treatments of red mud, but significantly reduced content of Ca was observed in *A. tubingensis* treatment when compared to bacterial and control treatments of red mud (Table 7.1.6). Significantly increased Ca content (5.14 to 5.31%) was estimated in bacterial and *A. tubingensis* treatments of gypsum amended red mud when compared to control (1.75%) treatment (Table 7.1.6). Bacterial and control treatments had significantly same content of Ca in sludge amended red mud. Higher content of Ca was estimated in *A. tubingensis* treatment as compared with control (Table 7.1.6). Ca content was decreased significantly in bacterial and *A. tubingensis* treatments of topsoil and fly ash amended red mud. In these treatments, *A. tubingensis* had significantly increased Ca content when compared with bacterial treatments (Table 7.1.6). Gypsum with bacteria and *A. tubingensis* showed maximum content of soil Ca among all treatments in comparison to all other treatments (Table 7.1.6).

Soil potassium content

High potassium retention was observed in bacterial and control treatment of red mud. However, no significant difference was observed between these two treatments. *A. tubingensis* treatment had shown significantly less content of K when compared to control treatment. Potassium content was reduced significantly in bacterial and *A. tubingensis* treatments of gypsum-amended red mud and fly ash-amended red mud as compared to their controls. Moreover, bacterial treatment had significantly increased K level in fly ash-amended red mud as compared with *A. tubingensis* treatment. But, in gypsum-amended red mud *A. tubingensis* had more K content when compared with bacterial treatment (Table 7.1.6). In sludge-amended red mud, the bacterial treatment had significantly increased K levels as compared to control and *A. tubingensis* treatments. No significant difference of K content was observed in *A. tubingensis* and control treatments (Table 7.1.6). In topsoil amended red mud, bacterial and *A. tubingensis* treatments showed higher content of K were observed when compared to control. No significant

difference was observed in bacterial and *A. tubingensis* treatments (Table 7.1.6). The highest content of K was observed in red mud amended with topsoil. Bacterial and *A. tubingensis* treatments had no significant difference between both the treatments (Table 7.1.6).

Soil magnesium content

The magnesium content was significantly decreased in bacterial treatment of red mud as compared with the control. No significant difference was observed in control and *A. tubingensis* treatments of red mud (Table 7.1.6). In gypsum-amended red mud, Mg content was increased in both bacterial and *A. tubingensis* treatments as compared with control. No significant difference was observed in bacterial and *A. tubingensis* treatments (Table 7.1.6). In sludge-amended red mud, a slight increase of Mg was observed in bacterial treatments while *A. tubingensis* treatments showed less Mg when compared to control. A slight increase was recorded in bacterial and *A. tubingensis* treatments of topsoil-amended red mud as compared with the control. No significant difference of Mg was observed in bacterial, *A. tubingensis* and control treatments of fly ash amended red mud (Table 7.1.6). The highest content of magnesium was estimated in red mud amended with gypsum and bacteria; and *A. tubingensis* (Table 7.1.6). Significant increase of the Mg was observed in gypsum, sludge and topsoil with bacteria as compared to its respective control. The *A. tubingensis* treatment showed significant increase of Mg in two amendments such as gypsum and topsoil compared to their respective controls (Table 7.1.6).

Soil sodium content

Sodium is one of the major elements present in red mud after iron and aluminum. High content of Na (5%) was recorded in red mud after six months time. No significant difference was observed between control and *A. tubingensis* treatments of red mud. But, bacterial treatment had shown significant decrease in Na content when compared with control and *A. tubingensis* treatment (Table 7.1.6). In gypsum-amended red mud, no significant difference was observed between control and *A. tubingensis* treatments. But, marginal increase of Na content was estimated in bacterial treatments compared to the control (Table 7.1.6). A significant increase of Na content was observed in *A. tubingensis* treatment of sludge amended red mud as compared with control. A marginal decrease of

Na was estimated in bacterial treatment as compared to control treatment (Table 7.1.6). In topsoil-amended red mud, bacteria and *A. tubingenensis* treatments showed decreased Na retention as compared with control. Increased content of Na was estimated in bacterial treatment when compared with *A. tubingenensis* treatment (Table 7.1.6). Na content decreased in bacterial treatment and increased in *A. tubingenensis* treatment as compared to control in fly ash amended red mud.

The highest content of sodium was observed in red mud control (Table 7.1.6). The highest significant reduction of sodium was observed in fly ash with bacteria among all treatments. Bacterial treatments showed significant decreases in the sodium content in all treatments but in gypsum-amended red mud whereas bacterial inoculants did not show any significant decrease of Na compared to control (Table 7.1.6).

Soil aluminum content

Red mud had 8.0% of aluminum content after six months, which was significantly decreased in bacterial treatment of red mud as compared to control. Also, a slight reduction was observed in *A. tubingenensis* treatment than the control red mud. In gypsum-amended red mud, bacterial treatment had decreased retention of Al as compared to control and *A. tubingenensis* treatments. But, there were no significant differences between control and *A. tubingenensis* treatments. A decreased content of Al was observed in bacterial and *A. tubingenensis* treatments of sludge amended red mud compared to control. However, marginal increased retention of Al was observed in *A. tubingenensis* treatment as compared with the bacterial treatment. The bacterial treatment of topsoil amended red mud had a decreased content of Al as compared to control and *A. tubingenensis* treatments. No significant difference was observed in the control and *A. tubingenensis* treatments. The highest content of aluminum was recorded in fly ash amended red mud. No significant difference was observed in the control and *A. tubingenensis* treatments, while bacterial treatment had shown decreased content of Al when compared with control and *A. tubingenensis* treatments (Table 7.1.6). The maximum reduction of aluminum was observed in case of red mud with bacteria; and red mud amended with gypsum and bacteria. Soil aluminum reduction was observed more in all bacterial treatments when compared to control and *A. tubingenensis* treatments (Table 7.1.6).

Table 7.1.6: Effect of red mud bacterial consortia and *A. tubingensis* on the soil metal content (%) of different red mud amended treatments of nursery experiment.

	Iron	Calcium	Potassium	Magnesium	Sodium	Aluminum
Red mud						
Control	41.81±0.95ab	1.09±0.08cd	0.14±0.02fgh	0.38±0.02bcd	4.94±0.55a	8.02±0.5c
Bacteria	37.66±1.10c	1.06±0.10cd	0.1±0.05fgh	0.08±0.01d	2.87±0.61c	5.32±0.2g
<i>A. tubingensis</i>	39.68±0.85bc	0.94±0.04d	0.04±0.01h	0.29±0.01bcd	4.74±0.04a	7.31±0.2cd
Red mud with gypsum						
Control	24.43±2.38ef	1.75±0.04b	0.84±0.04b	0.41±0.01bcd	1.34±0.04fg	6.54±0.5defg
Bacteria	21.81±1.05f	5.14±0.05a	0.53±0.03cd	1±0.1a	1.50±0.05ef	5.59±0.4fg
<i>A. tubingensis</i>	27.06±0.65de	5.31±0.10a	0.63±0.03c	0.94±0.04a	1.34±0.04fg	6.06±0.2defg
Red mud with sludge						
Control	43.42±0.90a	1.13±0.04c	0.11±0.01fgh	0.13±0.03cd	2.88±0.07c	7.0±0.5cde
Bacteria	40.73±0.90abc	1.15±0.04c	0.25±0.04efg	0.15±0.05bcd	2.39±0.05cd	5.8±0.3efg
<i>A. tubingensis</i>	43.30±1.11a	0.94±0.04d	0.07±0.02gh	0.06±0.0d	3.85±0.05b	6.41±0.3defg
Red mud with top soil						
Control	27.85±1.02d	0.64±0.04ef	0.85±0.04b	0.28±0.02bcd	2.19±0.30cde	6.94±0.4cde
Bacteria	9.87±0.74h	0.35±0.04h	1.26±0.10a	0.55±0.05abc	1.63±0.30def	6.58±0.4def
<i>A. tubingensis</i>	8.55±0.40h	0.45±0.03gh	1.34±0.04a	0.61±0.02ab	1.31±0.30fg	6.84±0.7cde
Red mud with fly ash						
Control	21.51±1.05f	0.75±0.04e	0.63±0.15c	0.14±0.03cd	1.03±0.04fg	11.81±0.3a
Bacteria	9.49±0.40h	0.43±0.05gh	0.37±0.07de	0.11±0.02cd	0.57±0.06g	9.96±0.5b
<i>A. tubingensis</i>	14.80±1.05g	0.56±0.05fg	0.28±0.07ef	0.10±0.018cd	1.57±0.1ef	11.79±0.2a

Values sharing a common letter within the column are not significant at P<0.05. Values are mean ± SD (n = 3)

7.1.5 Plant growth and mineral nutrition

Establishment of vegetation on residues produced from the bauxite refining process is a beneficial part of their environmental management. Bermuda grass was chosen as a species for rehabilitation of amended bauxite residue, as the presence of alkaline grass can result in greatly increased dry matter production. After six months of growth of Bermuda grass, vegetation was rinsed thoroughly with deionized water and oven-dried to a constant dry weight prior to grinding. Samples were then digested on an open hot block for 3 hours by di-acid digestion (Page *et al.*, 1982), filtered, and analyzed by inductively coupled plasma emission spectroscopy equipped with mass spectroscopy.

The results showed that the grass was unable to grow in red mud and *A. tubingenisis* inoculated red mud, while bacterial (RMB) treatment had shown potential for grass growth (Table 7.1.7). In sludge-amended red mud, *A. tubingenisis* treatment had higher biomass of grass as compared to bacteria and control treatments. Even the bacterial treatment had significantly higher grass biomass as compared to control (Table 7.1.7). In fly ash-, gypsum- and topsoil-amended red mud, bacterial and *A. tubingenisis* treatments showed more grass biomass than control. But, no significant difference was observed between the bacterial and *A. tubingenisis* treatment (Table 7.1.7).

7.1.6 Plant metals

Plant metal contents were analyzed for Al, Fe, K, Mg and Ca. In top soil amended red mud, bacterial and *A. tubingenisis* treatments had shown significantly increased accumulation of Al content in aerial part of grass as compared to control. When compared between bacterial and *A. tubingenisis* treatments, bacterial treatment had more accumulation of Al in grass. In sludge-amended red mud, bacterial treatment had more Al uptake in grass as compared to control and *A. tubingenisis* treatments. But, the *A. tubingenisis* treatment

Table 7.1.7: Effect of bacterial consortia and *A. tubingenis* on the growth of Bermuda grass in different amendment of red mud treatments.

Treatment	Grass dry wt. (g)
Red mud	
Control	NG
Bacteria	8.7±0.51h
<i>A. tubingenis</i>	NG
Red mud with sludge	
Control	16.65±0.35ef
Bacteria	33.16±0.92b
<i>A. tubingenis</i>	41.51±0.32a
Red mud with fly ash	
Control	14.78±0.96fg
Bacteria	33.25±2.35b
<i>A. tubingenis</i>	31.3±1.98b
Red mud with gypsum	
Control	23.4±1.11d
Bacteria	12.42±0.35g
<i>A. tubingenis</i>	12.22±1.3g
Red mud with top soil	
Control	23.05±0.36e
Bacteria	28.08±0.35c
<i>A. tubingenis</i>	26.89±1.65c

Values sharing a common letter in the column are not significant at $P < 0.05$. Mean \pm SD; NG- No Growth.

showed a decreased content of Al in grass as compared with control (Table 7.1.8). In the *A. tubingensis* treatment of fly ash-amended red mud, Al content was higher in grass as compared to control and bacterial treatments. Bacterial treatment showed significantly decreased content of Al as compared to control (Table 7.1.8). In gypsum-amended red mud, bacterial and *A. tubingensis* treatments showed significantly decreased content of Al in grass as compared to the control. Furthermore, bacterial treatment showed significantly less Al content than the *A. tubingensis* treatment (Table 7.1.8). The highest uptake of Al was recorded in red mud amended with fly ash and *A. tubingensis* treatments (Table 7.1.8). This was followed by the gypsum-amended red mud control and *A. tubingensis* treatments. The lowest content of Al was accumulated in the grass of topsoil control treatment (Table 7.1.8). Red mud inoculated with bacteria showed higher content of grass Al and was similar to the bacterial treatment of gypsum-amended red mud.

Iron metal uptake was significantly higher in grass treated with bacterial and *A. tubingensis* in topsoil amended red mud as compared with control. The bacterial treatment showed highest Fe content than the other two treatments (Table 7.1.8). In sludge-amended red mud, bacterial treatments showed significantly more iron content as compared to control and *A. tubingensis* treatments. *A. tubingensis* treatment showed decreased grass iron content when compared with control treatment. In fly ash- and gypsum-amended red mud, *A. tubingensis* treatment showed more content of iron in grass as compared to bacterial and control treatments. But, bacterial treatments showed less uptake of iron as compared to control and *A. tubingensis* treatments (Table 7.1.8).

The highest levels of iron was accumulated in red mud amended with fly ash and *A. tubingensis* treatment followed by red mud amended gypsum control (Table 7.1.8). The *A. tubingensis* showed significantly higher uptake of iron in grass when compared to its controls and bacteria in fly ash- and gypsum- amended red mud treatments. The iron uptake in grass of red mud with bacterial treatment showed significantly higher uptake as compared to control of topsoil, sludge and fly ash (Table 7.1.8).

The potassium content was observed significantly higher in bacterial and *A. tubingensis* treatments of red mud when compared with red mud control. However, very

slight increase was recorded in *A. tubingensis* inoculated red mud treatment as compared to bacterial treatment (Table 7.1.8). In sludge-amended red mud, the bacterial treatment showed significantly more content of K than *A. tubingensis* treatment and control treatment.

In fly ash-amended red mud, bacterial and *A. tubingensis* treatments showed significantly higher content of K in grass as compared to control. However, *A. tubingensis* treatments showed significantly higher level of K as compared to bacterial treatment. In gypsum-amended red mud, both bacterial and *A. tubingensis* treatments showed significantly lesser K content as compared to control. In the bacterial and *A. tubingensis* treatments, *A. tubingensis* treatment showed significantly more content of K than the bacterial treatment (Table 7.1.8). The highest content of potassium was also accumulated in red mud amended with fly ash and *A. tubingensis* treatment followed by red mud amended with gypsum control (Table 7.1.8). Only in one treatment i.e. sludge amended red mud, bacterial treatment stimulated the potassium uptake in grass as compared to its control and *A. tubingensis* treatment. *A. tubingensis* stimulated the potassium uptake in topsoil and fly ash. Red mud inoculated with bacteria showed same content of K as that of bacterial treatment of topsoil amended red mud (Table 7.1.8).

Magnesium uptake was significantly more in grass of bacterial and *A. tubingensis* treatment of red mud as compared to control. Red mud inoculated with bacteria showed significantly more accumulation of Mg in grass compared to *A. tubingensis* inoculated red mud (Table 7.1.8). In sludge-amended red mud, the bacterial treatment showed significantly more accumulation and *A. tubingensis* treatment showed significantly less accumulation of Mg in grass when compared to control treatment. In fly ash-amended red mud, *A. tubingensis* treatment showed significantly more accumulation of Mg in grass as compared to control and bacterial treatments. However, bacterial treatment showed significantly less Mg content when compared to *A. tubingensis* and control treatments. In gypsum-amended red mud, bacterial and control treatments showed no difference in relation to Mg content. But *A. tubingensis* treatment showed less accumulation of Mg in grass as compared to the bacterial and control treatments.

The maximum uptake of magnesium was observed in red mud amended with sludge and bacteria followed by red mud amended with fly ash and *A. tubingensis* (Table 7.1.8). Bacteria facilitated the magnesium uptake in sludge, topsoil and gypsum treatment, which was significantly higher than their respective controls and *A. tubingensis* treatments. Bacterial inoculated red mud treatment showed significantly more accumulation of Mg in grass as compared to bacterial treatments of topsoil, fly ash and gypsum amended red mud (Table 7.1.8). A significantly increased content of calcium was recorded in bacterial and *A. tubingensis* treatments of red mud as compared to control. Furthermore, *A. tubingensis* treatment showed more accumulation of Ca in grass when compared with bacterial treatment. In sludge amended red mud, no significant difference was observed in control and bacterial treatments. But, significantly decreased Ca content in grass was observed in *A. tubingensis* treatment as compared with control and bacterial treatments. In fly ash and gypsum amended red mud, bacterial and *A. tubingensis* treatments showed significantly lower content of Ca when compared with its respective controls. However, *A. tubingensis* treatments showed significantly more accumulation of Ca as compared with its respective bacterial treatments (Table 7.1.8). Calcium uptake was maximum in control treatment of fly ash amended red mud followed by red mud amended with topsoil and *A. tubingensis* treatment (Table 7.1.8). Red mud inoculated with bacteria showed increased uptake of Ca in grass when compared to other bacterial treatments (Table 7.1.8).

Table 7.1.8: bacterial consortia and *A. tubingensis* on the grass metal content ($\mu\text{g}/2\text{mg}$ of dry wt. of grass) of different amendments of red mud treatments.

Treatments	Al	Fe	K	Mg	Ca
Red mud with top soil					
Control	335.6 \pm 9i	232.4 \pm 8k	568 \pm 8i	550.9 \pm 10k	782.7 \pm 9.46j
Bacteria	847.4 \pm 8de	692.9 \pm 8f	734.8 \pm 8h	919.6 \pm 9f	996.1 \pm 7.66g
<i>A. tubingensis</i>	508.6 \pm 11gh	458.2 \pm 12i	760.9 \pm 9gh	878.9 \pm 9g	1237.5 \pm 8.74b
Red mud with sludge					
Control	614.3 \pm 9fg	465.3 \pm 9i	793.9 \pm 8f	960.9 \pm 9e	1039.5 \pm 9ef
Bacteria	763.4 \pm 9def	572 \pm 9g	1020.2 \pm 5bc	1604.7 \pm 9a	1046.7 \pm 10ef
<i>A. tubingensis</i>	258.7 \pm 10i	272 \pm 7.8j	589.6 \pm 9i	694.2 \pm 9j	894.5 \pm 8h
Red mud with fly ash					
Control	693.6 \pm 8f	524.5 \pm 8.5h	869.4 \pm 9e	1069.4 \pm 9c	1306.7 \pm 10a
Bacteria	364.4 \pm 8hi	286.5 \pm 8.5j	949.4 \pm 9d	818.3 \pm 11h	862.3 \pm 8i
<i>A. tubingensis</i>	1518.3 \pm 9a	1453.7 \pm 7.9a	1504.3 \pm 8a	1415.7 \pm 10b	1097.5 \pm 8d
Red mud with gypsum					
Control	1179.6 \pm 10b	1247.6 \pm 8.7b	1037.9 \pm 10b	968.2 \pm 11e	1048.8 \pm 9e
Bacteria	876.8 \pm 7cd	893.4 \pm 8.05d	777.1 \pm 10fg	963.3 \pm 7e	873.4 \pm 3i
<i>A. tubingensis</i>	1022 \pm 10c	1020.7 \pm 5.9c	993.7 \pm 10c	932.5 \pm 10f	1031.3 \pm 10f
Red mud					
Control	NG	NG	NG	NG	NG
Bacteria	946.5 \pm 9cd	761.5 \pm 9.7e	826.7 \pm 10h	1022.7 \pm 11d	1143.7 \pm 10c
<i>A. tubingensis</i>	NG	NG	NG	NG	NG

Values sharing a common letter within the column are not significant at $P < 0.05$. NG, no growth. Values are mean \pm SD (n = 3)

Salient features

Bacterial isolates and *Aspergillus tubingensis* were used in combinations with chemical treatments for remediation of red mud in nursery trial. Gypsum amended red mud showed highest reduction of pH. Red mud with topsoil and *A. tubingensis* treatment showed maximum reduction of electrical conductivity. The maximum increase of organic carbon, organic matter and available phosphorus was observed in red mud amended with sludge and bacterial treatment. Red mud amended with gypsum and bacterial treatment showed maximum increase of total nitrogen. Red mud with top soil and *A. tubingensis* showed the highest number of neutrophilic, bacteria. Iron, aluminum and sodium were greatly reduced in all treatments and an increase in magnesium, potassium and calcium was observed. The maximum growth of Bermuda grass was observed in red mud amended with sludge and *A. tubingensis* treatment. Red mud inoculated with bacteria showed grass growth, but red mud control and red mud inoculated with *A. tubingensis* did not support the grass growth.

Chapter 5

Discussion

Isolation and characterization of red mud isolates

Bauxite residue (red mud) is unique and artificially induced environment. The high EC, pH and exchangeable Na values are indicative of the extreme salinity; alkalinity and sodicity of the red mud (Hunt and Gilkes, 1992). The presence of higher concentrations of heavy metal oxides makes this environment extremely alkaline and different from the natural soda lakes. The sub optimal level of nutrient status makes it harsher environment to grow microbes and plants. Organic carbon, nitrogen and available phosphorus are limiting conditions to grow any heterotrophic population of microbes. However, they are not analogous to soda lakes or non-saline alkaline environment as reported by Tiago *et al.*, (2004), because of different chemical composition and predominance of iron and aluminium oxide. Bauxite residue contained small numbers of metabolically inactive (injured) bacterial cells that grew in rich medium but not in minimal media and exhibited the light blue color of methylene blue in the hanging drop experiments. The methylene blue reduction test uses the change in methylene blue from blue in the oxidized state to colorless form in the reduced state (Fletcher and Wilson, 1994). Viable bacterial cells use methylene blue as a hydrogen acceptor via the dehydrogenases in the presence of NADH and appear colorless, whereas dead cells stain dark blue. Metabolically inactive bacterial cells exhibit shades of light blue depending on the level of active dehydrogenases (Hamdy and Williams, 2001).

The number of heterotrophic bacteria isolated was not dependent on the media composition as all the three media used for the isolation was complex basal media in this study. The number of isolated heterotrophic microbes appears to be lower than the known alkaline and hyper-saline environment; this might be due to the unique characteristic of the red mud (Hamdy and Williams, 2001). Alkaliphilic bacteria are not confined to a single group or to a defined set of phylogenetic lineage; instead they are distributed in various evolutionary branches. The real paradox is that all isolates were isolated at pH 10.5 but five bacteria (RM10E, RM10D, RM13Y, RM1A and RM6) could grow at pH

10.0 or less. However, this effect is relatively common and various isolates from extreme alkaline environments only proliferate under laboratory condition at more moderate pH values (Jones *et al.*, 1994). The red mud isolated microbes exuded organic acids mainly acetic acid and succinic acid in red mud extract media amended with sucrose; and were able to ferment different carbon sources. In order to maintain the homeostasis of the cytoplasm and the acidic environment near the cell wall, exudation of the organic acids is an important aspect of the microbial physiology. Organic acids also help in chelation of the metal present in the medium (Gadd and Griffiths, 1977). However, the extent and rate of pH reduction in the red mud will differ from pH reduction in fermentable carbon-amended medium, due to differences in the chemistry and buffering capacities of the substrates (Valarie, 1999). This demonstrated the possibility of using pure cultures of indigenous bacteria in the red mud to reduce the alkalinity. It would be possible to reduce the pH of red mud by bacterial metabolism by inoculating the red mud with pure cultures of indigenous bacteria and incorporating nutrients to encourage bacterial growth. The isolates were positive for one or more enzyme activities like cellulase, Dnase, xylanase and phosphatases at alkaline pH. The stability of these enzymes at alkaline pH attributed to their habitat and growth profile in wide range of pHs.

Carbon substrate utilization pattern

Species of heterotrophic bacteria differ in the specific organic substrates, which they can use as carbon and energy sources for growth. The substantial diversities in the substrates that are biodegradable, and the ability of individual species to catabolize specific substrates, have been used for many years to characterize and identify pure cultures of bacteria (Konopka *et al.*, 1998). The metabolic diversities of the red mud isolates were detected by the ability of isolates to metabolize any substrate, including amino acids and carboxylic acids on the basis of color development in different wells of Biolog plates. Previously, the Biolog system had been used to characterize pure cultures isolated on heterotrophic growth media from diseased animals and plants, and also from aquatic or terrestrial habitats. Red mud isolates have shown diverse pattern of carbon substrate utilization. To know the effect of culture condition on the carbon substrate utilization, red mud isolates have grown in different media such as Horikoshi alkaline

medium, modified tryptic soya medium (pH 7 and 10) and nutrient medium (pH 7 and 10) to be further inoculated in the Biolog plates. It was noted that these culture conditions were not affecting the carbon substrate utilization pattern of red mud isolates.

Biolog, which is based on carbon substrate utilization pattern to identify microbes faster than the routine cultivation based approach, was used for the characterization and identification of the red mud isolates. In many cases, >70%, but less than 100%, of isolates could be identified (Miller *et al.*, 1993). Isolates from aquatic or terrestrial ecosystems are less likely to be identified by the Biolog database (McCarthy and Murray, 1996); identification rates of 47–70% were reported for cultures isolated from surface or subsurface soils. Some bacteria gave very few reactions on the plates (Konopka *et al.*, 1998). Interestingly, in present study, only one red mud isolate showed similarity index of 30%, and rest have showed less than 30% similarity, which suggest that the isolated microbes might be uncharacterized before or new member of the known genera.

DNA fingerprinting and 16S rDNA analysis

Repetitive sequence based PCR (Rep PCR) was used successfully to generate DNA fingerprints to distinguish between genetically unrelated and closely related bacterial strains. It involves the use of primers based on the short repetitive element derived from highly conserved palindromic inverted repeat regions dispersed throughout the prokaryote kingdom (Laguette *et al.*, 1996). In the present study, unique Rep-PCR based genotypic fingerprints of different isolates from red mud samples were found. With Rep PCR, 10 representative red mud isolates were found to be genetically dissimilar. The genomic fingerprints with Rep PCR showed certain intense amplimers. These amplimers can serve as distinct molecular markers for the respective isolates to track them in the environment (Sadowsky *et al.*, 1996). The genetic variability in the representative red mud isolates, 16S rDNA was amplified and sequenced. All red mud isolates showed genetic dissimilarity of sequence data and none of the isolates were 99% similar among themselves. The biochemical, Rep PCR and 16S rDNA results were in concordance and showed variability at each level.

According to variability map of sequences of 16S rDNA of red mud isolates, over 10% of bases in the 16S rRNA gene are totally conserved (within a sample of 500

bacterial sequences). The majority of these conserved bases are, however, not adjacent to each other and thus form no continuous conserved regions for universal priming (Watanabe *et al.*, 2001). The longest string of variable bases was between positions 173 to 218 in red mud isolates when compared within them along with *E. coli*. None of the isolates showed more than 97% similarities of their 16S rDNA among themselves. A comparison of the diversity observed in this study with that observed in other studies is rather difficult because of lack of studies of similar environments (Tiago *et al.*, 2004). Novel *Bacillus* sp. was reported from the red mud (Agnew *et al.*, 1995), and the focus was concentrating on *Bacillus* sp., while analyzing natural alkaline environments. This is due to its adaptability to higher pH and production of several industrially important enzymes (Horikoshi, 1999). In the present study, three isolates namely RM10E, RM1A and RM6 showed similarity with *Bacillus cereus*, uncultured bacterium and *Bacillus* sp. Some of the representative isolates of red mud were same as reported by Tiago *et al.*, (2004) in non-saline alkaline environments. They reported the occurrence of *Agrococcus* spp., *Kocuria* spp. *Micrococcus* spp. in high G+C gram-positive bacteria and low G+C gram-positive *Bacillus* spp. While *Salinococcus* species, a novel alkaliphile and moderate halophile was reported by Zhang *et al.* (2002) from Baer Soda Lake in inner Mongolia autonomous region, China. *Planococcus* species, Gram-positive, aerobic, heterotrophic bacterium are abundant members of the bacterial community in a variety of marine environments, including some in sensitive Antarctic ecosystems, was also present in the bauxite residue and capable of growing at elevated temperature (45 °C). In the present study, two red mud isolates RM9P and RM11R showed similarity with *Planococcus* sp. and *Salinococcus* sp. The isolate RM11R was placed with *Saninococcus* sp. and RM9P with *Planococcus* sp. in the phylogenetic tree. We also found some of the bacteria, which belong to class Actinobacteria. The isolates RM1 and RM13Y were grouped with *Kocuria* sp., whereas RM10D with *Microbacterium* and RM8 with *Agromyces* sp. in the phylogenetic tree. A gram-negative bacterium that showed similarity with *Pseudomonas* sp. (RM12W) was also reported from the red mud. Previously, alkaliphilic *Pseudomonas pseudoalkaligenes* was cultivated from decomposing reed rhizomes in Hungarian soda lakes (Borsodi *et al.*, 2005). RM-1A showed similarity with the uncultured bacterium and *Bacillus* spp. Because of the striking similarity between the genus *Bacillus* and

Planococcus, RM-1A showed phylogenetic affiliation with *Planococcus* sp. in phylogenetic tree. As more studies have revealed considerable heterogeneity among motile Gram-positive cocci (Marquez *et al.*, 1993; Hao and Komagata, 1985), and this was supported by phylogenetic analyses based on comparison of ribosomal RNA sequences (Farrow *et al.*, 1992, 1994). These studies showed that the genus *Planococcus* was most closely related to the round-spore-forming bacilli (e.g. *Bacillus globiformis* and *B. psychrophilus*). These results showed that red mud isolates were related to alkaliphilic, halophilic and marine bacteria. It is noteworthy that none of the amplicon had 100% identity to sequences in the database. The clones that had sequence identities of over 98% to a known organism may represent the same species. These sequences that share an identity 88% to 98% are usually considered to be part of the same genus (Sadowsky *et al.*, 1996). On this basis, seven red mud isolates described here probably represent new members of the known genera.

Red mud being hard to process sample (for DNA extraction) may be accessed for genome information with the whole genome amplification (WGA) using ϕ 29 DNA polymerase by amplifying the extracted DNA from a sample and constructing environmental libraries from the amplified DNA (Gonzalez *et al.*, 2005). The environmental libraries gave contrasting results to the culture dependent method. This seems to be the case for many environments (Grey and Herwig 1996; Borneman and Triplett, 1997). Diverse bacteria were identified which belong to known genera with high and low similarities. A potential hazard associated with PCR amplification of target DNA of mixed sequences is the creation of recombinant or chimeric products that consist of mixtures of parts of different sequences (Choi *et al.*, 1994; Wang and Wang, 1997). The frequency of occurrence of chimeric sequences in clone libraries was reported, from independent studies (Barns *et al.*, 1994; Koczynski *et al.*, 1994). In the present study, two chimeric sequences were detected using Chimera check program of RDP II and discarded. Detection of chimeric SSU rRNA genes is difficult but of importance since they could otherwise lead to erroneous reports of novel lines of evolutionary descent, giving a false impression of biodiversity within microbial communities.

The 16S rDNA sequences of red mud clone library belonging to members of Beta- and Gamma- proteobacteria; and Bacteroidetes. Members of Beta- and Gamma-

proteobacteria are metabolically versatile. Multiple detection of the identical sequences in clone library may also reflect that these corresponding aerobic bacteria were significant and predominant members of the communities that were probably active in the decomposition and mineralization of the toxic content of red mud. To understand the roles and structures of microbial communities, sequence data only are not enough, but cultivability of microorganisms is very important (Borsodi *et al.*, 2005). The cultivable isolates of red mud are mostly belonging to the alkaline and saline environment. But the paradox is in the fact that no clone from clone library matches with the isolates of the red mud. This might be due to the microbial remediation process of the red mud in which, bacteria might be reaching to transition phase, which is hard to cultivate on synthetic media or due to bias associated with diversity analysis.

Out of ten representative red mud isolates, seven isolates (RM9P, RM12W, RM13Y, RM1, RM1A, RM6 and RM8) is belonging to the new members of the known genera. This is evidenced from the 16S rDNA sequence analysis that it had shown 98% similarity or less with the existing databases. Their closest relative in database have mainly alkaline and saline habitat. Screening of bacteria by culture dependent and independent methods resulted in completely different results with no overlapping of the species between these two methods. Interestingly, all sequences of clone library have 99% or more similarity with the cultivable isolates or uncultured clones. Detection of large number of clones which belong to Proteobacteria suggested that Proteobacteria might be predominant and metabolically engaged in reduction of pH and metal toxicity of the red mud.

Alkaline tolerance of red mud isolates

Bacteria have their cell walls, which are designed to obtain nutrients from the environment, and to protect the organism from harmful chemical changes such as alkalinity in the surrounding medium. As a consequence, the function and structure of the cell wall of a living cell may change during acid-base titrations, due to passive and active (energy consuming) processes. Some of the processes that may regulate the acid-base activity of live bacterial suspensions are as follows: 1) Deprotonation of functional groups: macromolecules of the cell wall contain exposed ionisable functional groups that protonate or deprotonate, according to their acid dissociation constant and the pH of the

medium, 2) Proton leakage: at high pH, hydrogen ions diffuse out of the cell as a result of the proton concentration gradient across the cell wall, 3) Exudation of organic acids: by producing and subsequently releasing organic acids, bacteria may attempt to buffer the pH increase of the external medium, and 4) Electrostatic destabilization: electrical charging of the cell wall may lead to a destabilization of its three dimensional structure, and may expose previously inaccessible functional groups, which may then deprotonate (Claessens *et al.*, 2004). Different events such as buffering capacity, organic acid exudation, glycolysis activity and surface negative charge (zeta potential) associated with the change in alkalinity of two representative facultative red mud isolates RM9P (*Planococcus sp.*) and RM8 (*Agromyces sp.*) were studied.

The buffering capacities were estimated in two representative isolates of red mud, which were grown at different alkaline pH (7.0 – 10.0). The smooth curves of total buffering capacity (Bt) and outer buffering capacity (Bo) were obtained from a polynomial regression. Several studies of buffering capacity of bacterial cells have suggested that total and outer buffering capacity values vary markedly between species. The buffering capacity was quite different in gram-positive bacteria (RM9P) with actinomycetes (RM8). The slight differences of buffering capacity of RM9P (13 mM H⁺/pH unit/mg of protein) and the results (14 mM H⁺/pH unit/mg of protein) obtained by other worker for the same species (Kannan *et al.*, 2006) could be due to the culture conditions used, the solutions employed to prepare bacterial suspensions, and the technical approaches used. As it had been shown that the buffering capacity varies from species to species and one strain to another. *Staphylococcus aureus* exhibited greater buffering capacity than the acidophilic bacterium *Lactobacillus acidophilus* (Rius *et al.*, 1994b) and different strains of *Serratia marcescens* (Rius *et al.*, 1994a). No comparison of the buffering capacity of *Agromyces sp.* RM8 could be done due to lack of studies on similar genus. At external pH values below 10.0, imposition of a valinomycin-mediated potassium diffusion potential is a way to energize ATP synthesis (Guffanti *et al.*, 1985). The H⁺ translocation involved in ATP synthesis could increase the buffering capacity values obtained by the acid pulse technique. In order to avoid this possibility, cells were allowed to equilibrate for about 2 hr. with intermittent mixing after valinomycin addition (Rius and Loren, 1996).

Planococcus sp. (RM9P) and *Agromyces sp.* (RM8) had buffering capacity higher when grown at pH 7.0 than the cells grown at pH 10.5. These results are in contrast to the buffering capacity of *Bacillus alcalophilus* cells grown at pH 10.5 was greater than the buffering capacity of cells grown at pH 8.5 (Rius and Loren, 1996). As it was evident from the study of Rius and Loren (1996) that *B. alcalophilus* cells grown in L-malate medium exhibited greater resistance to low external pH values than cells grown in other medium. They suggested that the resistance seemed to correlate with the pH values of the suspensions. It was shown that certain enzymatic activities can be induced by a change in external pH (Hickey and Hirshfield, 1990) and that surface characteristics of gram-positive bacteria depend on the pH of cultures and the energized state of the membrane.

Differences in the proton motive force between respiring and fermenting cells have been reported for gram-positive and gram-negative bacteria. Aerobically growing cells had a greater proton motive force than cells growing anaerobically, and these differences depended on the pH of the medium (Kashket, 1981a; 1881b). The diversity of alkali tolerance depended strongly on the cellular metabolism, especially on the glycolysis reactions of a given strain. In fact, the alkali-tolerant representative strain, RM9P and RM8, exhibited the maximum utilization of glucose at neutral pH in the presence or absence of valinomycin. The glycolysis activity profile of RM9P and RM8 have never shifted to the alkaline side in the presence or absence of valinomycin. A dramatic decrease in glycolysis activity of both the red mud isolates, occurred at pH 8.0. It is interesting that in RM9P and RM8, substantial glycolysis activity in the presence or absence of valinomycin was still observed at pH 7.0, beyond this red mud isolates have decreased glucose consumption. In valinomycin added cells, an interesting pattern of glucose utilization activity was observed. Though, the maximal utilization was at pH 7.0 and it was decreasing with the increase of alkalinity. But, at pH 9.0 both the red mud isolates have shown increased activity when compared to pH 8.0 and 10.0. These results suggest that in these isolates anabolic reactions may be more sensitive to an alkaline environment than catabolic reactions (glycolysis reactions).

Under aerobic conditions, *Escherichia coli*, releases succinate as the major product in the tricarboxylic acid (TCA) cycle (Lin *et al.*, 2005). *E. coli* can also grow under anoxic conditions by using sugars, for example glucose, as sole carbon and energy

source in fermentation reactions, releasing different organic acids, such as succinate, acetate, formate and lactate (Clark, 1989; Sanchez *et al.*, 2005). It is likely that the RM9P and RM8 release succinate as an intermediate product of the TCA cycle as is reported for *E. coli* (Lin *et al.*, 2005; Sanchez *et al.*, 2005). RM9P and RM8 may use cell material or internal reserves, such as glycogen, to neutralize the pH of the surrounding solution by releasing acids. The results showed that the rate of organic acid released by live cells of RM9P and RM8 depends on the pH. With the increase of the alkalinity, there was increased production of succinic acid in both RM9P and RM8. Interestingly, the production of succinic acid was maximum at pH 9.0 and decreased at pH 10.0, showed decreased intracellular metabolism for the production of organic acid beyond pH 9.0. It may also be possible that the internal homeostasis of the red mud isolates to combat the alkalinity was maximum at pH 9.0.

Surface charge was measured as zeta potential in both the representative red mud isolates at different pH. The negative charge was higher in RM9P than RM8 at various pH ranges 7.0 to 10.0. Charge was increasing and negativity was decreasing with the increase of the alkalinity in both the cases. It was reported that the cell wall of *B. subtilis* was less negatively charged when the bacteria were metabolizing a carbon source and creating a proton motive force than when the cells had de-energized membranes (Kemper *et al.*, 1993). This might not be true in this case as it was evident from the glucose utilization that both red mud isolates utilized less amount of carbon substrates at the higher pH with respect to neutral pH. The variation in the result from the study of Kemper *et al.* (1993) might be due to the different strain chosen and cultural conditions used. Aono *et al.* (1995) demonstrated that cell walls of *Bacillus lentus* C-125, an alkalophile, grown at pH 10.0 were about 20% thicker than the cell walls of organisms grown at pH 7.0 and that they had about three times the negative charge density of the cell walls of strain C-125 cells grown in a neutral environment. The cell wall of bacterial cells showing less negative charge at the higher alkaline pH in the present study might be due to the fact that negative ions of cell wall may get neutralized in alkaline conditions. Cultural conditions might have direct impact on the charge densities of bacterial cell wall. The extreme pH environment is reported to cause damage to cellular components and interferes with transport across cellular membranes in neutrophiles, whereas alkalophiles

maintain their internal pH near neutral and require high concentration of Na⁺ ions. This shows that the protein in the cellular surface must be adapted to pH extremes. The bacterial cells isolated from red mud environment, in the present study were more stable under high alkaline environment. The cell walls and membranes have greater adaptation to the alkaline pH.

In vitro* studies of *A. tubingensis

The high EC, pH and exchangeable Na values are indicative of the extreme salinity, alkalinity and sodicity of the red mud (McArthur, 1991; Hunt and Gilkes, 1992). The ability of *A. tubingensis* to produce acidic compounds in nutrient medium was tested in this study in order to check whether this organism could reduce the pH of the red mud amended soil. *A. tubingensis* was grown at different pH levels and it was observed that it was able to grow at pH values from 2.5 to 12.0. It had optimal growth at pH 3.5. The pH of the medium was drastically reduced indicating that the *A. tubingensis* is producing the acidic compounds able to reduce the pH. This *A. tubingensis* was already known to reduce the pH of the medium when amended with different rock phosphates and also as excellent rock phosphate solubilizer (Reddy *et al.*, 2002). In metal tolerance studies, the growth of *A. tubingensis* was significantly inhibited by Al at 200 µg/mL; however, at 50 µg/mL of Al, the growth was increased compared to control. To determine the maximum concentration of NaCl tolerance by *A. tubingensis*, different concentrations of Na were amended and the results showed that up to 50µg/mL of Na, the growth of *A. tubingensis* was not affected and at higher concentrations (150 and 200 µg/mL) the growth was significantly inhibited. The Na content increased in the mycelium as the concentration of Na increased in the growth medium and the maximum accumulation was found at 200 µg/mL. The ability of *A. tubingensis* to grow in presence of different Fe concentrations was tested and the results indicated that the best growth of *A. tubingensis* was achieved at concentrations of 400µg/mL. At higher Fe concentrations (500 and 600 µg/mL) the growth was slightly increased when compared to control. The level of Fe accumulation was also significantly increased as the concentration of Fe increased in the medium. Pagano *et al.* (2002) showed that the analytical and toxicity outcomes of red mud are based on ore composition and variable release of toxic contaminants from bauxite

byproducts, including their main components Al and/or Fe and some other minor components such as Mn, Zn and Pb. When *A. tubingenensis* was grown in presence of different concentrations of red mud, the mycelial growth increased with the increasing of the concentration of the red mud amended in the medium. At 10% concentration of red mud amended media, maximum the growth of *A. tubingenensis* was observed. It was able to reduce the pH to below 3.0 in all the treatments after the growth of the *A. tubingenensis*. The mycelium grown in presence of red mud was digested and analyzed for different elements such as Al, Na, and Fe as these are normally present in red mud at higher concentration. The Al and Fe level had increased in the mycelia as the concentration of the red mud increased, but Na concentration was decreased as the concentration of red mud increased in the growth medium. From these results, it was clear that *A. tubingenensis* could tolerate high levels of Na, Fe, Al and high pH when inoculated into the red mud soil. Furthermore, because of its growth at high pH, metals (Na, Fe and Al) and in red mud amended media; it could be used for remediation of red mud.

Remediation of red mud using bacterial isolates and *A. tubingenensis*

Hazardous waste sites often contain complex mixtures of pollutants, which include both organic contaminants and heavy metals (Kovalick, 1992). Microbial bioremediation of pollutants is a promising method of environmental cleanup. However, if the metals in soils are toxic to the microbes, removal of pollutants is slowed or prevented. Many reports have shown that (i) the short-term response to toxic metals is a large reduction in microbial activity and (ii) habitats that have had high levels of metal contamination for years still have microbial populations and activities that are smaller than the microbial populations and activities in uncontaminated habitats (Hutchinson and Symington, 1997; Kuperman and Carreiro, 1997). Reclamation of red mud ponds is difficult because of high alkalinity, sodicity, salinity, high concentration of soluble ions, which are toxic and competitively inhibit the uptake of nutrients in plants and microbes (Thakur and Das, 1994). The high alkalinity also reduces the water availability to the plants. These properties when coupled with lack of nutrients and anoxic conditions can prevent vegetation. Hence, remediation of bauxite residue must be a way that can promote the plant growth and improve the nutritional status. In the present study,

reduction of alkalinity was achieved up to about pH 8.0 in red mud amended gypsum treatment of nursery experiment after six month. In four years of leaching and precipitation study, it was observed that pH reduced in red mud from 10 to 8.8 (Gherardi and Rengel, 2003). Conversely, in another study, within 5 years of planting, the pH of the red mud under vegetation had decreased from 10.5 to 9.5 (Hinz, 1982). In the current study, pH values recorded after six months in all treatments of red mud had reductions of more than 1.5 pH units. Except red mud-amended gypsum, all other treatments had more than 10.0 pH. Plants growth may not be favored at this elevated pH. Hence, long-term studies are needed to study the decrease in alkalinity of various treatments.

The electrical conductivity of all the treatments was below 0.5 except in gypsum amended red mud. The gypsum sample, in addition to Ca and SO₄, contained Si and small amounts of Na, Fe, K, etc. The high electrical conductivity of gypsum amended red mud might be due to presence of high concentration of Ca in treatment, which is exchanged, with the sodium ion of the red mud (Xenidis *et al.*, 2005). The increase in levels of soluble and exchangeable calcium and magnesium are attributed to the probable high content of these elements in the gypsum used. Exchangeable calcium is the major reserve of soil calcium available to plant roots (Haby *et al.*, 1990) as it acts as a supply of calcium over a longer period. Levels of exchangeable calcium cited as low or deficient will vary between plants and soil types. Substrate levels recorded in the current study show gypsum treatments have considerably higher concentration of Ca (1.5%).

Sewage sludge being a source of rich organic carbon was frequently used in reclamation schemes for its nutrient supply (Wong and Lai, 1982; Munshower, 1994). Whilst many workers have achieved plant growth in bauxite residue in pot trials, there is limited knowledge of long-term growth of vegetation in red mud at field level. Organic carbon values were recorded in the range of 0.4 to 3.5% which is much greater than 0.58–1.88% range reported by Ye *et al.* (2002) for sparsely and densely vegetated tailings.

Nitrogen is usually deficient in mine soils, which limits vegetation establishment and sustained productivity. Nitrogen contents of unamended bauxite residues are low with levels ranging from trace to 0.02% (Wong and Ho, 1993). Values found in the current study show a significant increase in total nitrogen levels of some treatments while

some were devoid of nitrogen. In red mud amended with gypsum and bacteria, nitrogen was about 388 mg/Kg. It ranged 0 to 40 mg/Kg in other treatments of red mud.

Bauxite residue contains large amounts of sesquioxides, and thus had a very high phosphorus retention capacity (Snars *et al.*, 2004). Analysis with acidic extracting reagent (pH 4.8) showed high values of greater than 150 mg/kg phosphorus as described by Courtney *et al.*, (2008). The available phosphorus was low in all treatments in the present study, with values corresponding to 0.2 to 5.9 mg/kg. Similarly, Meecham and Bell (1977) found available levels of 8.2 mg/kg phosphorus with an 8-fold increase in acid extract. These results indicate that although phosphorus reserves may be high, available amounts are low. This might be due to high pH values of the red mud.

Aluminum levels in bauxite residue are closely correlated with pH (Fuller *et al.*, 1982), with solubility increasing above pH 9.2. Wong and Ho (1993) reported soluble aluminum levels of 1.04 mg/kg (pH 10.5) in unamended residue with reductions to 0.08–0.24 mg/kg (pH 8.56–9.98) with increasing gypsum addition. Levels of 20 mg/kg are reported to be toxic to red clover and other legumes (Alam and Adams, 1979). Results reported in the current study are 5.3 to 11.7% of aluminum concentration in various treatments. Contrast to other reports, the pH directly does not affect the aluminum concentration of different treatments. Gypsum treatments have more pH reduction than the other treatments but have aluminum concentration in the range of other treatments such as sewage sludge, top soil and without chemical amendments. The fly ash treated red mud had more aluminum concentration than the rest, because initial level of aluminum was higher than the control red mud.

The decrease in soluble iron levels can be attributed to an improvement in substrate conditions. The relatively insoluble ferric ion (Fe^{3+}) is associated with well-drained soils, compared to ferrous iron (Fe^{2+}) associated with waterlogged soils (Troeh and Thompson, 1993). Topsoil and fly ash amended red mud along with bacteria and *A. tubingensis* have been recorded for the lower concentration of iron. Exchangeable forms of potassium are considered the primary source of K for plant uptake (Knudsen *et al.*, 1982). Typically, sludge and without any chemical amended treatments had marginally lower, but significantly, exchangeable potassium compared to other treatments. Gypsum,

sludge and fly ash amended treatments had significantly higher concentration of K. This indicates that the exchange of K is complex in different treatments.

Availability of major cations is a concern in revegetating bauxite residues due to excessive levels of exchangeable sodium. Previous studies indicated deficient levels of potassium in amended residue (Courtney and Timpson, 2004). Calcium and potassium levels in the amended residue are within the satisfactory limits reported for mine soils (Monterroso *et al.*, 1999). Available magnesium levels recorded for all treatments are within the slight to severe deficiency range reported by Monterroso *et al.* (1999) for mine soils. In the present study, Mg concentration was significantly increased in all treatments but a steep rise was recorded in gypsum and topsoil with bacteria and *A. tubingensis*. The results showed that microbes carry out the mobilization of Mg more efficiently. Sodium concentration was lesser than 3% in all bacterial and fungal treatments combined with chemical amendments, except that of red mud amended with sludge and *A. tunigensis*, where sodium concentration was reported to be 3.8%. Lower concentration of sodium is necessary for the adequate growth of plants.

The growth of Bermuda grass was observed in all red mud treatments except that of red mud control and red mud inoculated with *A. tubingensis*. This shows that indigenous bacteria played important role in plant growth promotion without addition of any chemical substrate for the reduction of pH. The maximum growth was observed in the sludge with *A. tubingensis* treatment. Bacterial inoculation had more growth promoting activity in all treatments of red mud except that of sludge treatment where *A. tubingensis* had shown more grass growth than the bacteria. This gives the clear evidence that these indigenous bacteria play important growth promoting activity in red mud treatments. Plants growing in high Na content substrates can have impaired uptake and a subsequent low Ca concentration (Chang and Dregne, 1955; Gupta and Abrol, 1990). Results from this study contrast with such finding, as none of the treatments can be considered deficient in calcium. It can be deduced that sodium levels in the substrate were not affecting ion regulation in the plant cells.

The high accumulation of aluminum and iron metal did not hamper the uptake of magnesium, potassium and calcium. For example, fly ash with *A. tubingensis* had Al 1518, Fe 1453, K 1504, Mg 1415 and Ca 1097 ($\mu\text{g}/2\text{mg}$ of soil). Interestingly, the lower

uptake of iron and aluminium also did not have effect on the Mg, Ca and K accumulation. This was observed in case of fly ash with bacterial treatments Al 364, Fe 286, K 949, Mg 818 and Ca 862 ($\mu\text{g}/2\text{mg}$ of soil). The soil of Damanjodi (Orissa) had a high content of Fe and aluminum from where the Bermuda grass was collected to use in this study. The high iron and aluminum content had resulted the Bermuda grass to be acclimatized to grow in high metal concentration. Because of this acclimatization, grass might not have shown impaired metal uptake activities when grown in red mud treatments. Although revegetation of bauxite mine sites is relatively straightforward, the bauxite residue (red mud) is difficult to revegetate. The refinery residue is characterized by high pH (pH >10), high electrical conductivity (EC > 30 mS/cm), and high exchangeable sodium percentage (>70%) (Meecham and Bell, 1977; Hinz, 1982; Wong and Ho, 1993). On the other hand, concentrations of plant nutrients such as calcium, magnesium, manganese, nitrogen and phosphorus are low (Meecham and Bell, 1977; Hinz, 1982) and the fine texture obstructs penetration of plant roots (Meecham and Bell, 1977). Consequently, the chemical and physical limitations of the red mud must be addressed prior to revegetation if the refinery residues are to form part of the plant growth medium. Inclusion of gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) and other material (sewage sludge, fly ash, chicken manure) is used to improve the physical characteristics and reduce nutrient deficiencies (Fuller *et al.*, 1982; Ward, 1986; Wong and Ho, 1994, 1993; Wehr *et al.*, 2006).

Amelioration of the surface layer has been widely attempted using gypsum (Wong and Ho 1991), manure/ compost, sewage sludge (Wong and Ho 1991; Courtney *et al.*, 2003; Courtney and Timpson 2004), soil (Wong and Ho 1991), and combinations thereof. Vegetation survival was only good at the highest application rates of these substrates. Most researchers attributed the positive effect of high addition rates for improvement of the physical structure (bulk density, porosity) of red mud, thereby improving drainage and leaching of salts from red mud (Wehr *et al.*, 2006). However, there are no indications that vegetation would have survived over longer timescales or with minimal input, or that roots penetrated into the unamended red mud. Furthermore, the quantities suggested by these studies are economically unlikely to be viable (Wehr *et al.*, 2006). Courtney *et al.* (2003) demonstrated that the establishment of clover species on red mud and process sand can be achieved without the addition of gypsum and that

thermally dried sewage sludge is a beneficial organic amendment. A period of leaching is however required to effectively lower pH and sodium levels. Revegetation is only achievable when sodium and pH levels are sufficiently low. Such threshold levels have yet to be established. But, microbial remediation is not studied so far in red mud. As soil microorganisms play a crucial role in increasing soil fertility and accelerating revegetation process through their activities in disturbed soils (Visser *et al.*, 1983). Hence, use of microbes in combination with chemical amendment in the current study, has helped in sufficient reduction of pH. Other parameters such as levels of the soil nutrient (organic C, total N and available P) were improved and promoted growth and activities of different microbial groups, thus showed both the nutrient pools and the stability of the soil ecosystem in the revegetated sites were gradually reestablished by inoculated microbes.

Microbial analysis of red mud treatments of nursery trial

Clear differences in the abundance of neutrophilic and alkaliphilic bacteria were observed in different treatments of the field trials. Addition of different substrates and the growth of Bermuda grasses after six months have facilitated the increase in microbial abundance. Though, there was no clear trend that indigenous bacteria or *A. tubingensis* had triggered the increase of microbial load. In some treatments like gypsum, sludge and without chemical; bacterial inoculation had increased alkaliphilic bacteria than the control or *A. tubingensis* treatments. But, in topsoil and fly ash treatments *A. tubingensis* inoculation had more numbers of alkaliphilic bacteria than the bacterial and control treatments. The same is true for actinomycetes but not for the neutrophilic bacteria. Neutrophilic bacteria were significantly more or same in *A. tubingensis* inoculated treatments except that the control red mud where no chemical amendment was made. In this treatment, red mud with bacteria had more neutrophilic bacteria than the *A. tubingensis* and control treatments.

pH, electrical conductivity, organic carbon and nitrogen might have direct impact on the structure and abundance of the microbial community, which is not directly correlated in this case. For example, the maximum organic carbon was observed in sludge amended red mud; but the maximum abundance of neutrophilic alkaliphilic and

actinomycetes bacteria was not observed in the same treatment. Microbial growth and activity in soil is often limited by the availability of carbon (Lynch and Whipps, 1990) and nitrogen (Wardle, 1992) and it may respond to a varying supply of substrates either by physiological adaptation or by changes in the community composition. Even small additions of organic substrates (in the form of sludge, fly ash, gypsum and topsoil) may trigger a shift in the composition of the microbial community and an accompanying change in the relative abundance of specific hydrolytic ecto-enzymes (Pinhassi *et al.*, 1999).

Conclusions

This is the first report of bacterial diversity analysis of red mud. Bacterial diversity seems to be complex of this extreme site as revealed by the cultivation based and culture independent approach. Cultivation based approach, revealed the presence of low bacteria load, predominance of gram-positive bacteria, which belonged to mainly alkaline, saline and contaminated habitat. These isolates mainly belonged to Phylum-Firmicutes, Actinobacteria and Proteobacteria. Sequence alignment of closely related bacteria and red mud bacterial isolates showed that similarity varied from 97 to 99%, but none of the isolates had shown 100% sequence similarity with the bacteria of existing database. On this basis, seven bacteria might be new member of existing genus.

Clone library showed the presence of bacteria inhabiting in contaminated sites. Clones mainly belonged to Proteobacteria and Bacteroidetes; and red mud isolates were related to Firmicutes, Actinobacteria and Proteobacteria. There was no commonality of identical species in clone library and red mud isolates which shows the complex community structure of this extreme environment.

Two of the bacterial isolates (RM9P and RM8), which were studied for alkaline tolerance showed that buffering capacity and organic acid exudation was more in RM9P which was identified as *Planococcus sp.* than the RM8 (*Agromyces sp.*). These results in combination with zeta potential and glucose consumption at different pH have shown that both the isolates have different strategies to combat the alkaline pH.

Isolated bacteria and *A. tubingensis* were used for the remediation of bauxite residue in combination with different substrates such as gypsum, sewage sludge, topsoil and fly ash. pH reduction was found maximum in gypsum amended red mud. Reduction of alkalinity was achieved maximally in gypsum amendment of red mud. Red mud amended with sewage sludge and bacteria treatment had shown maximal organic carbon, organic matter and available phosphorus. Growth and survival of alkali tolerant *Bermuda* grass which was native of NALCO Damanjodi, Orissa was studied in these treatments. Interestingly, red mud control and red mud inoculated with *A. tubingensis* did not support the growth of *Bermuda* grass, but red mud inoculated with bacteria had increased the growth of grass, but red mud control and red mud inoculated with *A. tubingensis* did not

support the grass growth. It showed that indigenous bacterial consortia played an important role in the growth promotion of *Bermuda* grass without any chemical amendment. *A. tubingenensis* did not promote the growth of *Bermuda* grass when used alone (without any chemical amendment). It had promoted grass growth, which was highest among all treatments when used in combination with sludge. Overall, the present study showed that indigenous bacteria and *A. tubingenensis* played an important role in reducing the pH of the red mud, reducing the soil metal content, promoting the grass growth, increasing the nutrient status, though bauxite residue is a poor substrate for plant growth because of very high pH, salinity and sodicity. Long term studies might be required for the reduction of alkalinity, uptake of metal contents, availability of nutrients and survival of plants. To best of our knowledge it the first report of amelioration of red mud using different biological means in nursery trial.

Summary

Red mud (bauxite residue) is waste produced during aluminum extraction from bauxite ore with concentrated sodium hydroxide at elevated temperature in the Bayer process (Evans, 1993). The major components in the red mud are iron oxide, silica, un-reacted alumina and residual NaOH as Na_2CO_3 as well as alkali bound in the form of sodalite, ferrite etc. The residual alkali content makes the red mud alkaline with a pH range from 9 to 13, and disposed off by putting them into red mud ponds. Reclamation of red mud ponds are difficult because of high pH, high concentration of soluble ions such as sodium and carbonate which are toxic and competitively inhibit the uptake of nutrients in plants and microbes.

In microbiological analysis, it was found that the bacterial count was very less, which varied from 5 to 100 cfu per gram of red mud. Ten bacterial isolates, which were studied, had different Rep-PCR profile and 16S rDNA sequences. 16S rDNA sequences showed that these isolated closely related to *Planococcus sp.*, *Bacillus sp.*, *Pseudomonas sp.*, *Kocuria sp.*, *Micrococcus sp.*, *Agromyces sp.* and *Salinococcus sp.* These isolates mainly belong to Phylum- Firmicutes, Actinobacteria and Proteobacteria. Multiple alignment of closely related bacteria and red mud bacterial isolates showed that similarity varied from 97 to 99%, but none of the isolates showed 100% sequence similarity with the bacteria of existing database. The clones that had sequence identities of over 98% to a known organism may represent the same species. These sequences that share an identity 88% to 98% are usually considered to be part of the same genus (Sadowsky *et al.*, 1996). On this basis, seven red mud isolates described here probably represent new members of the known genera. Furthermore, none of the isolates showed homology with the Biolog database when grown on Biolog plates, which depicts that these strains are not characterized previously.

Screening of the clone library resulted in distinct 16S rDNA sequences as compared to isolated cultures. These were mainly belonging to Proteobacteria and Bacteroidetes. These bacterial clones were affiliated to Beta- (34%) and Gamma- (56%) Proteobacteria while 4.8% clones represent Bacteroidetes. Interestingly, all sequences of clone library had 99% or more similarity with the uncultured and cultivable isolates. But, these clones

showed no homology with the cultivable isolates of red mud. These results showed that the bacterial diversity is complex in this environment. The present study gives the first insight about the microbiological (bacterial diversity) analysis of red mud.

The indigenous bacterial isolates and exogenous *Aspergillus tubingensis* were used in nursery trial for the remediation and revegetation of red mud in combination with fly ash, topsoil, gypsum and sewage sludge. Although, gypsum amended red mud showed maximum reduction of alkalinity (pH) and attained pH about 8.0, but the level of soluble ion in this treatment was relatively high as revealed by the measurement of electrical conductivity. Red mud amended sewage sludge and bacterial treatment had highest level of organic carbon, organic matter and available phosphorus. Red mud amended sewage sludge and bacterial treatment showed maximum metabolic diversity of bacteria on Biolog plates. Curiously, red mud inoculated with bacteria also showed growth of grass, but red mud control and red mud inoculated with *A. tubingensis* did not support the grass growth. It showed that indigenous bacterial consortia played an important role in the growth promotion of *Bermuda* grass without any chemical amendment. *A. tubingensis* did not promote the growth of *Bermuda* grass when used alone (without any chemical amendment). It promoted grass growth, which was highest when used in combination with sludge. Overall, the present study showed that *A. tubingensis* and indigenous bacteria play an important role in reducing the pH of the red mud and also promote the plant growth, though bauxite residue is a poor substrate for plant growth because of very high pH, salinity and sodicity. To best of our knowledge it the first report of amelioration of red mud using biological means in nursery trial.

Alkali tolerance mechanism were studied on two red mud isolates RM9P and RM8, which had shown that the buffering capacities are influenced by the pH of the medium and culture conditions. RM8 showed more buffering capacity than the RM9P. Alkali tolerance is strongly dependent on the cellular metabolism, as it was evident from the glycolysis reactions and organic acid production of RM9P and RM8 at different pH.

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Appendix I

Alkaline nutrient agar

Ingredients	Quantity (g/L)
Casein Hydrosylates	15.0
Peptone	5.0
NaCl	5.0
Agar	20.0

pH adjusted with 10N NaOH to 10.5 before autoclaving. Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Alkaline tryptic soya agar

Ingredients	Quantity (g/L)
Casein enzyme hydrosylates	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Agar	20.0

pH adjusted with 10N NaOH to 10.5 before autoclaving. Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Alkalophilic Horikoshi agar medium

Ingredients	Quantity (g/L)
Sucrose	10.0
Yeast extract	5.5
Polypeptone	5.5
K ₂ HPO ₄	1.1
Mg ₂ SO ₄ .7H ₂ O	0.2
Na ₂ CO ₃	10.0
Agar	20.0

Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Pikovskaya's agar

Ingredients	Quantity (g/L)
Agar	15.0
Ammonium sulphate	0.5
Calcium phosphate	5.0
Dextrose	10.0
Ferrous sulphate	0.0001
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Potassium chloride	0.2
Yeast extract	0.5

Suspended 31.3 g in 1000 ml distilled water. Boiled to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Czapek Dox medium (modified)

Ingredients	Quantity (g/L)
Glucose	10.0
Potassium nitrate	3.0
Magnesium sulfate	0.5
Potassium chloride	0.5
Iron(III)sulfate	0.01
di-potassium hydrogen phosphate	1.0

pH adjusted with 1N HCl to 5.5 and autoclaved (15 min at 121 °C).

Red mud extract media

Red mud was mixed with water in 1:5 ratio, kept at shaker (120 rpm) overnight and then filtered to separate water soluble part. To this extract following compound were mixed-

Ingredients	Quantity (g/L)
Glucose/Sucrose	10.0
KH ₂ PO ₄	5.0
Yeast extract	5.0
Casamino acid	5.0
Agar	20.0

Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Luria-Bertani (LB) medium

Ingredients	Quantity (g/L)
NaCl	10.0
Beef extract	5.0
Tryptone	10.0
Agar	10.0

pH 7 adjusted with 5N NaOH. Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min. (Added filter sterilized ampicillin 50 µg/ml to prepare LB - Ampicillin plates)

Actinomycete agar

Ingredients	Quantity (g/L)
Agar	15.0
Dipotassium phosphate	0.5
Ferrous sulphate	0.001
L-Asparagine	0.1
Magnesium sulphate	0.1
Sodium caseinate	2.0
Sodium propionate	4.0

Suspended 22 g in 1000 ml distilled water containing 5.0 ml glycerol. Boiled to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Nitrate agar

Ingredients	Quantity (g/L)
Agar	12.0
Beef extract	3.0

Peptic digest of animal tissue 5.0
 Potassium nitrate 1.0
 Suspended 21 g of nitrate agar in 1000 ml distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

Modified Universal buffer (5X)

Tris (hydroxyl methyl) amino methane 3.025 g
 Maleic acid 2.90 g
 Citric acid 3.50 g
 Boric acid 1.57 g
 NaOH (1N) 122 ml
 Water up to 250 ml
 pH 5.5

Phosphate buffer

Stock solution A

2 M monobasic sodium phosphate, monohydrate (276 g/L)

Stock solution B

2 M dibasic sodium phosphate (284 g/L).

Mixing an appropriate volume (ml) of A and B as shown in the table below and diluting to a total volume of 200 ml, a 1 M phosphate buffer of the required pH at room temperature.

A (mL)	B (mL)	pH
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5

Carbonate and bicarbonate buffer

Stock solution A

0.1 M Sodium carbonate. 10.599 g anhydrous Na₂CO₃ are dissolved in water and diluted to 1 L.

Stock solution B

0.1 M Sodium bicarbonate. 8.4 g NaHCO₃ are dissolved in water and diluted to 1 L.

Solution A and B were mixed in following way:

A (mL)	B (mL)	pH
5	5	9.90
6	4	10.14
7	3	10.28
8	2	10.53
9	1	10.83

TBE buffer (10x)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

Molybdate-tartarate solution

- i) Dissolved 12 g of ammonium molybdate in about 250 ml distilled water.
- ii) Dissolved 0.291 g of antimony potassium tartarate in 100 ml of distilled water. Added the two solutions to 1L of 5N H₂SO₄. Mixed thoroughly and made the volume to 2L with distilled water.

Bray's P-1 extractant

Dissolved 1.110 g of AR grade ammonium fluoride in one liter of 0.025N HCl.

Plasmid extraction solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na ₂ EDTA	10mM

Plasmid extraction solution II

NaOH	5M
SDS	10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

Ligation reaction of amplicon in pTZ57R/T

Plasmid pTZ57R/T (50ng/μl)	3μl
Amplicon (75ng/μl)	4μl
Buffer (10X)	3μl
T4 Ligase	1μl
H ₂ O	19μl

Ligation reaction of amplicon in pGEM-Teasy

Plasmid pGEM-Teasy (50ng/μl)	1μl
Amplicon (75ng/μl)	1μl
Buffer (2X)	5μl
T4 Ligase	1μl
H ₂ O	2μl

Primers

M13 forward primer	5'-GTAAAACGACGGCCAGT-3'
M13 reverse primer	5'-CAGGAAACAGCTATGAC-3'
T7 primer	5'-TAATACGACTCACTATAGGG-3'
SP6 primer	5'-ATTTAGGTGACACTATAG-3'
Rep forward primer	5'-IIICGICGICATCIGGC -3'
Rep reverse primer	5'-ICGICTTATCIGGCCTAC - 3'
16S rDNA forward primer	5'-AGAGTTTGATCCTGGCTCAG-3'
16S rDNA reverse primer	5'-ACGGGCGGTGTGTTC-3'
16S rDNA forward primer (V3 region)	5'-GACTCCTACGGGAGGCAGCAG -3'
16S rDNA reverse primer (V3 region)	5'-ATTACCGCGGCTGCTGG -3'

Appendix II

16S rDNA sequences of red mud isolates

16S rDNA sequences of red mud isolate RM9P

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SOURCE Planococcus sp. RM9P
ORGANISM [Planococcus sp. RM9P](#)
Bacteria; Firmicutes; Bacillales; Planococcaceae; Planococcus.
REFERENCE 1 (bases 1 to 1511)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic population of bacteria from extremely alkaline bauxite residue (red mud)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1511)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
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16S rDNA sequences of red mud isolate RM10E

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 VERSION EF675620.1 GI:151549011
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 Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
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 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic population of bacteria from extremely alkaline bauxite residue (red mud)
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1514)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Direct Submission
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ORIGIN

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16S rDNA sequences of red mud isolate RM10D

LOCUS EF675621 1486 bp DNA linear BCT 16-JUL-2007
 DEFINITION Microbacterium sp. RM10D 16S ribosomal RNA gene, partial sequence.
 ACCESSION EF675621
 VERSION EF675621.1 GI:151549012
 KEYWORDS .
 SOURCE Microbacterium sp. RM10D
 ORGANISM [Microbacterium sp. RM10D](#)
 Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
 Micrococccineae; Microbacteriaceae; Microbacterium.

REFERENCE 1 (bases 1 to 1486)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic population of bacteria from extremely alkaline bauxite residue (red mud)
 JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1486)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Direct Submission
 JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

FEATURES
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16S rDNA sequences of red mud isolate RM11R

LOCUS EF675622 1512 bp DNA linear BCT 16-JUL-2007
DEFINITION *Salinicoccus* sp. RM11R 16S ribosomal RNA gene, partial sequence.
ACCESSION EF675622
VERSION EF675622.1 GI:151549013
KEYWORDS .
SOURCE *Salinicoccus* sp. RM11R
ORGANISM [Salinicoccus sp. RM11R](#)
Bacteria; Firmicutes; Bacillales; *Salinicoccus*.
REFERENCE 1 (bases 1 to 1512)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic population of bacteria from extremely alkaline bauxite residue (red mud)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1512)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
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16S rDNA sequences of red mud isolate RM12W

LOCUS EF675623 1499 bp DNA linear BCT 16-JUL-2007
 DEFINITION Pseudomonas sp. RM12W 16S ribosomal RNA gene, partial sequence.
 ACCESSION EF675623
 VERSION EF675623.1 GI:151549014
 KEYWORDS .
 SOURCE Pseudomonas sp. RM12W
 ORGANISM [Pseudomonas sp. RM12W](#)
 Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
 Pseudomonadaceae; Pseudomonas.
 REFERENCE 1 (bases 1 to 1499)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic
 population of bacteria from extremely alkaline bauxite residue (red
 mud)
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1499)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Direct Submission
 JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and
 Environmental Sciences, Thapar University, Bhadson Road, Patiala,
 Punjab 147004, India
 FEATURES Location/Qualifiers
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16S rDNA sequences of red mud isolate RM13Y

LOCUS EF675624 1498 bp DNA linear BCT 16-JUL-2007
 DEFINITION Kocuria sp. RM13Y 16S ribosomal RNA gene, partial sequence.
 ACCESSION EF675624
 VERSION EF675624.1 GI:151549015
 KEYWORDS .
 SOURCE Kocuria sp. RM13Y
 ORGANISM [Kocuria sp. RM13Y](#)
 Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
 Micrococccineae; Micrococccaceae; Kocuria.
 REFERENCE 1 (bases 1 to 1498)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic
 population of bacteria from extremely alkaline bauxite residue (red
 mud)
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1498)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Direct Submission
 JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and
 Environmental Sciences, Thapar University, Bhadson Road, Patiala,
 Punjab 147004, India
 FEATURES Location/Qualifiers
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16S rDNA sequences of red mud isolate RM1

LOCUS EF675625 1488 bp DNA linear BCT 16-JUL-2007
DEFINITION Kocuria sp. RM1 16S ribosomal RNA gene, partial sequence.
ACCESSION EF675625
VERSION EF675625.1 GI:151549016
KEYWORDS .
SOURCE Kocuria sp. RM1
ORGANISM [Kocuria sp. RM1](#)
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Micrococcineae; Micrococcaceae; Kocuria.
REFERENCE 1 (bases 1 to 1488)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic
population of bacteria from extremely alkaline bauxite residue (red
mud)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1488)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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16S rDNA sequences of red mud isolate RM1A

LOCUS EF675626 1515 bp DNA linear BCT 16-JUL-2007
DEFINITION Bacillus sp. RM1A 16S ribosomal RNA gene, partial sequence.
ACCESSION EF675626
VERSION EF675626.1 GI:151549017
KEYWORDS .
SOURCE Bacillus sp. RM1A
ORGANISM [Bacillus sp. RM1A](#)
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 1515)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic population of bacteria from extremely alkaline bauxite residue (red mud)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1515)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
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661 agaggaaagc ggaattccac gtgtagcggg gaaatgcgta gagatgtgga ggaacaccag
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1261 cgaacccgag agggggagcc aatcccataa aaccgttctt agttcggatt gcaggctgca
1321 actcgcctgc atgaagccgg aatcgcctag aatcgcggat cagcatgccg cggtgatac
1381 gttccggggc cttgtacaca ccgcccgta caccacgaga gtttgtaaca cccgaagtcg
1441 gtggggtaac cttttggga gccagccgac gaagggtggga cagatgattg ggggtgaagtc
1501 gtaacaaggt aacca
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16S rDNA sequences of red mud isolate RM6

LOCUS EF675627 1512 bp DNA linear BCT 16-JUL-2007
DEFINITION Bacillus sp. RM6 16S ribosomal RNA gene, partial sequence.
ACCESSION EF675627
VERSION EF675627.1 GI:151549018
KEYWORDS .
SOURCE Bacillus sp. RM6
ORGANISM [Bacillus sp. RM6](#)
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 1512)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic population of bacteria from extremely alkaline bauxite residue (red mud)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1512)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
source 1..1512
/organism="Bacillus sp. RM6"
/mol_type="genomic DNA"
/strain="RM6"
/isolation_source="bauxite residue (red mud)"
/db_xref="taxon:[410796](#)"
[rRNA](#) <1..>1512
/product="16S ribosomal RNA"

ORIGIN

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121 acctgctgt aagattggga taactcggg aaaccggagc taataccgga taacattttg
181 aaccgcatgg ttcaaaattg aaaggtggct ttgtctatca cttacagatg gacccgcggc
241 gcattagcta gttggtgagg taacggctca ccaaggcaac gatgctgtag cgacctgaga
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361 ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc cgcgtgaacg atgaaggcct
421 tgggtcgta aagttctggt gttagggag aacaagtacc agagtaactg ctgggtacctt
481 gacgtacct aaccagaaag ccacggctaa ctacgtgcca gcagccgagg taatacgtag
541 gtggcaagcg ttgtccggaa ttattgggag taaagcgtac gcagggcggt tcttaagtct
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661 gaagaggaga gtggaattcc acgtgtagcg gtgaaatgcg tagagatgtg gaggaacacc
721 agtggcgaag gcgactctct ggtctgtaac tgacgctgag gtacgaaagc gtggggagcg
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901 aagactgaaa ctcaaaggaa ttgacggggg cccgcacaag cgggtggagca tgtgggttaa
961 ttcgaagcaa cgcgaagaac cttaccaggt cttgacatcc cactgcccgg tatagagata
1021 tacctttccc ttcgggggaca gtgggtgacag gtgggtgcatg gttgtcgtca gctcgtgtcg
1081 tgagatggtg ggttaagtcc cgcaacgagc gcaacccttg atcttagttg ccagcattta
1141 gttgggcact ctaagggtgac tgccgggtgac aaaccggagg aagggtggga tgacgtcaaa
1201 tcatcatgcc cttatgacc tgggctacac acgtgctaca atggatggta caaagggctg
1261 caagaccgag aggtcaagcc aatcccataa aaccattctc agttcggatt gcaggctgca
1321 actcgcctgc atgaagctgg aatcgctagt aatcgcggat cagcatgccg cgggtgaatac
1381 gttccggggc cttgtacaca ccgcccgtca caccacgaga gtttgtaaca cccgaagtgc
1441 gtggggtaac cgtaaggagc cagccgccta aggtgggaca gatgattggg gtgaagtcgt
1501 aacaaggtaa cc
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16S rDNA sequences of red mud isolate RM8

LOCUS EF675628 1489 bp DNA linear BCT 16-JUL-2007
DEFINITION Agromyces sp. RM8 16S ribosomal RNA gene, partial sequence.
ACCESSION EF675628
VERSION EF675628.1 GI:151549019
KEYWORDS .
SOURCE Agromyces sp. RM8
ORGANISM [Agromyces sp. RM8](#)
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Micrococccineae; Microbacteriaceae; Agromyces.
REFERENCE 1 (bases 1 to 1489)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Phylogenetic and physiological diversity of a heterotrophic aerobic
population of bacteria from extremely alkaline bauxite residue (red
mud)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1489)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (13-JUN-2007) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..1489
/organism="Agromyces sp. RM8"
/mol_type="genomic DNA"
/strain="RM8"
/isolation_source="bauxite residue (red mud)"
/db_xref="taxon:[453989](#)"
[rRNA](#)
<1..>1489
/product="16S ribosomal RNA"
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121 cctgcctggg actctgggat aaccccgaga aatcggagct aataccggat aggacctttc
181 ctgcgatgag gtttgggtga aagtttttcg gtctgggatg gactcgcggc ctatcagctt
241 gttggtgagg taatggctca ccaaggcgtc gacgggtagc cggcctgaga gggtgaccgg
301 ccacactggg actgagacac ggcccagact cctacgggag gcagcagtgg ggaatattgc
361 acaatgggag caagcctgat gcagcaacgc cgcgtgctgg atgacggcct tcgggttgta
421 aaccgctttt agtagggaag aagggtctcg gcttgacggt acctgcagaa aaaggaccgg
481 ctaactacgt gccagcagcc gcggaatac gtaggggtccg agcgttgtcc ggaattattg
541 ggcgtaaaga gctcgtaggc ggtttgtcgc gtctgctgtg aaaactagag gctcaacctc
601 tagcctgcag tgggtacggg cagacttgag tgggtgtagg gagactggaa ttctggtgtg
661 agcgggtgaa tgcgcagata tcaggaggaa caccgatggc gaaggcaggt ctctgggac
721 ttactgacgc tgaaggacga aagcgtgggg agcgaacagg attagatacc ctggtagtcc
781 acgccgtaaa cgttggggcg tagatgtggg gacctttcca cggtttccgt gtcgtagcta
841 acgcattaag cgcccgcct ggggagtacg gccgcaaggc taaaactcaa aggaattgac
901 gggggcccgc acaagcggcg gagcatgcgg ataatcga tgcaacgcga agaaccttac
961 caaggcttga catacagag aacgggccag aaatgggtcaa ctctttggac actcgtatac
1021 aggtggtgca tgggtgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga
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1201 tcacgcatgc tacaatggcc ggtacaaagg gctgcatgt cgtaaggcgg agcgaatccc
1261 aaaaagccgg tctcagttcg gattgaggtc tgcaactcga cctcatgaag tcggagtcgc
1321 tagtaatcgc agatcagcaa cgctgcgggtg aatacgttcc cgggccttgt acacaccgcc
1381 cgtcaagtca tgaagtcggg taacaccgga agccgggtggc ctaacccttg tggaggggagc
1441 cgccaagggt gggatcgggt attagatta agtcgtaaca aggtaacca

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16S rDNA sequences of red mud clones directly retrieved form red mud

16S rDNA sequences of red mud isolate RMCL-2

LOCUS EU665639 705 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-2 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665639
VERSION EU665639.1 GI:187960736
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 705)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 705)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..705
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-2"
/environmental_sample
[rRNA](#) <1..>705
/product="16S ribosomal RNA"
ORIGIN
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61 ggcagcagct ttatagcaat atagaggctg gcgaccggca aacgggtgcg gaacacgtac
121 acaaccttcc ttgaataggg ggatagccca tagaaatgtg gattaatacc ccgtaata
181 gcggtgtggc atcacactgt tattatagtt tcggcgattc gagatgggtg tgcggctgat
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301 gatcagccac acgggcaactg agacacgggc ccgactccta cgggaggcag cagtaaggaa
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421 gattgtaaac ttcttttata gggggcgaaa aaaggtcttt ctagatcact tgacagtacc
481 ctatgaataa gcaccggcta actccgtgcc agcagccgcg gtaatacggg ggggtgcaagc
541 gttatccgga ttcaactgggt ttaaagggtg cgtaggcggg taggtaagtc agaggtgaaa
601 tcctggagct taactccaga actgcctttg atactatcta tcttgaatat ggtggaggtg
661 agcggaaatg gtcgatgtagc ggtgaaatgc atagatatga catag

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16S rDNA sequences of red mud isolate RMCL-202

LOCUS EU665640 705 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-202 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665640
VERSION EU665640.1 GI:187960737
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 705)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 705)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..705
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-202"
/environmental_sample
[rRNA](#) <1..>705
/product="16S ribosomal RNA"
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601 tcctggagct taactccaga actgcctttg atactatcta tcttgaatat ggtggaggtg
661 agcggaatat gtcatgtagc ggtgaaatgc atagatatga catag

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16S rDNA sequences of red mud isolate RMCL-107

LOCUS EU665641 672 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-107 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665641
VERSION EU665641.1 GI:187960738
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 672)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 672)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..672
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-107"
/environmental_sample
[rRNA](#) <1..>672
/product="16S ribosomal RNA"

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121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgtcg
181 caagaccaa gagggggacc ttcgggcctc ttgacatcag atgtgccag atgggattag
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361 ttcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggt
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541 gcgttaatcg gaattactgg gcgtaaagcg cacgcagggc gtttggttaag tcagatgtga
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661 ggggtagaat tc
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16S rDNA sequences of red mud isolate RMCL-143

LOCUS EU665642 692 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-143 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665642
VERSION EU665642.1 GI:187960739
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 692)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 692)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..692
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-143"
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[rRNA](#) <1..>692
/product="16S ribosomal RNA"
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121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgtcg
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361 cacaatgggc gcaagcctga tgcagccatg ccgcgtgtgt gaagaaggcc ttcggggtgt
421 aaagcacttt cagcgaggag gaaggtgggtg agcttaatac gctcatcaat tgacgttact
481 cgcagaagaa gcaccggcta actccgtgcc agcagccgcg gtaatacggg ggggtgcaagc
541 gttaatcgga attactgggc gtaaagcgca cgcagggcgg ttgttaagtc agatgtgaaa
601 tccccgggct caactggga actgcatttg aaactggcaa gctagagtct cgtagagggg
661 ggtagaattc caggtgtagc ggtgaaatgc gt
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16S rDNA sequences of red mud isolate RMCL-63

LOCUS EU665643 678 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-63 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665643
VERSION EU665643.1 GI:187960740
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 678)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 678)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..678
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-63"
/environmental_sample
[rRNA](#) <1..>678
/product="16S ribosomal RNA"
ORIGIN
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541 gcgttaatcg gaattactgg gcgtaaagcg cacgcaggcg gtttggttaag tcagatgtga
601 aatccccggg ctcaacctgg gaactgcatt tgaaaactggc aagctagagt ctcgtagagg
661 ggggtagaat tccagggtg

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16S rDNA sequences of red mud isolate RMCL-89

LOCUS EU665644 709 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-89 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665644
VERSION EU665644.1 GI:187960741
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 709)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 709)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..709
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-89"
/environmental_sample
[rRNA](#) <1..>709
/product="16S ribosomal RNA"

ORIGIN

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1 agagtttgat cctggctcag attgaacgct ggcggcaggc ttaacacatg caagtcgagc
61 ggtagcacag gggggcttgc tcctggggtg acgagcggcg gacgggtgag taatgtctgg
121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgctg
181 caagaccaa gaggggacct tcgggcctct tgccatcaga tgtgcccaga tgggattagc
241 tagtaggtgg ggtaatggct cacctaggcg acgatcccta gctgggtctga gaggatgacc
301 agccacactg gaactgagac acggtccaga ctctacggg aggcagcagt ggggaatatt
361 gcacaatggg cgcaagcctg atgcagccat gccgcgtgtg tgaagaaggc cttcggggtg
421 taaagcactt tcagcgagga ggaaggtggt gagcttaata cgttcatcaa ttgacgttac
481 tcgcagaaga agcaccggct aactccgtgc cagcagccgc ggtaatacgg aggggtgcaag
541 cgtaaatcgg aattactggg cgtaaagcgc acgcaggcgg ttgttaagt cagatgtgaa
601 atcccggggc tcaacctggg aactgcattt gaaactggca agctagagtc tcgtagaggg
661 gggtagaatt ccaggtgtag cggtgaaatg cgtagagatc tggaggaat
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16S rDNA sequences of red mud isolate RMCL-61

LOCUS EU665645 687 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-61 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665645
VERSION EU665645.1 GI:187960742
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 687)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 687)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..687
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-61"
/environmental_sample
[rRNA](#) <1..>687
/product="16S ribosomal RNA"

ORIGIN

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121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgtcg
181 caagaccaa gagggggacc ttcgggcctc ttgccatcag atgtgccag atgggattag
241 ctagtaggtg gggtaatggc tcacctaggg gacgatccct agctgggtctg agaggatgac
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggtt
421 gtaaagcact ttcagcgagg aggaaggtgg tgagcttaat acgttcatca attgacgtta
481 ctgcgagaag aagcaccggc taactccgtg ccagcagccg cggtaatagc gaggggtgcaa
541 gcgttaatcg gaattactgg gcgtaaagcg cacgcaggcg gtttgttaag tcagatgtga
601 aatccccggg ctcaacctgg gaactgcatt tgaaaactggc aagctagagt ctcgtagagg
661 ggggtagaat tccaggtgta gcggtga
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16S rDNA sequences of red mud isolate RMCL-121

LOCUS EU665646 703 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-121 16S ribosomal RNA gene, partial sequence.
ACCESSION EU665646
VERSION EU665646.1 GI:187960743
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic bacteria of aluminum processing industry waste bauxite residue (red mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
source 1..703
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-121"
/environmental_sample
[rRNA](#) <1..>703
/product="16S ribosomal RNA"

ORIGIN

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121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgctg
181 caagaccaa gagggggacc ttcgggcctc ttgccatcag atgtgccag atgggattag
241 ctagtaggtg gggtaatggc tcacctagcc gacgatccct agctgggtctg agaggatgac
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggt
421 gtaaagcact ttcagcgagg aggaaggtgg tgagcttaat acgttcatca attgacgtta
481 ctgcgagaag aagcaccggc taactccgtg ccagcagccg cggtaatagc gaggggtgcaa
541 gcgttaatcg gaattactgg gcgtaaagcg cacgcagggc gtttggttaag tcagatgtga
601 aatccccggg ctcaacctgg gaactgcatt tgaaactggc aagctagagt ctcgtagagg
661 ggggtagaat tccaggtgta gcggtgaaat gcgtagagat ctg
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//

16S rDNA sequences of red mud isolate RMCL-14

LOCUS EU665647 742 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-14 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665647
VERSION EU665647.1 GI:187960744
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 742)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 742)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..742
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-14"
/environmental_sample
[rRNA](#) <1..>742
/product="16S ribosomal RNA"
ORIGIN
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61 ggaggttggt cccttgaggc gtggcttccg gagctaacgc gttaaatcga ccgcctgggg
121 agtacggccg caaggttaaa actcaaatga atgacggggg gcccgcaaa gcgggtggagc
181 atgtggttta attcgatgca acgcgaagaa ccttacctac tcttgacatc cagagaactt
241 tccagagatg gattggtgcc ttcgggaact ctgagacagg tgctgcatgg ctgtcgctcag
301 ctctgtttgt gaaatggttg gttaagtccc gcaacgagcg caacccttat cctttgttgc
361 cagcggttcg gccgggaact caaaggagac tgccagtgat aaactggagg aagggtgggga
421 tgacgtcaag tcatcatggc ccttacgagt agggctacac acgtgctaca atggcatgta
481 caaagagaag cgacctcgcg agagcaagcg gacctcataa agtatgtcgt agtccggatt
541 ggagtctgca actcgactcc atgaagtcgg aatcgctagt aatcgtagat cagaatgcta
601 cgggtaatac gttcccgggc cttgtacaca ccgcccgtca caccatggga gtgggttgca
661 aaagaagtag gtagcttaac cttcgggagg gcgcttacca ctttgtgatt catgactggg
721 gtgaagtcgt aacaaggtaa cc

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16S rDNA sequences of red mud isolate RMCL-19

LOCUS EU665648 711 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-19 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665648
VERSION EU665648.1 GI:187960745
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 711)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 711)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..711
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-19"
/environmental_sample
[rRNA](#) <1..>711
/product="16S ribosomal RNA"
ORIGIN
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61 agctaacgcg ttaaatcgac cgcctgggga gtacggccgc aaggttaaaa ctcaaataaa
121 ttgacggggg cccgcacaag cggtaggagca tgtggtttaa ttcgatgcaa cgcgaagaac
181 cttacctact cttgacatcc agagaacttt ccagagatgg attggtgcct tcgggaactc
241 tgagacaggt gctgcatggc tgtcgtcagc tcgtgttgtg aaatggtggg ttaagtcccg
301 caacgagcgc aacccttacc ctttgttgcc agcggttcgg ccgggaactc aaaggagact
361 gccagtgata aactggagga agtggggat gacgtcaagt catcatggcc cttacgagta
421 gggctacaca cgtgctacaa tggcatatac aaagagaagc gacctcgcga gagcaagcgg
481 acctcataaa gtatgtcgta gtccggattg ggtctgcaa ctcgactcca tgaagtcgga
541 atcgctagta atcgtagatc agaatgctac ggtgaatacgt ttcccgggccc ttgtacacac
601 cgcccgtcac accatgggag tgggttgcaa aagaagtagg tagcttaacc ttccggaggg
661 cgcttaccac tttgtgattc atgactgggg tgaagtcgta acaaggtaac c

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16S rDNA sequences of red mud isolate RMCL-90

LOCUS EU665650 714 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-90 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665650
VERSION EU665650.1 GI:187960747
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 714)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 714)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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/mol_type="genomic DNA"
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/db_xref="taxon:[77133](#)"
/clone="RMCL-90"
/environmental_sample
[rRNA](#) <1..>714
/product="16S ribosomal RNA"

ORIGIN

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121 gaattgacgg gggcccgcac aagcgggtga gcatgtgggt taattcgatg caacgcgaag
181 aaccttacct actcttgaca tccagagaac ttccagaga tggattgggt ccttcgggaa
241 ctctgagaca ggtgctgcat ggctgtcgtc agctcgtggt gtgaaatggt ggggtaagtc
301 ccgcaacgag cgcaaccctt atcctttggt gccagcgggt cggccgggaa ctcaaaggag
361 actgccagtg ataaactgga ggaaggtggg gatgacgtca agtcatcatg gcccttacga
421 gtagggttac acacgtgctg caatggcata taaaaagaga agcgacctcg cgagagcaag
481 cggacctcat aaagtatgct gtagtcggga ttggagtctg caactcgact ccatgaagtc
541 ggaatcgcta gtaatcgtag atcagaatgc tacgggtgaat acgttcccgg gccttgata
601 caccgccgtg cacacatgg gagtgggttg caaaagaagt aggtagctta accttcggga
661 gggcgcttac cactttgtga ttcagtactg gggggaagtc gtaacaaggt aacc
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16S rDNA sequences of red mud isolate RMCL-102

LOCUS EU665651 706 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-102 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665651
VERSION EU665651.1 GI:187960748
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 706)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 706)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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/organism="uncultured bacterium"
/mol_type="genomic DNA"
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/db_xref="taxon:[77133](#)"
/clone="RMCL-102"
/environmental_sample
[rRNA](#) <1..>706
/product="16S ribosomal RNA"
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61 acgcgttaaa tcgaccgcct ggggagtacg gccgcaaggt taaaactcaa atgaattgac
121 gggggcccgc acaagcgggt gagcatgtgg ttaattcga tgcaacgcga agaacttac
181 ctactcttga catccagaga actttocaga gatggattgg tgccttcggg aactctgaga
241 caggctgtgc atggctgtcg tcagctcgtg ttgtgaaatg ttgggttaag tcccgcacgc
301 agcgcaacc ttatcctttg ttgccagcgg ttcggccggg aactcaaagg agactgccag
361 tgataaactg gaggaagggt gggatgacgt caagtcatca tggcccttac gagtagggct
421 acacacgtgc tacaatggca tatacaaaga gaagcgacct cgcgagagca agcggacctc
481 ataaagtatg tcgtagtccg gattggagtc tgcaactcga ctccatgaag tcggaatcgc
541 tagtaatcgt agatcagaat gctacgggtg atacggtccc gggccttgta cacaccgcc
601 gtcacacat gggagtgggt tgcaaaaaga gtaggtagct taaccttcgg gggggcgctt
661 accactttgt gattcatgac tgggggtgaag tcgtaacaag gtaacc

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16S rDNA sequences of red mud isolate RMCL-125

LOCUS EU665652 709 bp DNA linear ENV 18-MAY-2008
 DEFINITION Uncultured bacterium clone RMCL-125 16S ribosomal RNA gene, partial sequence.
 ACCESSION EU665652
 VERSION EU665652.1 GI:187960749
 KEYWORDS ENV.
 SOURCE uncultured bacterium
 ORGANISM [uncultured bacterium](#)
 Bacteria; environmental samples.
 REFERENCE 1 (bases 1 to 709)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic bacteria of aluminum processing industry waste bauxite residue (red mud) impoundment of extremely alkaline nature
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 709)
 AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
 TITLE Direct Submission
 JOURNAL Submitted (21-APR-2008) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
 FEATURES Location/Qualifiers
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 /organism="uncultured bacterium"
 /mol_type="genomic DNA"
 /isolation_source="extremely alkaline bauxite residue (red mud) waste generated during the alumina extraction"
 /db_xref="taxon:[77133](#)"
 /clone="RMCL-125"
 /environmental_sample
[rRNA](#) <1..>709
 /product="16S ribosomal RNA"

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 121 gacgggggccc cgcacaagcg gtggagcatg tggtttaatt cgatgcaacg cgaagaacct
 181 tacctactct tgacatccag agaactttcc agagatggat tggcgccttc gggaaactctg
 241 agacaggtgc tgcattggctg tcgtcagctc gtgttgtgaa atgttggggtt aagtcccgcgca
 301 acgagcgcaa cccttatcct ttgttgccag cggttcggcc gggaaactcta aggagactgc
 361 cagtataaaa ctggaggaag gtggggatga cgtcaagtca tcatggccct tacgagtagg
 421 gctacacacg tgctacaatg gcatatacaa agagaagcga cctcgcgaga gcaagcggac
 481 ctcataaagt atgtcgtagt ccggattgga gtctgcaact cgactccatg aagtccggaat
 541 cgctagtaat cgtagatcag aatgctacgg tgaatacgtt cccgggcctt gtacacaccg
 601 cccgtcacac catggggagtg ggttgcaaaa gaagtaggta gcttaacctt cgggagggcg
 661 cttaccactt tgtgattcat gactgggggtg aagtcgtaac aaggtaacc

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16S rDNA sequences of red mud isolate RMCL-142

LOCUS EU665653 704 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-142 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665653
VERSION EU665653.1 GI:187960750
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 704)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 704)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..704
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-142"
/environmental_sample
[rRNA](#) <1..>704
/product="16S ribosomal RNA"
ORIGIN
1 acgctgtaaa cgatgtcgat ttggagggtg tggccttgag gcgtggcttc cggagctaac
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121 gggcccgcac aagcgggtgga gcatgtggtt taattcgatg caacgcgaag aaccttacct
181 actcttgaca tccagagaac tttccagaga tggattggtg ccttcgggaa ctctgagaca
241 ggtgctgcat ggctgtcgtc agctcgtggt gtgaaatggt gggttaagtc ccgcaacgag
301 cgcaaccctt atcctttggt gccagcgggt cggccgggaa ctcaaaggag actgccagtg
361 ataaactgga ggaaggtggg gatgacgtca agtcatcatg gcccttacga gtagggctac
421 acacgtgcta caatggcata tacaagaga agcgacctcg cgagagcaag cggacctcat
481 aaagtatgtc gtatccgga ttggagtctg caactcgact ccatgaagtc ggaatcgcta
541 gtaatcgtag atcagaatgc tacggtagaat acgttcccgg gccttgata caccgcccgt
601 cacaccatgg gtaggggtg caaagaagt aggtagctta accttcggga gggcgcttac
661 cactttgtga ttcatgactg gggtagagtc gtaacaaggt aacc

//

16S rDNA sequences of red mud isolate RMCL-66

LOCUS EU665654 426 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-66 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665654
VERSION EU665654.1 GI:187960751
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 426)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 426)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..426
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/db_xref="taxon:[77133](#)"
/clone="RMCL-66"
/environmental_sample
[rRNA](#) <1..>426
/product="16S ribosomal RNA"
ORIGIN
1 agagtttgat cctggctcag attgaacgct ggcggcaggc ttaacacatg caagtcgagc
61 ggtagcaciaa gggagcttgc tccctggggtg acgagcggcg gacgggtgag taatgtctgg
121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgtcg
181 caagacaaa gagggggacc ttcggggctc ttgccatcag atgtgcccag atgggattag
241 ctagtaggtg gggtaatggc tcacctaggc gacgatccct agctggtctg agaggatgac
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggt
421 gtaaag
//

16S rDNA sequences of red mud isolate RMCL-3

LOCUS EU665655 703 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-3 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665655
VERSION EU665655.1 GI:187960752
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..703
/organism="uncultured bacterium"
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mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-3"
/environmental_sample
[rRNA](#) <1..>703
/product="16S ribosomal RNA"

ORIGIN

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121 ggcccgcaca agcgggtggag catgtggttt aattcgatgc aacgcgaaga accttaccta
181 ctcttgacat ccagagaact ttccagagat agattggtgc cttcgggaac tctgagacag
241 gtgctgcatg gctgtcgta gctcgtggtg tgaatggtg ggtaagtcc cgcaacgagc
301 gcaaccctta tccttggtg ccagcggttc ggccgggaac tcaaaggaga ctgccagtga
361 taaactggag gaagtgggg atgacgtcaa gtcacatggt cccttacgag tagggctaca
421 cacgtgctac aatggcatat acaaagagaa gcgacctcgc gagagcaagc ggacctcata
481 aagtatgtcg tagtccggat tggagtctgc aactcgactc catgaagtgc gaatcgctag
541 taatcgtaga tcagaatgct acggtgaata cgttcccggg cttgtacac accgcccgtc
601 acaccatggg agtgggttgc aaaagaagta ggtagcttaa cttcgggag ggcgcttacc
661 actttgtgat tcatgactgg ggtgaagtgc taacaaggta acc
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16S rDNA sequences of red mud isolate RMCL-4

LOCUS EU665656 694 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-4 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665656
VERSION EU665656.1 GI:187960753
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 694)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 694)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..694
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-4"
/environmental_sample
[rRNA](#) <1..>694
/product="16S ribosomal RNA"
ORIGIN
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121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgtcg
181 caagaccaa gagggggacc ttcgggcctc ttgccatcag atgtgccag atgggattag
241 ctagtaggtg gggtaatggc tcacctagcc gacgatccct agctggctctg agaggatgac
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggt
421 gtaaagcact ttcagcgagg aggaaggtgg tgaacttaat acgttcatca attgacgtta
481 ctgcgagaag aagcaccggc taactccgtg ccagcagccg cggtaatagc gaggggtgcaa
541 gcgttaatcg gaattactgg gcgtaaagcg cacgcagggc gtttggttaag tcagatgtga
601 aatccccggg ctcaacctgg gaactgcatt tgaaactggc aagctagagt ctcgtagagg
661 ggggtagaat tccaggtgta gcggtgaaat gcgt

//

16S rDNA sequences of red mud isolate RMCL-95

LOCUS EU665657 697 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-95 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665657
VERSION EU665657.1 GI:187960754
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 697)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 697)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..697
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-95"
/environmental_sample
[rRNA](#) <1..>697
/product="16S ribosomal RNA"

ORIGIN

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61 ggtagcacag gggagcttgc tccctggggtg acgagcggcg gacgggtgag taatgtctgg
121 gaaactgcct gatggagggg gataactact gaaacggta gctaataccg cataacgctg
181 caagaccaa gagggggacc ttagggcctc ttgccatcag atgtgccag atgggattag
241 ctagtaggtg gggtaatggc tcacctagcc gacgatccct agctggctctg agaggatgac
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggt
421 gtaaagcact ttcagcgagg aggaaggtgg tgaacttaat acgttcatca attgacgtta
481 ctgcgagaag aagcaccggc taactccgtg ccagcagccg cggtaatagc gaggggtgcaa
541 gcgttaatcg gaattactgg gcgtaaagcg cacgcagggc gtttggttaag tcagatgtga
601 aatccccggg ctcaacctgg gaactgcatt tgaaactggc aagctagagt ctcgtagagg
661 ggggtagaat tccaggtgta gcggtgaaat gcgtaga
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16S rDNA sequences of red mud isolate RMCL-93

LOCUS EU665658 701 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-93 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665658
VERSION EU665658.1 GI:187960755
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 701)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 701)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..701
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-93"
/environmental_sample
[rRNA](#) <1..>701
/product="16S ribosomal RNA"
ORIGIN
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61 ggtagcacag gggagcttgc tccctggggtg acgagcggcg gacgggtgag taatgtctgg
121 gaaactgctt gatggagggg gataactact gaaacggta gctaataccg cataacgctg
181 caagaccaa gagggggacc ctcgggcctc ttgccatcag atgtgccag atgggattag
241 ctagtaggtg gggtaatggc tcacctaggc gacgatccct agctggctctg agaggatgac
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcggggt
421 gtaaagcact ttcagcgagg aggaaggtgg tgaacttaat acgttcatca attgacgtta
481 ctgcgagaag aagcaccggc taactccgtg ccagcagccg cggtaatagc gagggtgcaa
541 gcgttaatcg gaattactgg gcgtaaagcg cacgcagggc gtttggttaag tcagatgtga
601 aatccccggg ctcaacctgg gaactgcatt tgaaactggc aagctagagt ctcgtagagg
661 ggggtagaat tccaggtgta gcggtgaaat gcgtagagat c

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16S rDNA sequences of red mud isolate RMCL-103

LOCUS EU665659 712 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-103 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665659
VERSION EU665659.1 GI:187960756
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 712)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 712)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..712
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-103"
/environmental_sample
[rRNA](#) <1..>712
/product="16S ribosomal RNA"

ORIGIN

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61 gtagcacagg ggagcttgct ccctgggtga cgagcggcgg acgggtgagt aatgtctggg
121 aaactgcctg atggaggggg ataactactg gaaacggtag ctaataccgc ataacgtcgc
181 aagaccaaag agggggacct tcgggcctct tgccatcaga tgtgcccaga tgggattagc
241 tagtaggtgg ggtaatggct cacctaggcg acgatcccta gctgggtctga gaggatgacc
301 agccacactg gaactgagac acggtccaga ctctacggg aggcagcagt ggggaatatt
361 gcacaatggg cgcaagcctg atgcagccat gccgcgtgtg tgaagaaggc cttcgggttg
421 taaagcactt tcagcgagga ggaaggtggt gaacttaata cgttcatcaa ttgacgttac
481 tcgcagaaga agcaccggct aactccgtgc cagcagccgc ggtaatacgg aggggtgcaag
541 cgттаатсgg aattactggg cgtaaagcgc acgcaggcgg ttgtttaagt cagatgtgaa
601 atccccgggc tcaacctggg aactgcattt gaaactggca agctagagtc tcgtagaggg
661 gggtagaatt ccaggtgtag cggtgaaatg cgtagagatc tggaggaata cc
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16S rDNA sequences of red mud isolate RMCL-115

LOCUS EU665660 658 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-115 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665660
VERSION EU665660.1 GI:187960757
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 658)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 658)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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/organism="uncultured bacterium"
/mol_type="genomic DNA"
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/db_xref="taxon:[77133](#)"
/clone="RMCL-115"
/environmental_sample
[rRNA](#) <1..>658
/product="16S ribosomal RNA"
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61 aatgaattg acggggggccc gcacaagcgg tggagcatgt ggtttaattc gatgcaacgc
121 gaagaacctt acctactctt gacatccaga gaactttcca gagatggatt ggtgccttcg
181 ggaactctga gacagggtgt gcatggctgt cgtcagctcg tgttggtgaaa tgttgggtta
241 agtcccgcaa cgagcgcaac ccttatcctt tgttgccagc ggttcggccg ggaactcaaa
301 ggagactgcc agtgataaac tggaggaagg tggggatgac gtcaagtcac catgaccctt
361 acgagtaggg ctacacacgt gctacaatgg catatacaaa gagaagcgac ctcgcgagag
421 caagcggacc tcataaagta tgtcgtagtc cggattggag tctgcaactc gactccatga
481 agtcggaatc gctagtaatc gtagatcaga atgctacggt gaatacgttc ccgggccttg
541 cacacaccgc ccgtcacacc atgggagtggt gttgcaaaaag aagtaggtag cttaaccttc
601 gggagggcgc ttaccacttt gtgattcatg actgggggtga agtcgtaaca aggtaacc

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16S rDNA sequences of red mud isolate RMCL-16

LOCUS EU665661 684 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-16 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665661
VERSION EU665661.1 GI:187960758
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 684)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 684)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..684
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/db_xref="taxon:[77133](#)"
/clone="RMCL-16"
/environmental_sample
[rRNA](#) <1..>684
/product="16S ribosomal RNA"

ORIGIN

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121 gcatgtggtt taattcgatg caacgcgaag aaccttacct actcttgaca tccagagaac
181 tttccagaga tggattggtg ccttcgggaa ctctgagaca ggtgctgcat ggctgtcgtc
241 agctcgtggt gtgaaatggt gggttaagtc ccgcaacgag cgcaaccctt atcctttggt
301 gccagcggtt cggccgggga ctcaaaggag actgccagtg ataaactgga ggaaggtggg
361 gatgacgtca agtcatcatg gcccttacga gtagggctac acacgtgcta caatggcata
421 tacaagaga agcgcacctg cgagagcaag cggacctcat aaagtatgtc gtagtccgga
481 ttggagtctg caactcgact ccatgaagtc ggaatcgcta gtaatcgtag atcagaatgc
541 tacggtgaat acgttcccgg gccttgata caccgccgt cacaccatgg gagggggttg
601 caaaagaagt agtagctta accttcggga gggcgcttac cactttgtga ttcattgactg
661 ggggtgaagtc gtaacaaggt aacc
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16S rDNA sequences of red mud isolate RMCL-25

LOCUS EU665662 387 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-25 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665662
VERSION EU665662.1 GI:187960759
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 387)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 387)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-25"
/environmental_sample
[rRNA](#) <1..>387
/product="16S ribosomal RNA"
ORIGIN
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61 gggttgtaaa gcccttttgt tgggaaagaa atccagctgg ttaataaccg gttgggatga
121 cggtacccaa agaataagca ccggctaact tcgtgccagc agccgcggta atacgaaggg
181 tgcaagcgtt actcgggaatt actgggcgta aagcgtgctt aggtggctgt ttaagtccgt
241 tgtgaaagcc ctgggctcaa cctgggaact gcagtggata ctgggcgact agagtgtggt
301 agagggtagc ggaattcctg gtgtagcagt gaaatgcgta gagatcagga ggaataccgg
361 tggcgaaggg ggccccctgg acgaaga

//

16S rDNA sequences of red mud isolate RMCL-144

LOCUS EU665663 693 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-144 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665663
VERSION EU665663.1 GI:187960760
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 693)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 693)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..693
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-144"
/environmental_sample
[rRNA](#) <1..>693
/product="16S ribosomal RNA"
ORIGIN
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61 cgcctgggga gtacggctgc aagactgaaa ctcaaaggaa ttgacggggg cccgcacaag
121 cggtaggagta tgtggtttaa ttcgatgcaa cgcgaagaac cttacctggc cttgacatgt
181 cgagaacttt ccagagatgg attggtgcct tcgggaactc gaacacaggt gctgcatggc
241 tgtcgtcagc tegtgtcgtg agatggtggg ttaagtcccc caacgagcgc aacccttgtc
301 cttagtggcc agcacgtaat ggtgggaact ctaaggagac cgccgggtgac aaaccggagg
361 aagtggggga tgacgtcaag tcatcatggc cttacggcc agggctacac acgtactaca
421 atggtagggga cagagggctg caagccggcg acggtaagcc aatcccagaa accctatctc
481 agtccggatt ggagtctgca actcgactcc atgaagtcgg aatcgctagt aatcgcatg
541 cagcattgct gcggtgaata cgttcccggg cttgtacac accgcccgtc acaccatggg
601 agtttgttgc accagaagca ggtagcttaa cttcgggag ggcgcttgcc acggtgtggc
661 cgatgactgg ggtgaagtcg taacaaggta acc

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16S rDNA sequences of red mud isolate RMCL-1

LOCUS EU665664 699 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-1 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665664
VERSION EU665664.1 GI:187960761
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 699)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 699)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..699
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-1"
/environmental_sample
[rRNA](#) <1..>699
/product="16S ribosomal RNA"

ORIGIN

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61 gtgaagttga cgcctggggg agtacggctc caagattaaa actcaaagga attgacgggg
121 acccgcaaaa gcggtggatg atgtggatta attcgatgca acgcgaaaaa ccttacctac
181 ccttgacatg cactaacga agcagagatg catcagggtc ccgaaagga aagtggacac
241 aggtgctgca tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga
301 gcgcaaccct tgtctttagt tgctacgcaa gagcactcta gagagactgc cggtgacaaa
361 ccggaggaag gtggggatga cgtcaagtcc tcatggccct tatgggtagg gcttcacacg
421 tcatacaatg gtgcgtacag agggttgcca acccggaag gggagctaata cccagaaaac
481 gcatcgtagt ccggatcgta gtctgcaact cgactacgtg aagctggaat cgctagtaat
541 cgcggatcag catgccgcgg tgaatacgtt cccgggtcct gtacacaccg cccgtcacac
601 catgggagtg ggttttgcca gaagtagtta gcctaaccgc aaggaggcg attaccacgg
661 cagggttcat gactgggggtg aagtcgtaac aaggaacc
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16S rDNA sequences of red mud isolate RMCL-7

LOCUS EU665665 694 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-7 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665665
VERSION EU665665.1 GI:187960762
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 694)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 694)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-7"
/environmental_sample
[rRNA](#) <1..>694
/product="16S ribosomal RNA"
ORIGIN
1 taaacgatgt caactagttg ttggggattc atttcttcag taacgtagct aacgcggtgaa
61 gttgaccgcc tggggagtac ggtcgcgaaga ttaaaactca aaggaattga cggggacccg
121 cacaagcggg gatgatgtg gattaattcg atgcaacgcg aaaaacctta cctacccttg
181 acatgccact aacgaagcag agatgcatca ggtgcccga aaggaaagtg gacacaggtg
241 ctgcatggct gtcgtcagct cgtgtcgtga gatggtgggt taagtcccgc aacgagcgca
301 acccttgtct ttagttgcta cgcaagagca ctctagagag actgccgggtg acaaaccgga
361 ggaaggtggg gatgacgtca agtccctcatg gcccttatgg gtagggcttc acacgtcata
421 caatgggtgcg tacagagggg tgccaaccgg cgaggggggag ctaatcccag aaaacgcatc
481 gtagtccgga tcatagtctg caactcgact acgtgaagct ggaatcgcta gtaatcgcg
541 atcggcatgc cgcggtgaat acgttcccgg gtcttgatca caccgcccgt cacaccatgg
601 gagtggggtt tgccagaagt agttagccta accgcaagga gggcgattac cacggcaggg
661 ttcgatgactg ggggtgaagtc gtaacaaggt aacc

//

16S rDNA sequences of red mud isolate RMCL-18

LOCUS EU665666 678 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-18 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665666
VERSION EU665666.1 GI:187960763
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 678)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 678)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..678
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-18"
/environmental_sample
[rRNA](#) <1..>678
/product="16S ribosomal RNA"

ORIGIN

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61 gtacggtcgc aagattaaaa ctcaaaggaa ttgacgggga cccgcacaag cggtggtatga
121 tgtggattaa ttcgatgcaa cgcgaaaaac cttacctacc cttgacatgc cactaacgaa
181 gcagagatgc atcagggtgcc cgaaagggaa agtggacaca ggtgctgcat ggctgtcgtc
241 agctcgtgtc gtgagatggt gggttaagtc ccgcaacgag cgcaaccctt gtctttagtt
301 gctacgcaag agcactctag agagactgcc ggtgacaaac cggaggaagg tggggatgac
361 gtcaagtctc catggccctt atgggtaggg cttcacacgt catacaatgg tgcgtacaga
421 gggttgcaa cccgcgaggg ggagctaatc ccagaaaacg cgctcgtagtc cggatcgtag
481 tctgcaactc gactacgtga agctggaatc gctagtaatc gcggatcagc atgccgcggg
541 gaatacgttc ccgggtcttg tacacaccgc ccgtcacacc atgggagtgg gttttgccag
601 aagtagttag cctaaccgca aggagggcga ttaccacggc agggttcatg actgggggtga
661 agtcgtaaca aggtaacc
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16S rDNA sequences of red mud isolate RMCL-58

LOCUS EU665667 734 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-58 16S ribosomal RNA gene, partial sequence.
ACCESSION EU665667
VERSION EU665667.1 GI:187960764
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 734)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic bacteria of aluminum processing industry waste bauxite residue (red mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 734)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
source 1..734
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/isolation_source="extremely alkaline bauxite residue (red mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-58"
/environmental_sample
[rRNA](#) <1..>734
/product="16S ribosomal RNA"
ORIGIN
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61 ttggggattc atttcttcag taacgtagct aacgcgtgaa gttgaccgcc tggggagtag
121 ggtcgcaaga ttaaaactca aaggaattga cggggaccgc cacaagcggg ggatgatgtg
181 gattaattcg atgcaacgcg aaaaacctta cctacccttg acatgccact aacgaagcag
241 agatgcatca ggtgcccgaaggaaagtg gacacaggtg ctgcatggct gtcgtagct
301 cgtgtcgtga gatgttgggt taagtccgc aacgagcgca acccttgtct ttagttgcta
361 cgcaagagca ctctagagag actgcccggg acaaaccgga ggaaggtggg gatgacgtca
421 agtcctcatg gcccttatgg gtagggcttc acacgtcata caatgggtgcg tacagagggg
481 tgccaacccg cgagggggag ctaatcccag aaaacgcatc gtagtccgga tcgtagtctg
541 caactcgact acgtgaagct ggaatcgcta gtaatcgcg atcagcatgc cgcggtgaat
601 acgttcccgg gtcttgtaga caccgcccgt cacaccatgg gtagtggggtt tgccagaagt
661 agttagccta accgcaagga gggcgattac caccgagggg ttcatgactg gggtagaagt
721 gtaacaaggt aacc

//

16S rDNA sequences of red mud isolate RMCL-88

LOCUS EU665668 705 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-88 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665668
VERSION EU665668.1 GI:187960765
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 705)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 705)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..705
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-88"
/environmental_sample
[rRNA](#) <1..>705
/product="16S ribosomal RNA"

ORIGIN

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61 taacgcgtga agttgaccgc ctggggagta cggtcgcaag attaaaactc aaaggaattg
121 acggggaccc gcacaagcgg tggatgatgt ggattaattc gatgcaacgc gaaaaacctt
181 acctaccctt gacatgccac taacgaagca gagatgcac aggtgcccga aagggaaagt
241 ggacacaggt gctgcatggc tgtcgtcagc tcgtgctcgt agatggtggg ttaagtcccc
301 caacgagcgc aacccttgct tttagttgct acgcaagagc actctagaga gactgccggt
361 gacaaaccgg aggaagggtg ggatgacgtc aagtoctcat ggcccttatg ggtagggcct
421 cacacgtcat acaatggtgc gtacagaggg ttgccaaccc gcgaggggga gctaatacca
481 gaaaacgcat cgtagtccgg atcgtagtct gcaactcgac tacgtgaagc tggaatcgct
541 agtaatcgcg gatcagcatg ccgcggtgaa tacgttcccg ggtcttgtag acaccgcccg
601 tcacaccatg ggagtgggtt ttgccagaag tagttagcct aaccgcaagg agggcgatta
661 ccacggcagg gttcatgact ggggtgaagt cgtaacaagg taacc
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16S rDNA sequences of red mud isolate RMCL-92

LOCUS EU665669 669 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-92 16S ribosomal RNA gene, partial sequence.
ACCESSION EU665669
VERSION EU665669.1 GI:187960766
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 669)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic bacteria of aluminum processing industry waste bauxite residue (red mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 669)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
source 1..669
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red mud) waste generated during the alumina extraction"
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/clone="RMCL-92"
/environmental_sample
[rRNA](#) <1..>669
/product="16S ribosomal RNA"
ORIGIN
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61 caagattaaa actcaaagga attgacgggg acccgcaaca gcggtggatg atgtggatta
121 attcgatgca acgcgaaaaa ccttacctac ccttgacatg ccactaacga agcagagatg
181 catcagggtgc ccgaaagggg aagtggacac aggtgctgca tggctgtcgt cagctcgtgt
241 cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct tgtctttagt tgctacgcaa
301 gagcactcta gagagactgc cggtgacaaa ccggaggaag gtggggatga cgtcaagtcc
361 tcatggccct tatgggtagg gcttcacacg tcatacaatg gtgctgtacag aggggtgcca
421 acccgcgagg gggagctaat cccagaaaaa gcatcgtagt ccggatcgta gtctgcaact
481 cgactacgtg aagctggaat cgctagtaat gcgagatcag catgccgcgg tgaatacgtt
541 cccgggtcct gtacacaccg cccgtcacac catgggagtg ggttttgcca gaagtagtta
601 gcctaaccgc aaggagggcg attaccacgg cagggttcat gactgggggtg aagtcgtaac
661 aaggtaacc

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16S rDNA sequences of red mud isolate RMCL-98

LOCUS EU665670 703 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-98 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665670
VERSION EU665670.1 GI:187960767
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
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/environmental_sample
[rRNA](#) <1..>703
/product="16S ribosomal RNA"
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121 ggggaccgcg acaagcgggtg gatgatgtgg attaattcga tgcaacgcga aaaaccttac
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241 acacaggtgc tgcattggctg tcgtcagctc gtgtcgtgag atgttggggtt aagtcccgca
301 acgagcgcaa cccttgtctt tagttgctac gcaagagcac tctagagaga ctgccgggtga
361 caaacgggag gaaggtgggg atgacgtcag gtccctcatgg cccttatggg tagggcttca
421 cacgtcatac aatggtgcgt acagaggggtt gccaacccgc gaggggggagc taatcccaga
481 aaacgcatcg tagtccggat cgtagtctgc aactcgacta cgtgaagctg gaatcgctag
541 taatcgcgga tcagcatgcc gcggtgaata cgttcccggg tcttgtacac accgcccgtc
601 acaccatggg agtgggtttt gccagaagta gttagcctaa ccgcaaggag ggcgattacc
661 acggcagggg tcatgactgg ggtgaagtcg taacaaggta acc

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16S rDNA sequences of red mud isolate RMCL-114

LOCUS EU665671 703 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-114 16S ribosomal RNA gene, partial sequence.
ACCESSION EU665671
VERSION EU665671.1 GI:187960768
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic bacteria of aluminum processing industry waste bauxite residue (red mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 703)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
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/db_xref="taxon:[77133](#)"
/clone="RMCL-114"
/environmental_sample
[rRNA](#) <1..>703
/product="16S ribosomal RNA"
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61 acgcgtgaag ttgaccgcct ggggagtacg gtcgcaagat taaaactcaa aggaattgac
121 ggggaccgcg acaagcgggtg gatgatgtgg attaattcga tgcaacgcga aaaaccttac
181 ctacccttga catgccacta acgaagcaga gatgcatcag gtgcccgaaa gggaaaagtgg
241 acacaggtgc tgcattggctg tcgtcagctc gtgtcgtgag atggtgggtt aagtcccgca
301 acgagcgcaa cccttgtctt tagttgctac gcaagagcaa tataaagaga ctgccgggtga
361 caaacgggag gaaggtgggg atgacgtcaa gtcctcatgg cccttatggg tagggcttca
421 cacgtcatac aatggtgctg acagagggtt gccaacccgc gaggggggagc taatcccaga
481 aaacgcatcg tagtccggat cgtagtctgc aactcgacta cgtgaagctg gaatcgctag
541 taatcgcgga tcagcatgcc gcggtgaata cgttcccggg tcttgtacac accgcccgtc
601 acaccatggg agtgggtttt gccagaagta gttagcctaa ccgcaaggag ggcgattacc
661 acggcagggg tcatgactgg ggtgaagtcg taacaaggta acc
//

16S rDNA sequences of red mud isolate RMCL-31

LOCUS EU665672 710 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-31 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665672
VERSION EU665672.1 GI:187960769
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 710)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 710)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..710
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-31"
/environmental_sample
[rRNA](#) <1..>710
/product="16S ribosomal RNA"

ORIGIN

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61 gtagctaacg cgtgaagttg accgcctggg gagtacggtc gcaagattaa aactcaaagg
121 aattgacggg gaccgcaca agcgggtggat gatgtggatt aattcgatgc aacgcgaaaa
181 accttaccta cccttgacat gccactaacg aagcagagat gcatcagggtg cccgaaaggg
241 aaagtggaca caggtgctgc atggctgtcg tcagctcgtg tcgtgagatg ttgggttaag
301 tcccgaacg agcgaaccc ttgtcttttag ttgctacgca agagcactct agagagactg
361 ccggtgacaa accggaggaa ggtgggggatg acgtcaagtc ctcatggccc ttatgggtag
421 ggcttcacac gtcatacaat ggtgctgaca gagggttgcc aaccgcgag ggggagctaa
481 tcccagaaaa cgcacgtag tccggatcgt agtctgcaac tcgactacgt gaagctggaa
541 tcgctagtaa tcgcgatca gcatgccgcg gtgaatacgt tcccgggtct tgtacacacc
601 gcccgtcaca ccatgggagt gggttttgcc agaagtagtt agcctaaccg caaggagggc
661 gattaccacg gcagggttca tgactggggt gaagtcgtaa caaggtaacc
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16S rDNA sequences of red mud isolate RMCL-28

LOCUS EU665673 711 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-28 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665673
VERSION EU665673.1 GI:187960770
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 711)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 711)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..711
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-28"
/environmental_sample
[rRNA](#) <1..>711
/product="16S ribosomal RNA"

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61 ggcagcacgg gagcaatcct ggtggcgagt ggcgaacggg tgagtaatac atcggaacgt
121 gccctgtcgt ggggataaac tagtcgaaag attagctaat accgcatacg acctgagggt
181 gaaagcgggg gaccgtaagg cctcgcgcga taggagcggc cgatgtctga ctagctagtt
241 ggtggggtaa aggcccacca aggcgacgat cagtagctgg tctgagagga cgatcagcca
301 cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga attttgaca
361 atgggggcaa ccctgatcta gcaatgccgc gtgtgtgaag aaggccttcg ggttgtaaag
421 cacttttgtc cggaaagaaa tccttgccc taatacggcg ggggatgac ggtaccggaa
481 gaataagcac cggctaacta cgtgccagca gccgcggtaa tacgtagggt gcgagcgtaa
541 atcgaatta ctggcgtaa agcgtgcgca ggcggttttg taagacaggc gtgaaatccc
601 cgagctcaac ttgggaatgg cgcttgtagc tgcaaggcta gagtatgtca gaggggggtt
661 agaattccac gtgtagcagt gaaatgcgta gagatgtggg aggaataccg a
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16S rDNA sequences of red mud isolate RMCL-68

LOCUS EU665674 711 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-68 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665674
VERSION EU665674.1 GI:187960771
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 711)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 711)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..711
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-68"
/environmental_sample
[rRNA](#) <1..>711
/product="16S ribosomal RNA"

ORIGIN

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61 ggcagcacgg gagcaatcct ggtggcgagt ggcgagcggg tgagtaatac atcggaacgt
121 gccctgtcgt ggggataaac tagtcgaaag attagctaat accgcatacg acctgagggg
181 gaaagcgggg gaccgtaagg cctcgcgcga taggagcggc cgatgtctga ttagctagtt
241 ggtggggtaa aggccacca aggcgacgat cagtagctgg tctgagagga cgatcagcca
301 cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga attttgaca
361 atgggggcaa ccctgatcca gcaatgccgc gtgtgtgaag aaggccttcg ggttgtaaag
421 cacttttgtc cggaaagaaa tccttgccc taatacggcg ggggatgac ggtaccggaa
481 gaataagcac cggctaacta cgtgccagca gccgcggtaa tacgtagggt gcgagcgtaa
541 atcgaatta ctggcgtaa agcgtgcgca ggcggttttg taagacaggc gtgaaatccc
601 cgagctcaac ttgggaatgg cgcttgtagc tgcaaggcta gagtatgtca gaggggggta
661 gaattccacg ttagcagtg aatgcgtag agatgtggag gaataccgat g
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16S rDNA sequences of red mud isolate RMCL-112

LOCUS EU665675 706 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-112 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665675
VERSION EU665675.1 GI:187960772
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 706)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 706)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..706
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
/db_xref="taxon:[77133](#)"
/clone="RMCL-112"
/environmental_sample
[rRNA](#) <1..>706
/product="16S ribosomal RNA"

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121 acgtgccctg tcgtggggga taactagtct aaagattagc taataccgca tacgacctga
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241 agttggtggg gtaaaggccc accaaggcga cgatcagtag ctggtctgag aggacgatca
301 gccacactgg gactgagaca cggcccagac tcctacggga ggcagcagtg gggaaatttg
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481 ggaagaataa gcaccggcta actacgtgcc agcagccgcg gtaatacgta gggtagcagc
541 gttaatcgga attactgggc gtaaagcgtg cgcagggcgt tttgtaagac aggcgtgaaa
601 tccccgagct caacttggga atggcgcttg tgactgcaag gctagagtat gtcagagggg
661 ggtagaattc cacgtgtagc agtgaaatgc gtagagatgt ggagga
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16S rDNA sequences of red mud isolate RMCL-62

LOCUS EU665676 681 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-62 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665676
VERSION EU665676.1 GI:187960773
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 681)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 681)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..681
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[rRNA](#) <1..>681
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421 cacttttgtc cggaaagaaa tccttgccc taatacggcg ggggatgac ggtaccggaa
481 gaataagcac cggctaacta cgtgccagca gccgcggtaa tacgtagggt gcgagcgtaa
541 atcgaatta ctggcgtaa agcgtgcgca ggcggttttg taagacaggc gtgaaatccc
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661 gaattccacg tgtagcagtg a
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16S rDNA sequences of red mud isolate RMCL-105

LOCUS EU665677 712 bp DNA linear ENV 18-MAY-2008
DEFINITION Uncultured bacterium clone RMCL-105 16S ribosomal RNA gene, partial
sequence.
ACCESSION EU665677
VERSION EU665677.1 GI:187960774
KEYWORDS ENV.
SOURCE uncultured bacterium
ORGANISM [uncultured bacterium](#)
Bacteria; environmental samples.
REFERENCE 1 (bases 1 to 712)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Bacterial diversity and metabolic activity of heterotrophic aerobic
bacteria of aluminum processing industry waste bauxite residue (red
mud) impoundment of extremely alkaline nature
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 712)
AUTHORS Krishna,P., Babu,A.G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (21-APR-2008) Department of Biotechnology and
Environmental Sciences, Thapar University, Bhadson Road, Patiala,
Punjab 147004, India
FEATURES Location/Qualifiers
source 1..712
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="extremely alkaline bauxite residue (red
mud) waste generated during the alumina extraction"
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/clone="RMCL-105"
/environmental_sample
[rRNA](#) <1..>712
/product="16S ribosomal RNA"

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121 gccctgtcgt ggggataaac tagtcgaaag attagctaat accgcatacg acctgagggt
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301 cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga attttgaca
361 atgggggcaa ccctgatcca gcaatgccgc gtgtgtgaag aaggccttcg ggttgtaaag
421 cacttttgtc cggaaagaaa tccttgccc taatacggcg ggggatgac ggtaccggaa
481 gaataagcac cggctaacta cgtgccagca gccgcggtaa tacgtagggt gcgagcgtaa
541 atcggatta ctggcgtaa agcgtgcgca ggcggttttg taagacaggc gtgaaatccc
601 cgagctcaac ttgggaatgg cgcttgtagc tgcaaggcta gagtatgtca gaggggggta
661 gaattccacg ttagcagtg aatgcgtag agatgtggag gaataccgat gg
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//