BIOREMEDIATION OF BAUXITE RESIDUE (RED MUD) USING MICROBES

A Dissertation
In the Partial Fulfillment of
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Supervised by

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled **"BIOREMEDIATION OF RED MUD (BAUXITE RESIDUES) USING MICROBES"** in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY**, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala is an authentic record to my own work during a period of five months from January 2003 to May 2003, under the supervision of Dr. M S Reddy, Department of Biotechnology & Environmental Sciences, Thapar Institute of Engineering & Technology, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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Dedicated to my guide

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Chapter: 1

Introduction

Chapter: 1

Introduction

To meet the ever-growing demand of materials, the natural resources are being exploited to the fullest extent. As a result of which there is depletion of these valuable resources as well as accumulation of different types of wastes. Red mud is one such waste produced during alumina extraction from bauxite ore with concentrated NaOH at elevated temperature in the Bayer's process, depending on raw material composition, red mud composition varies (**Das *et al*.**, 1995).

Aluminium is the most abundantly available metal and the third most plentiful element (8%). In the earth's crust, next only to oxygen and silicon. It is light as metal, tough as an alloy, has good thermal and electrical conductivity, easy to fabricate, non magnetic in nature, has excellent resistance to many chemicals and is non toxic. Because of these outstanding properties aluminium and number of aluminium based alloys are finding growing application in various fields of consumer goods. The utility of the metal is enhanced by its tendency to form a stable adherent oxide that resists corrosion. The only economic ore of aluminium is bauxite, $Al_2O_3.xH_2O$, which is always associated with silica, iron oxide, titanium dioxide and few other minor and trace impurities (**Thakur *et al*.**, 1994)

Bayer, a German scientist, in the year 1880 developed a method for producing alumina from bauxite by treating it with caustic soda (**Fathi Habashi**, 1995).

$Al_2O_3 + 2NaOH \longrightarrow 2NaAlO_2 + H_2O$

The iron oxide along with other impurities is filtered off as red mud. The sodium aluminate is converted into alumina (Al_2O_3) by precipitation, dehydration and calcinations.

$2NaAlO_2 + H_2O \longrightarrow NaOH + Al(OH)_3$

$Al(OH)_3 \longrightarrow Al_2O_3 + 3H_2O$

All commercial aluminium is produced electrolytically by a process discovered by Charles M. Hull (1863–1914) and independently in the same year by P.L.T. Heroult (1863–1914).

By the large Bayer's process for the production of aluminium and Hall–Heroult process for electrolytic production of aluminium metal have no alternatives for the commercial production **of aluminium anywhere in the world.**
The first commercial application was ventured in a small plant near Saint Peters berg for the production of alumina/aluminium salts by alkaline digestion of bauxite for use in textile industry (Report by **RRL, Bhuvneshwar).** With the introduction of Bayer's process to the industry the problem of red mud waste started growing along with the increasing rate of alumina production. Under normal conditions, when 1 tonne of alumina is produced, nearly a tonne of red mud is generated as a waste. In terms of metal production the ratio of alumina to the red mud is 1:2(**Karvy Research Desk**, 2003). This red mud, which is also called as bauxite residues, red slime, red sludge etc. is red in appearance due to the presence of iron oxide from the undigested bauxite. In other words, red mud is that part of bauxite which is inert towards alkali so far as the digestion and dissolution are concerned. By the middle of

twentieth century it was established that red mud is a pollutant and has a deterrent effect on the environment and human health. This waste material has been accumulating at an increasing rate throughout the world. In the world, nearly 30 million tonnes of red mud is produced annually. This figure is calculated on the basis that two tonnes of alumina are used to produce 1 tonne of aluminium and that 58% of alumina and 42% of red mud comes out from 1 tonne of bauxite approximately. At present around 70 plants are producing alumina generating the same amount of red mud by the Bayer's process all over the world with capacities ranging from 0.028 to 2.205 million tonnes per year. In India, at present, aluminium production per year is 0.46 million tonnes amounting to 1.4 million tonnes of red mud generation in the country (Report by **RRL**, Bhuvneshwar).

Disposal of any solid waste is associated with space/ real estate near industry, cost of disposal and pollution, which are now crucial factors. Obviously these three problems are also tagged with red mud disposal and the problem has become more acute with increasing amount of red mud, shortage of real estate around the industry and environmental awareness of the society. Disposal of wastes adds up to the cost of production in the range of 2–5%. Japan was reported to incur an expenditure of more than five dollars per tonne to throw the waste material into the sea, way back in 1973. One of the ways of disposal was not feasible for most of the alumina plants. 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 out to nearby estate dug into ponds where it is left for sun drying (**Wagh *et al.***, 1996).

Simultaneously it is deeply felt by the scientists and technologists that a good amount of metal value is thrown as a waste for which mining and transport cost have been paid. Hence soon after the acceptance of Bayer's process in industry, scientists and technologists directed their efforts possible utilization of red mud.

Antipollution laws are getting stricter is reflected from the instance that Kaiser Aluminium and Chemical Corporation which had been dumping red mud from two alumina plants in the Mississippi river was instructed under water quality control regulations to end this disposal by 1977(**Wagh *et al*.**, 1998).

MECHANISM OF METAL TOXICITY IN MICROBES

Mechanism of toxicity

Toxic metals can exert harmful effects in many ways but principally as a result of their strong coordinating abilities(**Ochiai**,1987). Toxic effects include the blocking of functional groups of biologically important molecules (e.g. Enzyme and transport systems for essential nutrients and ions), the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units, conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (**Ochiai**, 1987). Because of the wide spectrum of potentially toxic interactions between metals and fungi, almost every aspect of their metabolism, growth and differentiation may be affected, depending on the organism, metal species and concentration, and physico-chemical factors (**Gadd**, 1986*a,c*; **Gadd** and **White**, 1989a). Thus, toxic symptoms may vary widely between different

fungi and for different metal species. A prerequisite for direct toxic interactions is contact between the active metal species and cellular components (**Gadd** and **White**, 1989*a*). The cell membrane is an obvious initial site of an action for a toxic metal species and membrane damage can result in loss of mobile cellular solutes, e.g. K^+, and increased permeability of the cell to external materials (**Norris** and **Kelly**, 1977; **Kuypers** and **Roomans**, 1979; **Mowll** and **Gadd**, 1983, **White** and **Gladd**, 1987 a,b; **Laurence**, **Cooney** and **Gadd**, 1989)

Indirect mechanisms of metal toxicity may involve free radicals, which are deleterious to cells as they can take part in chain reactions, which involve the breakdown of biological macromolecules. Consequently, aerobic organisms possess protective enzymes such as superoxide dismutases, which are metalloenzymes containing Mn, Fe or Cu/Zn (**Greco *et. al.*,** 1990; **Galiazzo *et al.*,** 1991), which eliminate the radicals produced by normal metabolism. Major targets in cells are membranes, where lipidperoxidation, by the alkyl chains of lipids are converted to peroxyalkyl redicals and fatty acid hydroperoxides, is initiated (**Mehlhorn**, 1986). Lipid soluble complexes of transition elements such as Fe(II) may undergo the Fenton reaction with the hydroperoxides and accelerate this process.(**McCord & Day**, 1978)

$$Fe^{2+} + H_2O \longrightarrow Fe^{3+} + OH^- + OH^-$$

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

Complexes and free ions of these cations may also undergo this reaction in aqueous solution. In animal systems, metal

ions such as Hg^{2+}, Cd^{2+}, Pb^{2+} and Ag^{2+} induce free radical toxicity as a result of their reactions with thiols or enzymes which normally protect against these reactive species (**Mehlhorn,** 1986). Little work on this aspect has been carried out with fungi except for Cu^{2+} and *Saccharomyces cerevisiae (***Greco *et al.*,** 1990).

Organometallic compounds are of increasing environmental significance because of their use in the chemical and petroleum industries and as biocides. Organometals are generally more toxic towards fungi than corresponding free metal ions and the toxicity of their compounds varies with the number and with the identity of the organic groups (**Blunden, Hobbs** and **Smith,** 1984: **Cooney** and **Wuertz,** 1989). Major effects of organothins and organoleads are disruption of mitochondrial membranes and action as Cl^-/OH^- ionophores. In this way, they depolarize electrochemical gradients and consequently interfere with energy conservation (**Blunden *et al.*** 1984, **Cooney and Wuertz,** 1989). Organometals may also damage membranes by the production of free radicals since the carbon metal bond readily reacts with available radicals to produce peroxyalkyl radicals which can result in lipid peroxidation (**Mehlhorn,** 1986). As well as the mitochondrial membrane, organometallic compounds may also exert a disruptive effect on cell membranes and cause a loss of K^+ (**Cooney *et al.*,** 1989).

RESISTANCE AND TOLERANCE OF METALS IN MICROBES

Fungal survival in the presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetical adaptation, including morphological changes, and environmental modification of

metal speciation, availability and toxicity, the relative importance of each often being difficult to determine (**Gadd and Griffths** 1978, **Gadd** 1990b, 1992b). Arbitrary terms such as resistance and tolerance which are used rather loosely and often, interchangeably in the literature are generally based on the ability to grow on a certain metal concentration in laboratory media (**Trevors, Stratton and Gadd**, 1986: **Gadd** 1992b,c). It is probably more appropriate to define 'resistance' by means of a mechanism produced in direct response to the metal species concerned, e.g., synthesis of metallothioneins or ϒ-glutamyl peptides (**Mehra** and **Winge**, 1991). Metal tolerance may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (**Gadd**, 1992b,c). Intrinsic properties that can determine survival include possession of impermeable pigmented cell walls, extracellular polysaccharide and metabolite excretion, especially where this leads to detoxification of the metal species by, e.g., binding or perception (**Gadd**, 1990b). However, in many cases distinctions are difficult because of the involvement of several direct and indirect physio-chemical and biological mechanisms implicated in fungal survival (as distinct from environmental modification of toxicity) include extracellular precipitation, complextion and crystallization, transformation of metal species by e.g., oxidation, reduction, methylation and dealkylation, biosorption to cell walls, pigments and Extracellular polysaccharide, decreased transport or impermeability, efflux, intracellular compartmentation and precipitation and/or sequestration (**Ross**, 1975: **Gadd** and **Griffths** 1978 ;

Gadd 1988, 1990 a, b, 1992 a–c, **Brown** and **Hall** 1990; **Mehra** and **Winge**, 1991). A particular organism may directly and/or indirectly rely on several survival strategies. For example, metallothionein synthesis is a mechanism of Cu^{2+} resistance in *S.cerevisiae S.cerevisiae* yet Cu^{2+} binding or precipitation around the cell wall and intracellular transport are also components of the total cellular response (**Gadd** and **White**, 1989 a).

EXTRACELLULAR PRECIPITATION AND COMPLEXATION

Many Extracellular fungal products can complex or precipitate heavy metals. Citric acids can be an efficient metal–ion chelator and oxalic acid can interact with metal ions to form insoluble oxalate crystals around cell walls and in the external medium (**Murphy** and **Levy**, 1983; **Sutter**, **Jones** and **Walchli**, 1983). The production of H_2S by yeasts can result in precipitation of metals as insoluble sulphides predominantly in and around cell walls (**Ashida**, **Higashi** and **Kikuchi**, 1963; **Minney** and **Quirk** 1985). For strains of *S.cerevisiae* growing on a copper containing medium, resultant colonies may appear dark in color owing to formation of copper sulphide (**Ashida *et al.*,** 1963).

Iron is of fundamental importance to living cells and many filamentous fungi and yeasts release high affinity Fe–binding molecules called siderophores (**Winkelmann *et al*** 1987, **Winkelmann** 1992). The externally formed Fe^{3+} chelates may subsequently be taken up into the cell (**Raymond**, **Muller** and **Matzanke**, 1984 ; **Adjimani** and **Emery** 1987). In several fungi, the excretion of such iron binding molecules is markedly stimulated by Fe deficiency and such compounds may also bind Ga (**Adjimani** and **Emergy**, 1987). *Debaryomyces*

hansenii produced riboflavin or a related compound, when grown in Fe deficient media or in the presence of copper, cobalt and zinc. Polarographic analysis showed that the pigment was capable of Fe^{3+} binding (**Gadd** and **Edwards**, 1986).

INTERNATIONAL SCENE

The major producers of alumina are concerned because the red mud ponds are being filled up and environmental hazards may arise due to overflow of effluents. M/S Alumina Company of America TX point comfort is capping the bauxite residue with degraded materials and subsequently revegetating the area with the alkali tolerant plants. Here again they are concerned about the upward migration of electrolyte, dusting problem, erosion due to rainwater, seepage etc.

Kaiser Alumina Company using Zamaican bauxite neutralizes the red mud with hydrochloric acid. They buy extra HCl to neutralize the red mud. They follow total acid neutralization deep drew (TANDD) process to maintain pH 6–9 as desired by environmental protection agency.

In Australia at GOVE, they cap red mud surface to cover up the exposure of red mud pond to meet environmental regulations.

INDIAN SCENE

BALCO has initiated crop cultivation in central Indian bauxite mines at Amarkantak (**Das *et al.***, 1995).

HINDALCO has initiated work on red mud of pH 10.2 to 10.5 but the plants died. After addition of massive dosages of compost the Albizia plantation on red mud lake showed some promise.Major Alumina producers in India are

Industry	Alumina:Alumina (in 1000 tonnes)
NALCO (Bhubaneswar)	**218:800**
HINDALCO (Renukoot)	**170:350**
BALCO (Korba)	**100:200**
INDAL (Calcutta)	**117:295**
MALCO (Madras)	**25:60**

Out of these NALCO is the largest integrated Bauxite-Alumina-Aluminium producer in Asia. Present capacity of India to produce Aluminium is estimated to be 0.633 million tonnes (RRL, Bhuvneshwar,1996).

Utilization options including use as soil conditioner/fertilizer etc.

The application of red mud for the growth of the plants as a soil conditioner/fertilizer has drawn remarkable attention. The problems are similar to saline and alkaline soil of arid regions. High salts concentration lowers the soil/water potential which interferes with plant water uptake. High concentration of soluble ions such as sodium and carbonate may be toxic and competitively inhibit the uptake of nutrients. Without drainage and costly chemical neutralization and exchangeable sodium percentage, pH and soluble aluminium concentration remain high. These properties when coupled with lack of nutrients and anoxic conditions can prevent the vegetation. Similarly when exposed to air these barren surfaces dry up and become a source of highly alkaline wind blown dust.

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 down the pH to 8.3. for fine texture red mud, this amount was 34 tonnes/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. 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 been taken place. Attempts have also made with glucose, yeast and potassium acid phosphate (**RRL**, Bhuvneshwar, 1996).

NALCO has decided for the revegetation of the area due to public pressure. The red mud ponds are so designed that after final phase of treatment seawater is filled up and is covered with degraded materials so as to grow grasses.

Chapter: 2

Review

Of

Literature

Chapter: 2

POLLUTION, ECOLOGICAL, UTILIZATION AND AMELIORATION CONSIDERATIONS FOR RED MUD

The environmental problem associated with red mud is insignificant in Afro-Asian countries while in the European countries, Japan and US, it has become acute. An episode from France is of much significance. In the year 1965, public protests induced French experts from the Institut des Peches to give their decision to a red mud dumping project in the Bay of Cassis (Mediterranean Sea)(**Dass *et al.*,** 1995).

Red mud till today is disposed off from the plant in two conventional ways depending upon the facilities available and surrounding conditions (**RRL,** Bhuvneshwar 1995). Wherever real estate is available, red mud is disposed off to nearby pools or lagoons made for this purpose where slurry is left open for sun drying and overflowing water is taken back into the plant depending upon conditions. The main reasons for the popularity of this method are low cost and easy implementation. This practice is followed by all the Indian plants producing alumina from bauxite by Bayer's process save a few who have adopted dry disposal only recently (**Thakur *et al.*,** 1995). The other method of disposal into sea/bay is practiced by countries like France, England, Germany, Japan, etc. where availability of land for dumping is scarce and the sea is nearby. Red mud is piped directly into the sea at the disposal site (**Dass *et al.*,** 1995).

Whatever way this waste is disposed, it causes pollution to the surroundings. The environmental chemistry and toxicity of aluminium in red mud may be significant under such alkaline conditions. Red mud of similar composition may create different types of pollution under different environmental conditions. The conditions are available sunshine, annual rain fall, average temperature, wind velocity, soil permeability and so on for the land disposal while for the sea disposal it depends on specific zone, length of inlet pipe, depth at that point, variety of fish culture and under currents, if any. To use red mud as a soil conditioner, amelioration of red mud disposal sites are essential.

Halsband and **Halsband**(1971), **Paffenhoefer**(1971 and 1972), **Cole**(1973), **Blackman** and **Wilson**(1973), **Pafenhoefer**(1972), **Rosenthal *et al.*** (1971, 1972 and 1973) studied the physiological effects of red mud on marine organisms. It was observed in North Sea that fish was getting affected faster as compared to algae. However, a tolerant dose was arrived upto 1g/L, which was not damaging for either organisms. It was noticed that iron hydroxide part of red mud was particularly responsible for growth inhibiting effect on *C. helgolendices* cultured on phytoplankton. Red mud diluted 10^6 times resulted in 16% mortality. Mortality rate was also dependent on quantity of dry mud (micro gram/ animal) and also the stage of cultures. Further, it was also observed that ingestion of red mud in partial replacement created lower growth, lower body weight and restricted mobility resulting in higher mortality of copepods for phytoplankton. They also observed similar kind of effect on embryos and larvae of herring *Clupea harengus.*

It was established, in general, that waste which was found harmful to fish or shell fish will similarly affect other organisms also. Killing of fish or shell fish at any stage in their life cycle, i.e., as larvae, juvenile or adults; interference in biological processes such as growth physiology or breeding etc.; contaminations with persistent toxic substances so that fish and shell fish became unsafe to eat and were rendered unpalatable and temporarily unsaleable (**Cole**, 1973). The above said effects are directly due to red mud. However, indirect effects also take place, i.e., the environment of seawater or seabed is altered so that it's capacity to sustain fish is impaired. Another way of pollution is that certain metals may accumulate in fish, which may not affect physiology of fish at all but render the fish quite unsafe for human consumption. It is established that red mud affects aquaculture directly as well as indirectly. It is reported that permission for dumping red mud in North Sea was refused to an alumina industry based upon the observation that red mud severely harmed the different sea organisms around the dumping site.

Nauke (1973) investigated geological aspects of red mud dumping site. In North Sea at an experimental site 15000 tonnes of red mud were dumped and after several months it was observed at a dumping area, gray color of sand had changed to brown indicating that red mud changed to brown iron hydroxide which was found on the surface of sand grains. **Rushing** (1973) in his publication "Alumina plant tailing storage" discussed pollution due to red mud and suggested a way out by making dams and dikes with the help of red mud. It was stated that dumping the red mud into

river increased silt content, concentration of the heavy minerals and limited the downstream use of water. Dumping the tailing into the ocean ever over the continental shelf also causes problems with marine lives and could be swept back to land area during unusual current movements. The small amounts of caustic liquor in the slurry also can cause a fine precipitate of magnesium and calcium hydroxide. **Rushing**(1973) had also given a design for the dike preparation which prevents pollution by red mud. **Leslie**(1976) reported pollution of lime stone aquifer by red mud. It was observed that from red mud ponds pollutants like sodium, iron hydroxide and organic substances make way to ground water table. This polluted water becomes unfit for domestic, agricultural as well as few industrial uses. Near the disposal site five location were drilled for ground water and it was analyzed. It was found that two of them were already polluted. It was suggested that ponds should be made with utmost care and geological characteristics like ground water table location should be taken into account. **Adam**(1981) and **Toth**(1981) discussed the programmes of environmental protection due to aluminium industry in Hungary starting from bauxite mining, Al_2O_3 production and aluminium metallurgy. Publication included present and future plans to control and decrease the environmental pollution by NaOH, red mud, alumina dust, aluminium metallurgical waste gas and flue gas at Ajka plant in Hungary.

Barrow(1982) worked out a scheme for improving chemical and physical properties of sandy soils with the help of red mud in order to control pollution. Detailed studies were carried out to modify red mud in order to yield a material

capable of supporting plant growth. It was observed that cation exchange properties of red mud and adsorption of Cd increased in pH, but phosphate adsorption declined. In this system pH of the red mud was brought upto 8 by exposing red mud to CO_2 of air and mixing it with gypsum, which precipitated it as calcium carbonate. Leachable sodium sulphate, released from red mud could be washed out. The composition of the leftover material was found to be ideal for medic species along with requisite amount of phosphate, potassium and magnesium. It was pointed out that incomplete leaching of sodium sulfate was detrimental to growth of certain other species. They proposed to use this potential to revive sandy soils of Western Australia coastal plains.

Boriosco *et al*. (1981) 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 Na_2CO_3 per tonne of dry red mud. This red mud was mixed up with seawater to neutralize residual Na_2CO_3 by bringing pH to 9.5. This neutral material was disposed off to a large basin to settle down.

Williams and **Hamdy**(1982) introduced biological activities in bauxite residue in order to neutralize its extra alkali. So in an envisaged programme alfa alfa hey was mixed up 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. **Koch** and **Bell** (1983) used red mud along with lie and saw dust to treat low pH soils and make them suitable for plant

growth. Red mud around 18% was quite effective in reducing acidity level and levels of available aluminium in the overburden spoils of coalmines. It was further observed that red mud treatment produced ten-fold increase in plant dry matter accumulation as compared with $CaCO_3$. It was concluded that both $CaCO_3$ and red mud were suitable for amelioration of acidic overburdens in pot trials. The red mud had some inherent fertility especially in phosphorus but also had high adsorption capacities when phosphate was added as fertilizer. However, it was also recorded that since its neutralization capacity was less than that of $CaCO_3$, higher doses of red mud could become uneconomical.

Ward (1983) studied the conditions to utilize red mud as a new material for soil amendment and improve the soil condition for favorable crops. While studying for growth and fertilizer equivalent of annual legumes on sandy soil amended with fine red mud, they had taken red mud upto 1680 tonnes per hectare mixed up with 5.8% gypsum. Experiments were conducted on fields with number of variable parameters, i.e., sand to red mud ratio, red mud to gypsum ratio, depth of the soil used for amendment and addition of nutrients. The growth response in legumes was plotted against phosphorus addition, red mud level, potassium addition, etc. further plants were also analysed for uptake of different wanted/unwanted elements, such as F, Cr, Ni, Cd and Pb. Research observations were interesting and had different openings for future investigation. However, following conclusions were summarized. The highest level of red mud decreased yield slightly; adding gypsum increased yield; sodium salts were quickly leached by rain when highest value of red mud was used. Only normal agricultural

rates of potassium and 0–20 kg/hectare of phosphorus were required for maximum yield. It was further stated that foliar analysis showed low manganese concentration and an excessive uptake of molybdenum from fertilizer at high level of red mud addition. There was no accumulation of heavy metals or fluoride in the plant grown in amended soil. It was also observed that water-holding capacity of amended soil has gone up but could not be used due to poor drainage facilities. Lastly it was suggested that there might be more potential for the use of red mud as a soil conditioner where better drainage system were available.

Patel *et al*. (1986) have studied the concentration of pollutant fluorides in effluent and by product of alumina industry in India. The samples were obtained from effluent discharge from Alumina and smelter plants and solid wastes such as Vanadium sludge, cryolite mud and red mud. Collection of sample was carried out also after sufficient time gap. The micro pollutants of red mud and studied their effect on nearby soil and waters. Sampling was done from different points with intermittent time gap.

Wagh and **Thompson** (1988) studied inter-particle bonds in dry bauxite waste resulting in atmospheric sols. The authors stated that millions of tonnes of the waste produced in every country are stored in contaminant drains or natural valleys. This leads to ground water pollution, distraction of plant and bird lives and is hazardous to human settlements in earthquake-prone regions like Jamaica. However, when dry red mud is formed it may become a potential source of caustic dust. They have reported elemental composition of dust and its health hazards. Red mud was found to be a complex mixture of several oxides in

several mineralogical forms but could be classified simply as a lateritic soil. Ultraviolet radiation exposure of red mud resulted in hardening of material. It was suggested that possibly traces of organics existing in the mud have to play as catalyst. Number of elements were estimated in the dust out of which few were objectionable as pollutant like chromium, thorium, uranium, lanthanum, neodymium and sodium, etc.

Hughes *et al*. at **(CSIRO),** while comparing chemical properties of red mud from wet and dry disposal methods, looked into the economic and environmental considerations. An alternative method of dry disposal of red mud was proposed in order to avoid pollution problems. This dry disposal method involved dewatering of red mud prior to disposal at high density in dry beds. Advantages of dry disposal with respect to environmental protection were described and it was stated that the environmental advantages of the lower land use and the decrease in the risk of contaminating ground water were extremely important and economically significant factors in the long run. Even while the dry stakes were operational they had an advantage over the wet disposal system that they produced a higher density and more stable deposits which were readily accessible. **Summers *et al.*** (1984) at Alcoa Limited of Australia had been quite active in finding out a use of red mud as a soil conditioner. It was observed that in some areas of Australia sands lacked clay material to bind and hold nutrients and so rainfall leached them out. Peel Harvey study group, CSIRO, Alcoa and Murdoch university (1984) tackled the above said problem and found that the treated red mud had a texture similar to loam and helped to

hold more nutrients and water. When the red mud was incorporated into these sandy soils phosphorus loss was low and much of it was available to plants; hence it was suggested that productive pastures could be grown in otherwise poor countries. Number of field trials were carried out with different variables starting from 200 to 2000 t/ha treated red mud, 270 kg/ha of phosphorus, leached out solution, water/nutrient holding capacity, etc. it was suggested that if large scale treatments were carried out and programme was executed it may benefit tree groups. Alcoa would benefit because it would not have to store the residues. However, the cost of transporting residues to the catchment and of spreading it on land was higher than the cost of storing it. Farmers would benefit through increased productivity of their land although long term data were still required on this aspect. The increased productivity alone would not be enough to justify the extra cost. The community would benefit from a cleaner estuary.

Bell and **Meechan**(1978)reported the red mud consisted of hematite (Fe_2O_2), boehmite(AlOH), quartz($SiO2_2$), gibbsite[$Al(OH)_3$] in addition to amorphous ferric aluminium hydroxide. As the red mud dissicated in the field, it first formed a gel which eventually hardened the cracks as the amorphous iron and aluminium hydroxides dehydrates irreversibly to form a columnar structure. It was further recorded that saturated hydraulic conductivity of the mineral was so low that removal of excess salts in the field would be impracticable in the short term. Conceivably natural weathering might enable the waste to support a sparse cover of salt and alkali tolerant species after many years. But the erosion of the material posed a threat to

the adjacent to estuarine or urban environment and called for a rapid stabilization of the material. Number of trials with various variables like assessment of nutritional limitations, nutrient omission trials, and nutrient rate trials and methods material application to fields were carried out. Although sufficient reduction in the salinity and alkalinity of the waste could occur over a long term to permit the establishment of a plant cover, there appeared to be no prospect of a vegetation stabilization occurring in the short term without the use of soil-top dressing.

Hintz and **Dottling** (1979) studied rehabilitation programme 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. These ponds were built in such a way as to minimize the impact on environment by vegetation of the pond surfaces when they are filled and out of use. 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 after selection, elimination and combination trials of a number of plants, native as well as introduced and a few were marked for this purpose. These vegetation species were *Chloris gayana*, *Stylosanthes humilis*, *Cynodon dectylon*, *Acacia leptocarpa*, *Sporobulus virginis*, *Dolochos lab lab* and *Calapogonium mucuniodes* which tolerated highly alkaline

soil conditions and could grow into a stable and maintenance free vegetation over red mud ponds.

Summers *et al*. (1993) worked on different aspects of red mud utilization. They have worked on different heading such as " modifying sandy soils with the fine residue from bauxite refining to retain phophorus and increase plant yield", "bauxite residues increases phosphorus retention in sandy soil catchment in Western Australia", "phosphorus retention and leachates from sandy soil amended with bauxite residues", "bauxite residue improves pasture growth on sandy soil in Western Australia", "nutrient and metal content of water, sediment and soils amended with bauxite residue in the catchment of the peel inlet and Harvay estuary, Western Australia", "the phosphorus content in the run off from the coastal catchment of the peel inlet and Harvey estuary and its associations with land characteristic", "comparison of single superphosphate coated with bauxite residue for subterranean clover production on phosphorus-leaching soils", "effect of application of bauxite residues to very sandy soils on subterranean clover yield and P response". According to them production of alumina from bauxite in western Australia results in large quantities of processing residues. The fine portion of the residue has a high phosphorus (P) absorption capacity compared with the native sandy soils of the coastal plain. When neutralized with gypsum or acidic materials, the residue can be incorporated into, or spread on, the surface of the sandy soils for horticulture using simple agricultural equipment. Neutralization with gypsum is unnecessary for application to pasture at less than 100t ha^{-1}. Field and laboratory

experiments show that 10–80 t ha $^{-1}$ of bauxite residues, spread evenly over the surface of the soil, significantly reduced P leaching from coastal plain sands ferlizied with superphospate. Rates of 500 t ha^{-1}, or more, significantly increased the yield of pastures on well drained sandy soil, significantly increased the yield of pastures on well drained sandy soils, primarily due to the increased water holding capacity of the amended soils, while rates of 10–80 ha^{-1} significantly increased the yield of pastures primarily due to the increased pH. Analysis of the leachate from bauxite residue indicates that it is unlikely to cause adverse environmental impacts as a result of agricultural-scale amendment of sandy soils. Amendment with bauxite residue offers potential as a practical component of an integrated strategy to reduce P losses from sandy soils. Economic and logistic considerations indicate soil amendment may be most applicable to intensive land users as horticulture and for land treatment of wastewaters from animal industries and urban areas (**Summers *et al.***, 1995). However, economical methods are being developed to spread low rates of bauxite residue on land used for more extensive agriculture.

Bioremediation of red mud

Hamdy et al. (2001) demonstrated that low levels of injured bacterial cells in the bauxite residues actively grew using various added nutrients and/or hay. The organisms grew less than 10 to more than 10^9 cells g^{-1} bauxite residues and formed organic acids that lowered the pH from 13 to about 7.0. A total of 150 cultures were isolated from treated bauxite residues and included species of *Bacillus,*

Lactobacillus, Leuconostoc, Micrococcus, Staphylococcus, Pseudomonas, Flavobacterium and Enterobacter. Scanning electron micrograph demonstrated that untreated particles (control) of the bauxite residues were clumped together, and in the treated bauxite residues supported growth of several plants and earthworms that survive for over 300 days. In a test plot bioremediation on a residue deposit at Alcoa Point Comfort, TX, the Bermuda grass hay used, which was effective mulch material and encouraged water filtrations, leading to establishment and growth of salt – tolerant vegetative species.

Valerie Ee (1999) investigated the microbiology of red mud and ascertained the possibility of using bacteria to reduce the alkalinity of red mud. To achieve this, several properties of red mud were analyzed and 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.

The results from this project showed that the properties of red mud had an influence on the types of bacteria present. Different types of bacteria of varying degrees of coping with at high alkalinity and salinity were isolated from red mud. The project also demonstrated the possibility of using pure cultures of indigenous bacteria in red mud to reduce the alkalinity of the red mud. The experiments revealed that oxygen was necessary for pH reduction under the chosen conditions, and that the pH reduction was related to

bacterial growth. It would thus be possible to reduce the pH of red mud by bacterial metabolism in the presence of aerobic condition, by inoculating the red mud with pure cultures of indigenous bacteria, and incorporating nutrients to encourage bacterial growth.

Chapter: 3

Materials

&

Methods

Chapter: 3

MATERIALS AND METHODS

Red mud sample

The red mud was collected using alcohol-sterilized implements from the National Aluminium Company Limited situated at Damanjodi, Orrisa.

SOIL (RED MUD) CHEMICAL ANALYSIS

(A) Determination of Soil Reaction (pH)

(International Society of Soil Science, 1930)

Reagents:

Standard buffer solutions of 4, 7, and 9.2 pH

Procedure:

(I) Weighed 20 g air-dried soil passed through 2 mm sieve into a 100 mL beaker.

(ii) Added 50 mL distilled water to it.

(iii) Thoroughly stirred it for 10 seconds using a glass rod.

(iv) Further, stirred suspension four or five times during the next 30 minutes.

(v) Allowed suspension to settle for 30 minutes.

(vi) In the meanwhile, switched on the pH meter and after 10 minutes of warming up period, adjust the pH meter reading to the pH of the buffer solution with the help of standardization knob.

(vii) Checked the instrument with two buffer solutions of known pH viz. one acidic and other alkaline.

(viii) Rinsed the electrodes with distilled water and carefully wipe with filter paper.

(ix) Measured pH of the sample by immersing the combination electrode in supernatant solution.

(x) Recorded pH value when the reading has stabilized (usually after 1 minute)

(B) Determination of Total Soluble Salts in soil

(Measurement of Electrical Conductivity)

Reagent:

0.01 M KCl solution:

Dissolved 0.7456 g dry KCl in distilled water and make up the final volume 1L. This solution had an electrical conductivity of 1.413 dS m^{-1} at 25°C.

Procedure:

1. Weighed 20 g soil and transfer it to a 100 mL beaker.
2. Added 50 mL distilled water to it.
3. Shaked intermittently with glass rod for one hour and allow to stand.
4. (Alternatively, the clear extract after pH determination can also be used for EC measurement).
5. In the meanwhile, switched on the EC meter and allow it to warm for 20 minutes.
6. Use 0.01 M KCl solution (EC=1.413 dS m^{-1}) to calibrate the meter.
7. Dipped the electrode in the supernatant solution and record the reading displayed.

Calculation of Results:

Corrected the reading for cell constant and temperature (see table below) and express conductivity at 25°C as dS m^{-1} ($K_{25}^{\bullet}$).

$K_{25}^{\bullet}$ = reading (mS) x cell constant x temperature correction factor (f_1)

Temperature (°C)	Correction factor (f_1)	Temperature (°C)	Correction factor (f_1)
8	1.499	21	1.092
10	1.421	22	1.067
12	1.350	23	1.044
14	1.284	24	1.021
15	1.254	25	1.000
16	1.224	26	0.979
17	1.196	28	0.941
18	1.168	30	0.906
19	1.142	32	0.873
20	1.118	34	0.843

a. The values for saturation extract can be calculated from the 1:5 soil-to-water measurement by the equation:

$EC_{(Sat.Ext.)} = EC_{1:5} \times$ Water in soil at saturation (%)

b. Salt concentration, mg L^{-1}=640 x EC (mS cm^{-1})

c. Osmotic pressure of solution, bars at 25°C is 0.36 x EC (mS cm^{-1})

d. The SI unit for conductivity is Siemens per meter (S m^{-1}). In the past the results have been reported as mmho cm^{-1}, which is equal to mS cm^{-1} (1 mho = 1 Siemen). This manual therefore uses mS cm^{-1}. If required, the results can be converted to SI units as follows:

   mS cm^{-1} = dS m^{-1} = S m^{-1} x 10

   mS cm^{-1} x 0.1 = S m^{-1}

(C) Colorimetric method of determination of Organic Carbon:

(Dutta et al., 1962)

Reagents:

(i) *1 N Potassium dichromate solution*:

Dissolved 49.04 g of AR grade $K_2Cr_2O_7$ crystals in distilled water and make up the final volume 1 litre.

(ii) *Concentrated Sulphuric acid (sp. gr. 1.84)*.

(iii) *Sucrose (AR grade), anhydrous*.

Procedure:

1. Weighed accurately 1.0 g of soil sample and transfer it in a 150 mL conical flask.
2. Added 10 mL of 1N $K_2Cr_2O_7$ solution and shake it well to mix.
3. Prepared a blank in which all reagents except soil are added.

4. Kept the conical flask on teflon/asbestos sheet and add 20 mL concentrated H_2SO_4 carefully from the side of flask, swirl the flask during addition.
5. Allowed the flask to stand for 30 minutes and there after 70 mL of water is added.
6. Swirled the flask thoroughly and allow to settle the soil particles overnight.
7. Next morning switched on the spectrophotometer and let it warm for 15-20 minutes.
8. Adjusted the wavelength to 660 nm.
9. Washed the cuvettes with distilled water and wipe out the sides carefully with tissue paper.
10. Filled the cuvettes with blank solution, place them in cuvette wells and make the instrument to read 00 absorbance.
11. Now kept one cuvette filled with blank and wash and fill standard solution one by one in the other cuvette; recorded the respective readings and draw standard curve.
12. Once the standard curve is drawn and values are recorded, took readings for samples.

Preparation of Standard Curve:

1. In 6 different 100 mL volumetric flasks weigh out 0, 10, 20, 30, 40, 50 and 60 mg of sucrose.
2. Added 10 mL of 1N $K_2Cr_2O_7$ solution and shake well to dissolve sucrose crystals.
3. Kept the flasks on teflon/asbestos sheet and add 20 mL concentrated sulphuric acid. Swirl the flask during addition
4. Allowed the flasks to stand for 30 minutes.
5. Added distilled water and make up the volume up to the mark.
6. Let the flasks cool completely.

7. Took the reading one by one as given in the procedure.
8. Drew the curve between concentration of carbon in different sucrose standards and their respective absorbances.

Calculation:

Multiplied the quantities of sucrose (0, 10, 20, 30, 40, 50 & 60 mg) by 0.4207 and find out the quantities (%) of carbon present therein (see table):-

(Note: The quantities of sucrose are multiplied by 0.4207 because sucrose contains 42.07% carbon)

Sucrose (in mg.)	Carbon (in mg.)	Carbon (%)
0	0	0
10	4.207	0.42
20	8.414	0.84
30	12.621	1.26
40	16.828	1.68
50	21.035	2.10
60	25.242	2.52

Because 10 mg sucrose contains 4.207 mg C and if suppose 4.207 mg carbon is found in 1000 mg of soil, it means 100 mg soil will contain

$$= \frac{4.207 \times 100}{1000} \text{ mg carbon}$$

or = 0.4207 % carbon

Estimation of organic matter:

Because organic matter contains 58% carbon so,

$$\text{Organic matter} = \text{Organic carbon (\%)} \times \frac{100}{58}$$

or organic matter = org. C(%) x 1.724 (Van Bemmelen factor)

(D) Determination of Total Nitrogen in Soil

Digestion of soil samples

The method employs a digestion to convert nitrogen present in the sample to ammonium sulphate. The ammonium nitrogen is subsequently determined by *distillation* and *titration*.

Reagents:

(1) *Concentrated H_2SO_4 (18 M)*

(2) *Kjeldahl Catalyst Tablets. B.D.H. cat. No. 33064*. Each tablet contains 1 g sodium sulphate and 0.1 g copper sulphate.

Procedure:

(1) Weigh and transfer 1.0 g finely ground (air dry, <0.25 mm) soil into a digestion tube.

(2) Add 10 mL concentrated H_2SO_4; mix by swirling.

(3) Heated at 200°C in a digestion block until very white (about 30 minutes). "To avoid acid irritation to the analyst, the digestion block was loaded in a fumehood to ensure the removal of fumes and vapours released during digestion".

(4) Add one Kjeldahl catalyst tablet.

(5) Heated for 3-4 hrs. until tablet dissolves (200°C) and to get the precipitation.

(6) Removed the digestion tubes from the block and allow to cool for 5 minutes. "Did not allow to cool in the heating block - NH_3 from the $(NH_4)_2SO_4$ formed by digestion will be lost if heated."

(9) Transfer the whole sample into 50 mL volumetric flasks and make up the volume.

Determination:
(Distillation –Titration method)

Reagents:

1. *40% NaOH solution*: Dissolved 400 g NaOH pellets in distilled water and make up the volume 1 litre.
2. *4% Boric Acid solution*: Dissolved 40 g H_3BO_3 in a litre of distilled water.
3. *0.01 M HCl solution*:
4. *Mixed indicator*:0.066 g methyl red plus 0.099 g bromocresol green dissolved in 100 mL of 95% alcohol.

Procedure:

(1) Transferred 5 mL of sample digest or aliquot to distillation apparatus.

(2) Added 10 mL of 40% NaOH solution. Formation of a brown precipitate of ferric hydroxide, when the liquids are mixed, indicates neutralization of the acid.

(3) Added 10 mL of 4% Boric acid and 2 drops of mixed indicator to a 100 mL Erlenmeyer flask and place under the delivery tube of the condenser so that the tip is below the surface of the liquid.

(4) Closed sample inlet and drainage outlet and pass steam into the distillation flask. (The liquid will soon boil, and the indicator in the boric acid solution will change colour as soon as ammonia begins to distill over).

(5) After a minute or two, lowered the flask so that the tip of the deliver tube is clear of the liquid. (No ammonia will be lost if the condenser is efficient).

(6) When about 20 to 25 ml of distillate have collected, rinse the tip of the tube with a little water and remove the flask.

(7) Stopped the entry of steam. The distilling flask will empty automatically and the vacuum can be used to rinse the apparatus by immersing the tip of the delivery tube in distilled water.

(8) Titrate the distillate against 0.01 M HCl, to the pink colour of the indicator.

(9) Corrected titration for reagent blank.

Calculation:

$$\% \text{ N in soil} = \text{Volume (in mL) of 0.01 M HCl used} \times 0.014 \times \frac{50}{\text{Volume (in mL) of aliquot used}}$$

Used moisture factor to correct to oven-dry basis.

Determination of Available Phosphorus in soil

(I) Olsen's method of determination of available phosphorus:

(Suitable for calcareous and saline alkaline soils)

(Olsen et al. 1954)

Reagents:

1. *Extracting solution*: (0.5 M $NaHCO_3$**)**:

   Dissolved 84 g $NaHCO_3$ in water and make up to 2L. Mix thoroughly. [Adjusted to pH 8.5 with 1 M NaOH (4 g NaOH per 100 mL) solution]. Did not keep this solution more than one month in a glass bottle, otherwise use polythene bottle.

(2) Reagent A:

   Dissolved 12 g ammonium molybdate $[(NH_4)_6\ MO_7O_{24}.4H_2O]$ in 250 mL of distilled H_2O.

   Dissolved 0.2908 g antimony potassium tartrate $[K(SbO)\ C_4H_4O_6.1/2\ H_2O]$ in 100 mL of distilled water.

   Add these two solutions to 1000 mL of 2.5 M H_2SO_4, mix thoroughly and make to 2000 mL with distilled water. Store in pyrex glass bottle in a dark, cool place.

(3) Reagent B:

Dissolved 1.056 g ascorbic acid ($C_6H_8O_6$) in 200 mL of reagent A and mix. Prepare daily as required. This did not keep for more than 24 hours at room temperature.

(4) *Sulphuric acid (2.5 M)*:

Diluted 140 mL concentrated H_2SO_4 to 1L.

(5) Stock standard P solution (50 mg L^{-1} P):

Dissolved 0.2917 g KH_2PO_4 L^{-1}. Added five drops of toluene to diminish microbial activity. (KH_2PO_4 should be dried at 100°C for 1 hour and cooled in a desiccator before weighing).

(6) Working standard P solution (1 mg L^{-1} P):

Diluted 20 mL of 50 mg L^{-1} P solution (stock) to one litre. Mixed thoroughly.

Procedure:

(i) Transferred 2.5 g soil into 100 ml Erlenmeyer flask.

(ii) Added 50 mL extracting solution and shake on the reciprocating shaker for 30 minutes (soil-to-solution ration 1:20). The rate of shaking should be constant.

(iii) Filtered through Whatman 42 filter paper. Shake flask immediately before pouring suspension into funnel.

(iv) Transferred 10-mL aliquot of the filtrate to a 100-mL beaker.

(v) Added 1.0 mL 2.5 M H_2SO_4 to lower the pH to 8.5.

(vi) Added 15.5 mL distilled water.

(vii) Added 8 mL of reagent B.

(viii) Added 15.5 mL distilled water.

(ix) Prepared blank as above using 10 mL extracting solution in place of soil extract

(x) Standard Curve: measure 0, 2, 5, 10, 15 and 20 mL of standard (working) P solution in 6 different 50 mL volumetric flasks. Add 10 mL extracting solution and 1.0 mL 2.5 M H_2SO_4 to each flask. Add 8 mL reagent B. Add 31, 29, 26, 21, 16 and 11 mL distilled water, respectively (the total volume will be 50 mL in each flask). The P concentration of these solutions will be 0.04, 0.10, 0.20, 0.30 and 0.40 mg L^{-1}, respectively.

(xi) After 10 minutes (solution should be bluish purple), read P concentration at 882 nm after calibrating the spectrophotometer with standards.

Calculation:

P in soil (mg kg^{-1}) = P in extract (mg L^{-1}) x 20 (the standard soil-to-solution ratio).

Analysis of metal in the red mud soil sample

1 gm of red mud was digested with nitric acid and perchloric acid (3:1). After the digestion the volume made up to 50ml for Inductively coupled plasma emission spectroscopy analysis for quantitative detection of metals.

Microbiology of red Mud

Isolation of microbes from red mud

Microbes has been isolated from the red mud using serial dilution of red mud and then plated on nutrient media

(HiMedia, Bombay) and their variant (e.g. Nutrient media having pH 10)and incubated at 37^0C. Morphologically three distinct bacteria have selected for further study.

Morphological Characters

Gram staining has been done for all the red mud isolates. For this, a thin film of the bacteria of log phase was kept on a clean glass slide, using a sterile loop. Air-dried, then heat fixed the slide by passing it several times through a flame. Then flooded the slide with crystal violet solution for 1 minute. Then added iodine solution for 3 minutes. Added alcohol for 20 seconds. Lastly, the cells are restained with safranin. This results in gram-positive cells remaining purple and gram negative ones being red or pink (**Bradshaw,** 1973).

Growth kinetics of red mud isolates

All the isolates of red mud have been grown in the nutrient broth culture maintained at pH 10 (at 37^oC). Samples were taken out after every 2 hrs. for spectroscopic reading at 600nm. Simultaneously, the change in pH was also observed at 12 hrs. interval.

Physiological characterization of the microbes

All the microbes which has been isolated from the red mud studied for effect of sodicity, alkalinity, salinity and three different metal (Na, Al and Fe) tolerance.

Alkalinity

To know the survival of isolates at different pH, cells were grown in nutrient broth having different pH 7, 8, 9, 10, 11, 12, 13 and 14 and absorbance was taken at 600nm.

Sodicity and salinity

All the three bacteria has been grown in the nutrient broth culture (pH 10) at different concentration of sodium chloride (e.g. 0.5%, 1%, 1.5% 2%, 5% and 10%). Absorbance has been taken after 24 hrs. at 600nm for growth pattern. While bacterial cells have been separated by centrifuge at 20^0C at 10000rpm, fresh cells were collected, digested with nitric acid and perchloric acid (3:1). And after the digestion the volume made up to 50ml for atomic absorption spectroscopy analysis to know the uptake of Na.

Metal tolerance

Isolates have been grown in nutrient broth at different Ferric chloride and Aluminium sulphate concentration (100,200,300,600, 900 and 1500ppm). To check growth pattern, absorbance have been taken at 600nm. Also the media was centrifuged at 20^0C at 10000rpm, fresh cells were collected, digested with nitric acid and perchloric acid (3:1). After the digestion the volume made up to 50ml for atomic absorption spectroscopy and inductively coupled plasma emission spectroscopy (ICP) analysis to know the uptake of Fe and Al.

Potency of fungus *A. tubingensis* **to bioremediate the red mud**

Many exracellular fungal products can complex or precipitate heavy metals. Citric acid can be an efficient metal–ion chelator and oxalic acid can interact with matal ions to form insoluble oxalate crystals around cell walls.

Apart from these two acids, they also produces other organic acid such as malic acid, gluconic acid etc.(Sutter, Jones and Walchi, 1983). From previous studies, carried out for *A. tubingensis* it was found that it is efficient to produce organic acid production. So we have considered this fungus as a potent microbe for the overall bioremediation purpose. The strain of *A. tubingensis* phosphate solubilizer used for this purpose. It was isolated from the rhizospheric soil of *Eucalyptus* plantation near Patiala (**Reddy *et al.,*** 2002).

Aspergillus tubingensis

A. tubingensis was grown on potato dextrose agar medium and maintaining regularly at 30^{o}C.

Alkalinity

A. tubingensis was grown in Czapek modified broth having different pH 2.5 to 14 for three days, mycelia was filtered using preweighed filter paper, mycelia was dried at 80^{o}C for 48 hrs. to find the dry weight. The change in pH of culture filtrate was recorded.

Metal tolerance

A. tubingensis was grown in Czapek modified broth with different concentration of Na_2Co_3(considering only Na conc. For Na^{+}), Aluminium sulphate (Al^{3+}) and Ferric chloride (Fe^{3+}) of concentration 100,200 & 100,200 and 100,200,300,400,500 and 600ppm respectively. After three days of fungal growth, the mycelia was harvested, dried and digested with nitric acid and perchloric acid (3:1). After the digestion the volume was made up to 50ml for atomic

absorption spectroscopy analysis to know the uptake of Na, Al and Fe.

Growth on various concentrations of red mud

By varying the red mud concentration (1,2,5 and 10%) in modified pskovskaya broth where KH_2PO_4(1mg/ml) was used in place of tricalcium phosphate and yeast extract was eliminated from the content. Mycelia was filtered and digested and volume made to 50 mL and analysis of various metal has been done by ICP.

Nursery experiment

The effect of different concentration of red mud on the growth of maize (*Zea mays)* seedlings inoculated with *A. tubingensis* and the consortia. For this the soil (collected from Thapar campus) was mixed with red mud to obtain 0,25,50 and 75%. The spores of *A. tubingensis* was grown in the Potato dextrose broth, harvested and mixed with sterile soil rite (~359.49 cfu/cup). The bacterial isolates was grown in the nutrient broth (pH 10), centrifuged (20^{o}C, 10000rpm for 5 min.) and washed with sterile water for two times. This consortia (~334.84 cfu/cup) was mixed with the sterile soil rite and used as an inoculum. After 1-month shoot height, shoot dry weight, roots dry weight and soil characteristics like pH and organic carbon was recorded.

Chapter: 5

Results & Discussion

Chapter: 5

Result and discussion

Red mud soil properties

Red mud samples were very alkaline. The pH ranges from 8 to 10, and it is an indicative of the extreme alkalinity and sodicity. Organic carbon content, organic matter contents, available phosphorus and total nitrogen were significantly low and perhaps it creates the great limitation of nutrients to the microbes (**Table 1**).

Isolation and characterization of bacteria

Three mesophilic bacteria (**MSR–P**, **MSR–D** and **MSR–E)** bacteria were isolated from the red mud. All of them were extremophiles or alkalophiles surviving in the pH of 10. Although, when they were plated directly after serial dilution of red mud, they took initially more time to generate. But after 3 – 4 generations, they reduced generation time. This initial delay in growth is due to the extreme physical and chemical conditions of the red mud as they have high caustic soda and presence of heavy metals (**McArther**, 1991), which may result in creating stresses to the microbes.

Morphological identification

All the isolates were Gram stained and microscopic studies have shown that two of the isolates have found to be

Table 1. Physico-chemical characteristics of red mud collected from Damanjodi, Orissa

	Red mud soil
PH	**8.3** to **11.1**
Organic carbon	**0.0036%** per gm of red mud
Organic matter contents	**0.05792%** mg of red mud
Available phosphorus	**1.4616** mg per kg of red mud
Total nitrogen	not detectable

Microbiology

Table 2. Morphological characterizations and Gram staining of the bacterial isolates

Red mud Isolate	Morphology staining	Gram
MSR-P	reddish, pleomorphic	-VE
MSR-D	yellowish, cocci	+VE
MSR-E	whitish, pleomorphic	+VE

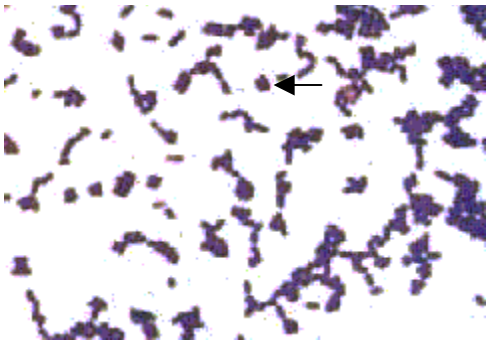

Fig. 1 –Microscopic view (10X) of red mud isolate MSR–P

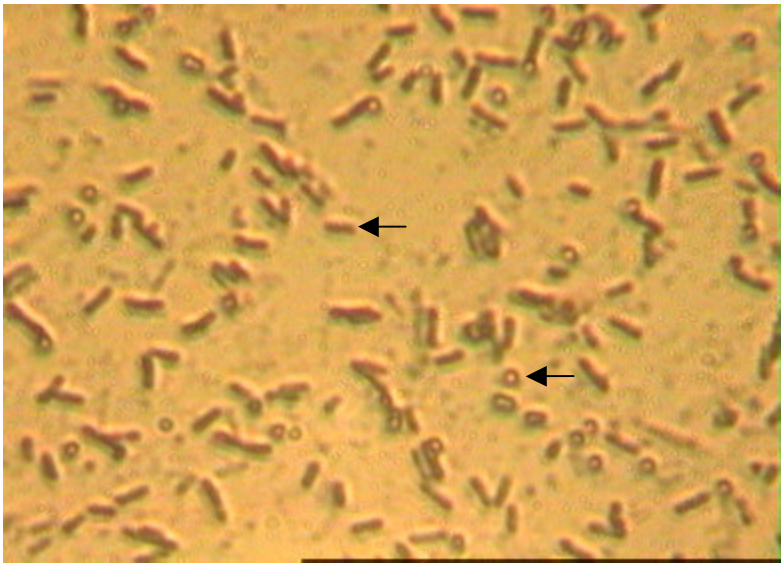

Fig. 2 – Microscopic view (40X) of red mud isolate MSR–E.

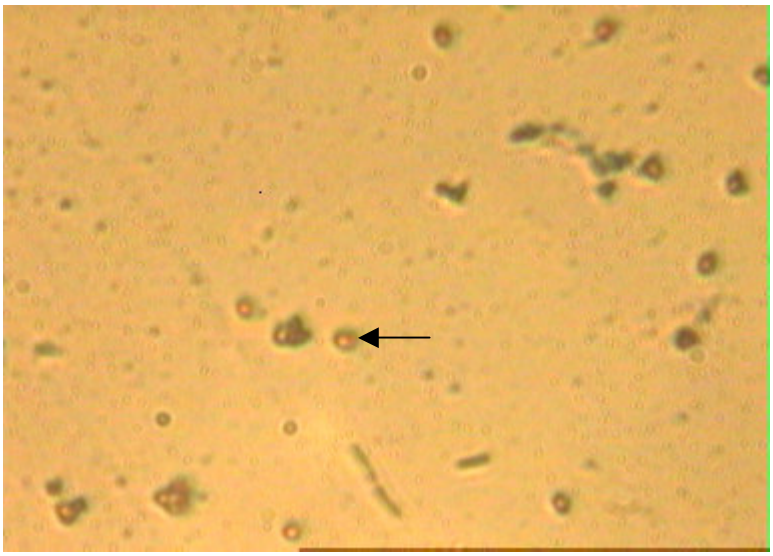

Fig. 3 –Microscopic view (40X) of red mud isolate MSR-D.

Gram+ve and one Gram-ve. **MSR-P** was reddish in colour and pleomorphic in shape and Gram-VE, **MSR-D** was yellowish in colour and cocci shaped and Gram+VE. **MSR-E** was whitish in colour, pleomorphic in shape and Gram+VE microorganism. Possibly MSR-P has a plasma membrane, a thin peptidoglycan layer, a periplasm, and an outer membrane. But on the other hand, in MSR-D and MSR-E only a plasma membrane and a thick layer of peptidoglycan can be expected. (**Table 2**, **Fig. 1,2 &3**).

Alkali tolerance of different red mud bacterial isolates

MSR-D could grow well at pH 8 to 10, but growth is retarded at pH 11 and no growth was observed at pH 12. MSR-P and MSR-E could grow vigorously at pH 8 to 11, though former can grow at the pH 12 but not the later (**Table 3).**

Growth kinetics of bacterial isolates

MSR-P/E and D isolates have lag phase up to 4,8 and 10 hrs., log phase of 4 to 12, 8 to 18 and 10 to 18 and stationary phase of 12 to 30, 18 to 32 and again 18 to 32 hrs. respectively. While MSR-P had very short log phase and take less time to assemble the nutrients while MSR-D & E has long lag phase (**Table 4,5 & 6**, **Fig. 4,5 & 6**).

Growth and tolerance of bacterial isolates towards Fe, Na and Al

Comparative study was made to study the effect of iron on the growth of bacterial isolates MSR-P, MSR-D and MSR-E to find whether these isolates were iron bacteria. They were grown on citrate agar (pH 10) and incubated at 37^{o}C, all the three isolates were well grown after four days, but there was no colour change of the solid citrate plate which is an

Table 3. Alkali tolerance of different red mud bacterial isolates.

	pH				
RM isolate	**8**	**9**	**10**	**11**	**12**
MSR-P	**+++**	**+++**	**+++**	**+++**	**++**
MSR-E	**+++**	**+++**	**+++**	**+++**	**--**
MSR-D	++	++	++	+	--

Table 4. Time Vs absorbance of red mud bacterial isolate MSR-P.

Hrs	OD
0.00	0.101500
2.00	0.101500
4.00	0.164500
6.00	0.291500
8.00	0.632500
10.00	0.963000
12.00	1.157500
18.00	1.206500
24.00	1.225000
30.00	1.223000
36.00	1.141000

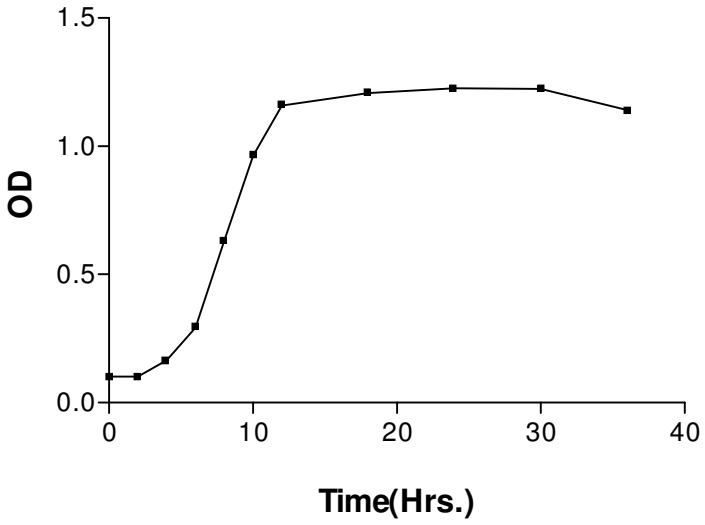

Fig. 4 - Growth curve of red mud isolate MSR-P.

Table 5. Time Vs absorbance of red mud bacterial isolate MSR-D

Hrs.	OD
2.0	0.360
4.0	0.360
6.0	0.360
8.0	0.364
10.0	0.364
12.0	0.610
14.0	1.180
16.0	1.632
18.0	1.873
24.0	1.953
36.0	1.916
40.0	1.760

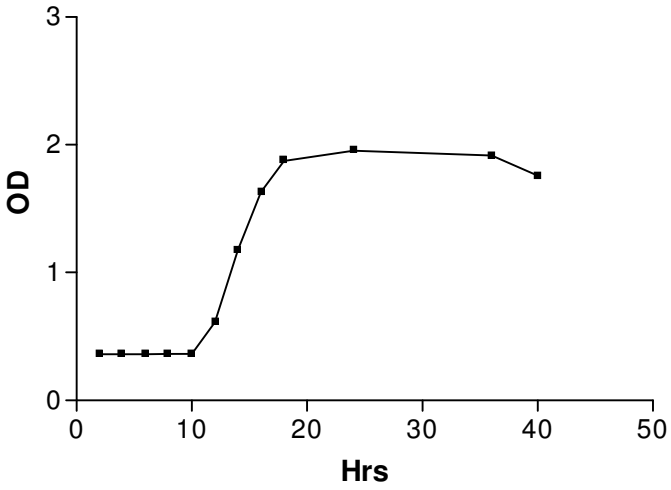

Fig. 5 – Growth curve of red mud isolate MSR-D

Table 6. Time Vs absorbance of bacterial isolate MSR-E

Hrs.	OD
0.00	0.07500
2.00	0.07500
4.00	0.07700
6.00	0.08500
8.00	0.24000
10.00	0.97100
12.00	1.55350
18.00	1.98150
24.00	2.04550
30.00	2.04000
36.0	1.82500

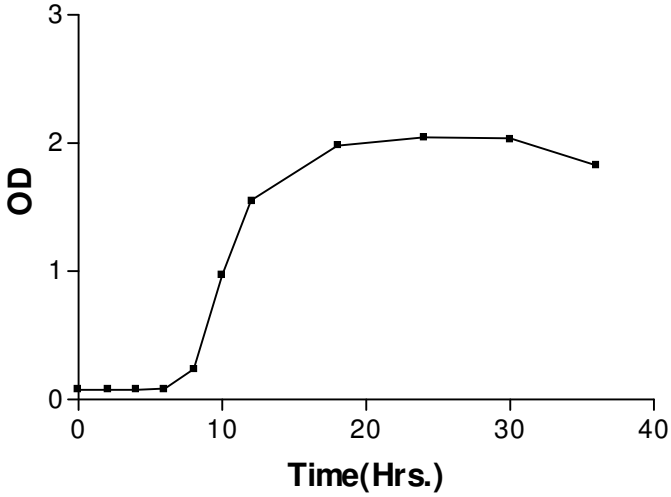

Fig. 6 – Growth curve of red mud isolate MSR-E

indicative of not using citrate as a sole source of carbon, but the isolates can use other form of carbon source too.
As the red mud has higher concentration of the iron oxide and it's colour also appears red due to this. All bacterial isolates were grown in the nutrient broth (pH 10) and ferric chloride which was membrane filtered and added to the media. The iron concentration in the media was 0, 100, 300, 500, 900, 1000 and 1500ppm. It was observed that MSR-P was growing in the higher concentration of Fe^{3+} than the other two bacterial isolates. Atomic absorption spectroscopy studies were made in order to know the intracellular uptake of the iron by these bacterial isolates. Results studies showed that among these isolates MSR-P has the maximum uptake of iron. Although, the uptake of iron in MSR-P and MSR-D is increasing while increasing the concentration of Fe^{3+} but in MSR-E, it decreases after 300ppm(**Table 7&8, Fig. 7&8**). Possibly, it is the biosynthesis and excretion of ferric specific ligands termed as siderophores which are responsible for the metabolism and transportation of iron from the external environment to the internal environment (**Neilands *et al.***, 1985) is more efficient and of universal type in MSR-P than the other two.

Because of the use of caustic soda and bauxite ore, the red mud has higher concentration of Al (~90ppm) and Na (~5ppm). To see the tolerance level of Al and Na, bacterial isolates were grown separately with different concentration of these metals.
When all the isolates were grown in the NaCl concentration to check whether they are halophiles or what is the salinity concentration in which they can survive. It was

Table 7. Effect of different concentration of Fe on the growth of the bacterial isolates from red mud (Fe Vs OD)

Absorbance						
Fe Concn.	**MSR-D**		**MSR-E**		**MSR-P**	
0.0000	1.9530	1.9490	1.8690	1.9060	1.1410	1.1030
100.0000	2.0030	1.9470	1.9240	1.9240	1.2300	1.2100
300.0000	1.8530	1.8570	1.7660	1.7780	1.3610	1.3700
600.0000	1.6400	1.6500	1.6680	1.6370	1.4940	1.4840
900.0000	0.1250	0.1050	0.1180	0.1250	0.6350	0.5640
1500.0000					0.1080	0.1060

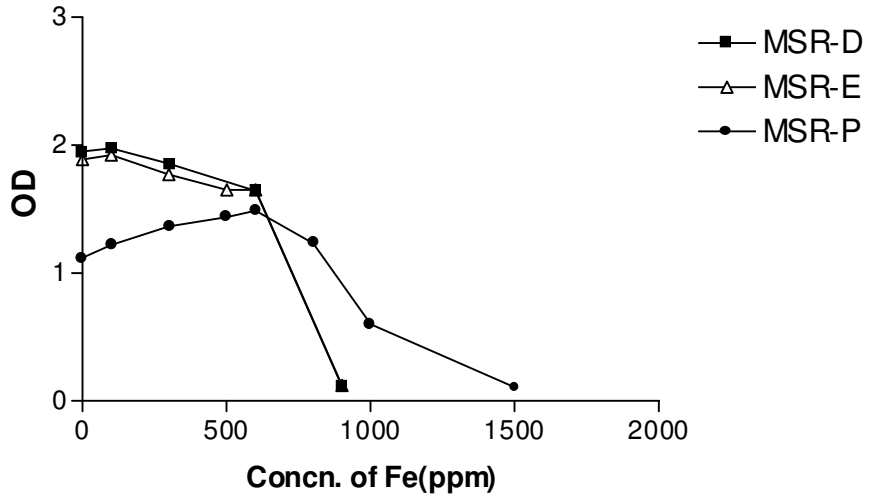

Fig. 7 – Effect of different concentration of Fe on the growth of the bacterial isolates from red mud.

Table 8. Red mud isolates (MSR-P/E/D) grown in different concentration of Fe and their intracellular Fe uptake in μg/mg of fresh wt. of cells

Fe Conc.	MSR-D	MSR-P	MSR-E
0.000	0.521300	0.787500	0.503700
100.000	6.630100	6.250000	11.133000
300.000	10.092100	10.295000	31.777000
500.000	21.732100	17.520000	22.506000
800.000		24.249000	
1000.000		35.670000	

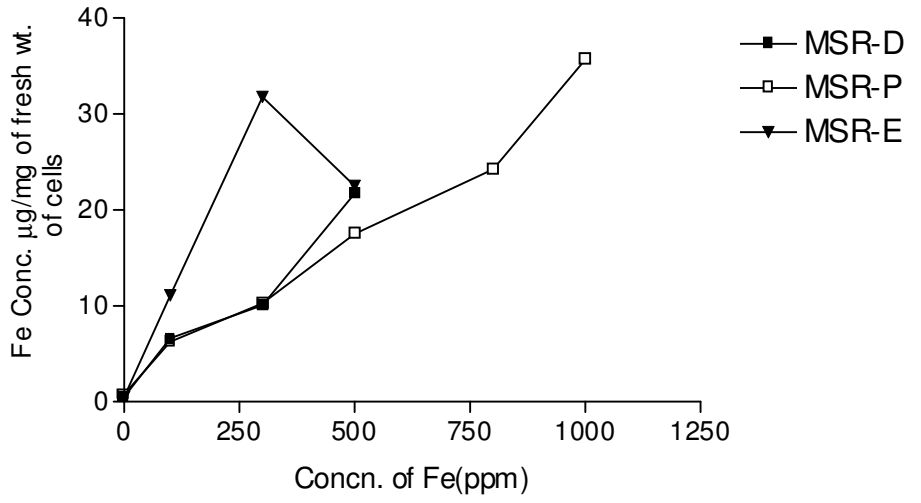

Fig. 8 – Red mud isolates (MSR-P/E/D) grown in different concentration of Fe and their intracellular Fe uptake in (μg/mg of fresh wt. of cells).

found that all the three bacteria can withstand 2% of NaCl concentration and only one isolate MSR-P was survived in much higher concentration of NaCl i.e. 10%. Pattern of growth in all three isolates vary. MSR-P has lowest growth at 0% and optimum at 5% but growth decreases in 10%. And for the MSR-E growth is almost same in 0% to 2%. But, it is MSR-D whose optimum growth were seen in 0% and almost much decreased growth in 2%, these results showed that it is only extreme alkalophiles but not extreme halophiles. Uptake of intracellular Na is also seen in MSR-P and that is corresponding to the growth of it in NaCl concentration. Because optimum growth is seen in 5%, and also the intracellular uptake of Na is higher and trend shows increase in cells from 0% to 5% and then decrease to 10%(**Table 9&10, Fig. 9&10**).

As aluminium is not the essential element for the growth and survival of the microbes but can interact with microbial cells and be accumulated by physico-chemical mechanisms and transport systems of varying specificity (Gadd, 1988). So, to know up to what level they can survive the Al^{3+} toxicity, the isolates were grown in nutrient broth (pH 10) and filter sterilized aluminium sulphate was added after sterilizing the media. The cells were harvested after 48 hrs. by centrifugation at 20°C at 10000 rpm and washed with the citrate buffer to remove the extracellular membrane bound aluminium and digested cells were analyzed by inductively coupled emission spectroscopy. In bacterial isolate MSR-P Al and Na uptake increases as the concentration increases but reverse is the case with Fe and S. However, sodium concentration slightly increases with the increased concentration. In case of isolate MSR-E, it is the Al and phosphorus whose uptake was almost same.

Table 9. Effect of different NaCl(%) concentration on the growth of different red mud isolates (MSR-P/E/D)(Na Vs OD)

NaCl Concn(%)	MSR-P	MSR-D	MSR-E
0.0	0.8655	1.9530	1.789
0.5	1.106	0.5730	1.790
1.0	1.233	0.1050	1.790
1.5	1.262	0.0750	1.793
2.0	1.490	0.0710	1.801
5.0	1.7165	--	--

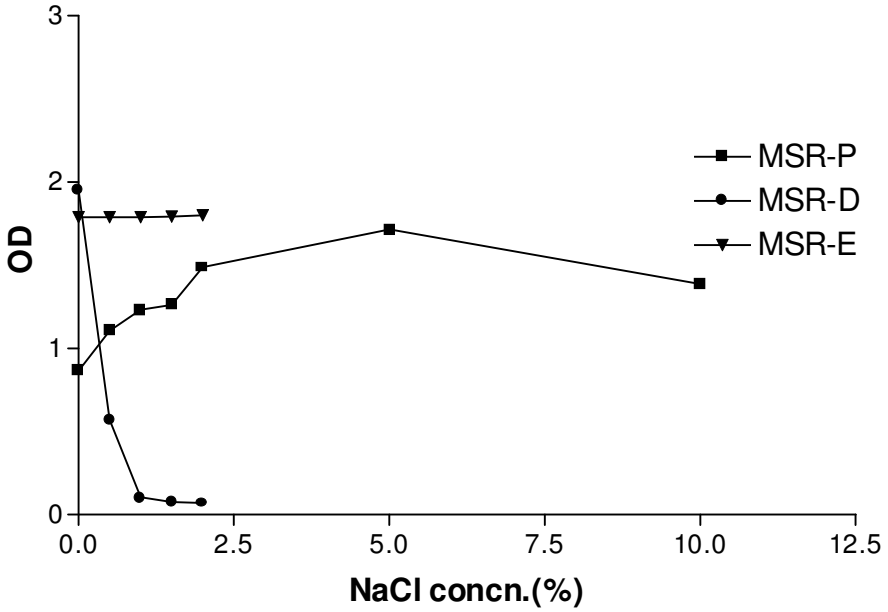

Fig. 9 - Effect of concentration of different NaCl (%)on the growth of different red mud isolates MSR-P/E/D.

Table 10. Intracellular uptake of Na in isolate MSR-P

(%)	Na in MSR-P
0.0	3.2050
2.0	10.7900
5.0	215.7500
10.0	198.2500

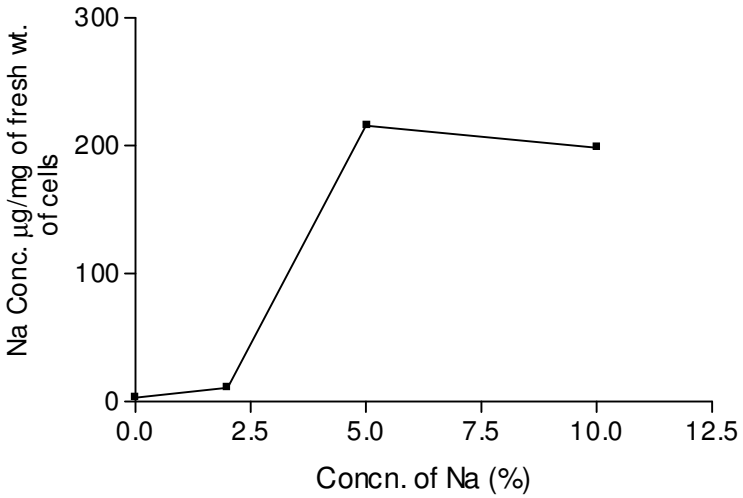

Fig. 10 – Intracellular uptake of Na in isolate MSR-P grown in different Na conc.

Here, again it is the Na, which increases slightly with the increased concentration of Al^{3+}. Iron metal uptake was sharply fallen down after 150ppm of Aluminium but Ca sharply increases after 150ppm. There might be some competition among these metals to be transported inside the cells so some metals are inhibited to be transported and some metals did not. That's why the accumulation of some metals was more than the others. (**Table: 11&12, Fig. 11,12,13&14**)

In the present study among the three different bacteria isolated from red mud, all the bacteria were able to tolerate high pH level. The high alkali content of red mud (bauxite residue) deposits from alumina production plants in developed nation poses a challenge to reestablish flora and fauna at the deposit sites. All the red mud and lake water samples contain small number of metabolically inactive (injured) bacterial cells that grew in enriched, but not in minimal media and exhibited the light blue colour of methylene blue in the hanging drop slide. These cells grew after inocubation at 37^0C in the desired medium followed by plating them on tryptic soy agar for aerobes and anaerobes (**Hamdy and Williums**, 2001). More than 150 bacterial cultures were isolated from the treated bauxide residues samples and most were identical to the genera: Bacillus, *lactobacillus*, *Leuconostoc*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Flavobacterium*, *Enterobacter* and *proteus* by Hamdy and Willium (2001). They reported that among the different bacteria, *Lactobacillus*, *Leuconostoc* and *Bacillus* were predominant and all played a role in the physical properties and acid production leading to the bioremediation of the bauxite residue treated samples. As

Table 11. Intracellular accumulation of Al, Fe, Na, Ca & P in μg/mg of fresh wt. of red mud isolate MSR-E grown in different Al conc

Al concn.	Al	Fe	Na	Ca	P
0.0	0.000	0.94170	3.63790	0.29460	0.189810
50.0	3.99785	0.90070	3.11715	0.92820	3.131900
100.0	12.5585	1.10090	5.65370	0.90600	11.483500
150.0	19.361	1.62680	7.74550	0.96225	17.937000
200.0	47.1115	0.97440	15.74900	1.79505	47.526000

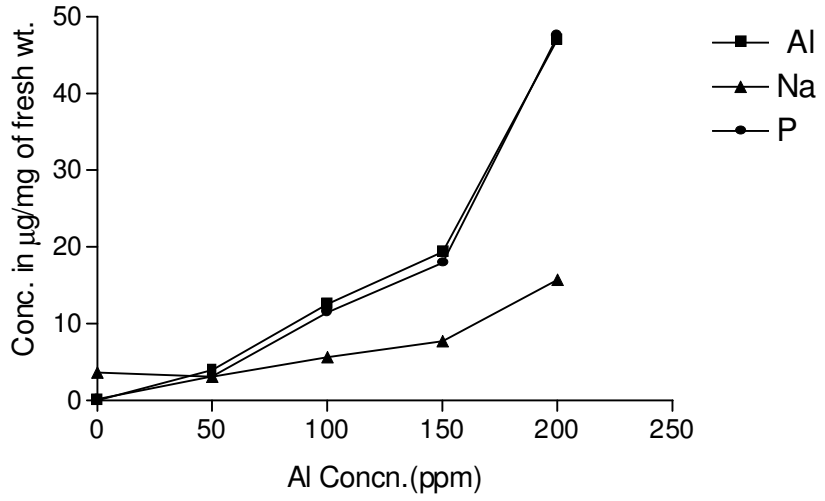

Fig. 11 – Concentration of intracellular Al, Na & P (μg/mg) of fresh wt. of red mud isolate MSR-E grown in various Al concentrations.

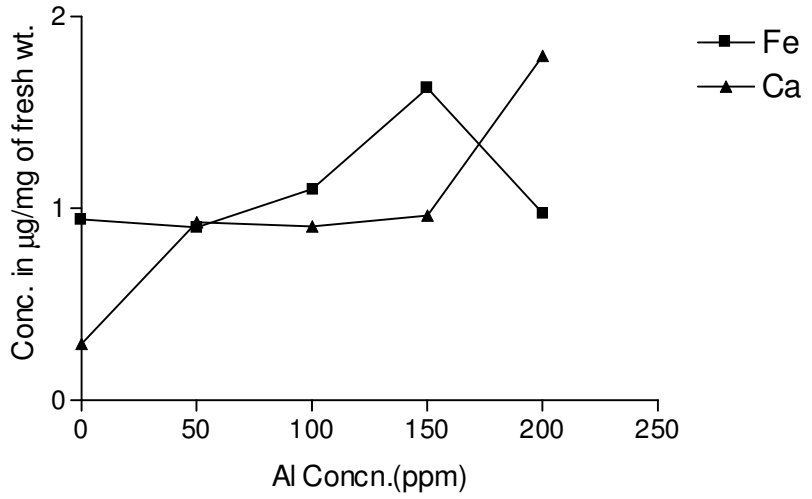

Fig. 12 – Concentration of intracellular Fe & Ca (µg/mg) of fresh wt. of red mud isolate MSR-E grown in various Al concentrations.

Table 12. Concentration of intracellular Al, Fe, Na, S & P in µg/mg of fresh wt. of red mud isolate MSR-P grown in various concentration of Al.

Al. Cocn.	Al	Fe	Na	P	S
0.0	0.0000	0.8299	8.010	0.0949	0.4865
50.0	6.3840	1.12275	3.3234	8.351500	0.64875
100.0	14.7730	0.82720	5.179	15.090000	0.3784

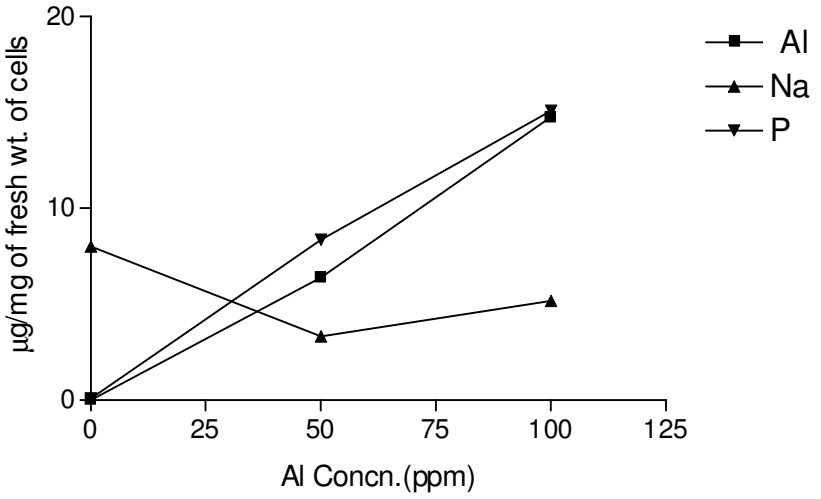

Fig. 13 – Concentration of intracellular Al, Na & P in µg/mg of fresh wt. of red mud isolate MSR-P grown in various concentration of Al.

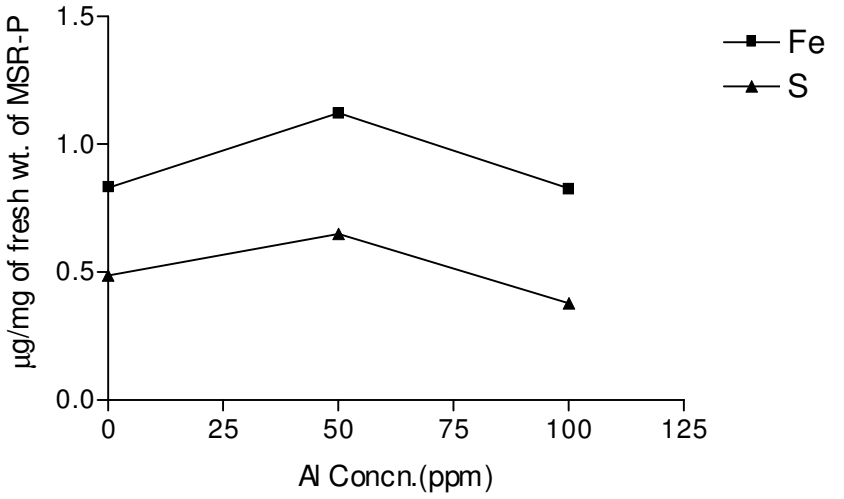

Fig. 14 – Concentration of intracellular accumulation of Fe & S in µg/mg of fresh wt. of red mud isolate MSR-P grown in various concentration of Al.

suggested by Hamdy and Williuums (2001) that bacterial cells in the red mud actively grew when using rich medium. This may be the reason why we have got only 3 different morphotypes of bacteria in this study. Though the bacterial isolate from the red mud were not identified in this study, they seems to play an important role in bioremediation of red mud ponds especially in reducing the pH of the red mud soils.

Valarie (1999) also isolated 16 isolates of facultative anaerobic bacteria from red mud. Most of them are rod shaped, Gram +ve, Gram –ve and able to tolerate the pH range between 7 and 10. she reported that most of the isolates are NaCl tolerants. In the present study also the isolated bacteria are able to tolerate high level of NaCl.

The bauxite residue is a highly alkaline mixture of fine particle sized metal oxides. It normalyy consists of Fe_2O_3, Al_2O_3, SiO_2 and CaO (**Summers *et al.*,** 1996) at higher levels. In the present study the effect of Al, Fe and Na on the growth of the bacterial isolates from red mud was studied to see whether these isolates can tolerate higher level of metals or not. The results showed that the bacterial isolate MSR-P in the present study showed higher tolerance to Na, Fe, Al and also the uptake in the cells when compared to the other two bacterial isolates. Hence, these isolates may play an important role in bioremediation of red mud ponds.

Aspergillus tubingensis

Filamentous fungi are widely used as producers of organic acids (**Matty**, 1992) and particularly black *Aspergillli* and some species of *Penicillium*. Most species have been tested for solubilizing insoluble phosphates as these microorganisms convert these insoluble phosphates into soluble form through the process of acidification, chelation and exchange reaction (**Gerke**, 1992). In the present study the highly efficient phosphate solubilizing fungus *Aspergillus tubingensis* (**Reddy *et al*.**, 2002) was used to explore the possibility of using this fungus as a remediating agent and also to reduce the pH of the red mud for possible vegetation.

A. tubingensis was tested for its tolerance to different pH. The fungus was grown in different pH viz., 2.5, 3.5, 4.5, 5.5, 6.5, 7, 8, 9, 10, 11,12 and 13. The mycelium was harvested after three days and dried the mycelium. The results showed that this fungus was able to grow up to pH 2.5 to 11 with a maximum growth at pH3.5 and also at pH 8 (**Table 13,Fig.15**).

The effect of aluminium on the growth of *A. tubingens* was studied to see whether this fungus can tolerate higher level of aluminium. The fungus was grown at different concentration of Al (0,50,100,200 and 500ppm). When Al in the form of $Al_2(SO_4)_2$ was added to the medium, the pH of the medium was reduced to 2 to 3. The fungus was grown at the pH of 2.5 where the growth was not inhibited. The results showed that the fungus was not able to grow at 200ppm of Al, which clearly showed that this fungus could not tolerate high level of Al. The mycelium harvested from this experiment was digested with nitric acid:perchloric acid

Table 13. Dry wt. of *A. tubingensis* at various pH

pH	Total Dry Wt
2.5	0.056
3.5	0.0796
4.5	0.0634
5.5	0.062
6.5	0.053
7	0.0595
8	0.099
9	0.0345
10	0.031
11	0.03
12	0.0175

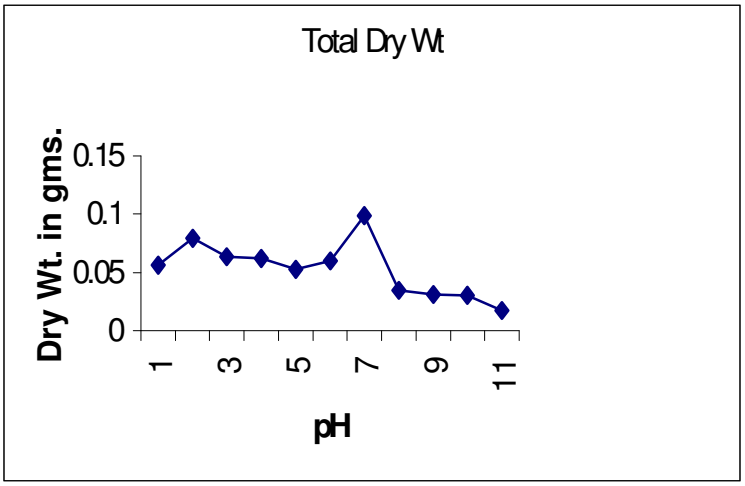

Fig. 15 – Dry wt. of *A. tubingensis* at various pH.

for elemental analysis by ICP. The results showed that the level of P and Al in the mycelium increased as the concentration of Al in the growth medium increases (**Table 16, Fig.16**). The levels Ca, Mg and K were decreased as the Al level increased in the growth medium (**Fig.17**)(**Table 18**).

The effect of iron on the growth of the mycelium and its uptake was also studied in this investigation. When grown in different concentration of Fe, *A. tubingensis* accumulates higher level of Fe as the concentration increases in the growth medium (**Table 18, Fig 18**). The maximum uptake of iron was observed at 600ppm of iron in this study. The fungus also showed higher accumulation of Na when grown at different concentration of Na. The maximum uptake of sodium was observed when the fungus was grown at 200ppm of Na (**Table 19, Fig.19**).

The fungus was grown in the medium containing different concentrations (0,1,2,5 & 10%) of red mud to see whether this fungus can grow in presence of red mud once it is inoculated. The mycelium was harvested and dried. The dried mycelium was digested with nitric acid and percholoric acid (3:1) for different elemental analysis. The growth of the fungus was not affected when different concentrations of red mud were amended in the medium. In fact the mycelial growth was increased in the red mud as compared to the control (**Table 20.2,Fig.20**) and the change in the pH of the supernatant was in the acidic range (**Table 20.1**).

The ICP analysis showed that this fungus was able to accumulate Al in the mycelium. The maximum accumulation was seen when grown in 2% of red mud (**Table 21,Fig. 21**). The Na levels were decreased in the mycelium as the concentration of red mud increased in the medium. The maximum Na level

Table 16. various metal uptakes by *A. tubingensis* when grown in different concentration of aluminium sulfate

Al conc.	Al	Fe	Na	Ca	P	S
0.0	0.0	1.84350	0.7270	5.51120	4.36530	0.648740
100.0	8.6236	1.88930	2.4253	1.74060	10.624	1.189400
200.0	21.542	2.14760	2.2004	1.638	24.296	1.513700

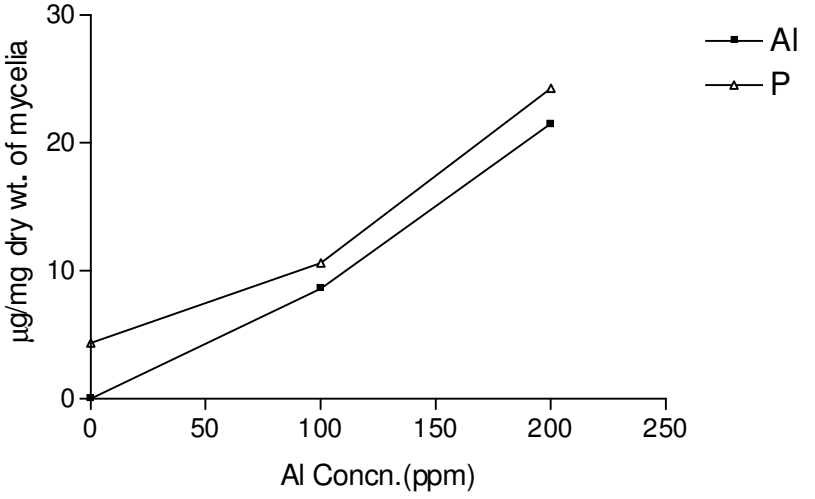

Fig. 16 – Al & P metal uptake by *A. tubingensis* when grown with different concentration of aluminium sulfate.

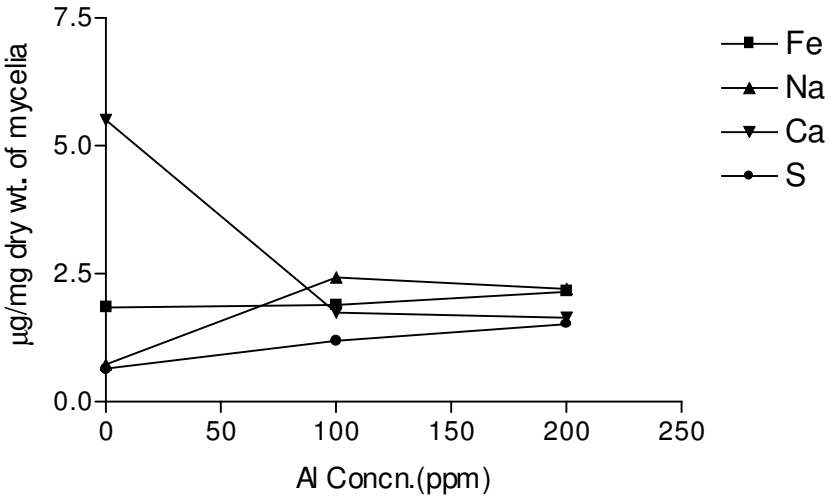

Fig. 17 – Fe, Na Ca and S metals uptake by *A. tubingensis* when grown with different concentration of aluminium sulfate.

Table 18. Fe uptake by *A. tubingensis* grown with different concentration of ferric chloride

Fe (ppm)	µg/mg of dry mycelia
0.0	2.250
50.0	1.010
100.0	3.810
200.0	3.855
300.0	3.875
400.0	6.430
500.0	11.340
600.0	25.650

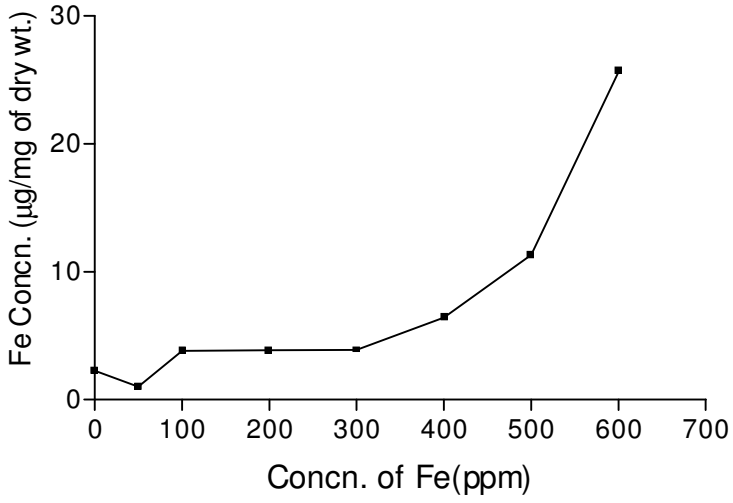

Fig. 18 – Fe uptake by *A. tubingensis* grown with different concentration of ferric chloride.

Table 19. Uptake of Na by *A. tubingensis* grown in different Na conc.

Na Conc.	µg/mg of dry mycelia
0.0	0.4650
50.0	0.4655
150.0	0.5095
200.0	0.6510

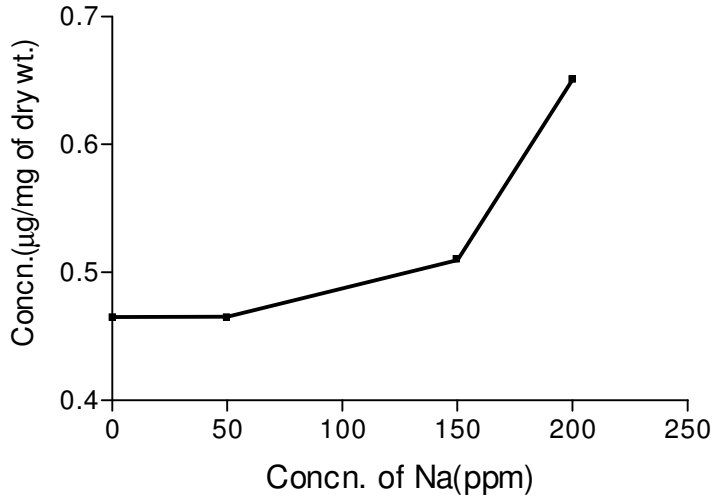

Fig. 19 – Uptake of Na by *A. tubingensis* grown in different Na conc.

Table 20.1. Change in pH of different red mud concentrations by *A. tubingensis*

Red mud conc.(%)	Initial pH	Final pH
0	7.0	2.55
1	7.68	7.27
2	7.72	2.53
5	7.87	2.71
10	8.25	2.77

Table 20.2. Dry wt. of *A. tubingensis* at different red mud concentrations

RM Conc.	Dry wt.	
0.0	0.1070	0.1190
1.0	0.2230	0.1740
2.0	0.4280	0.7520
5.0	0.4140	0.4720
10.0	0.6650	0.5080

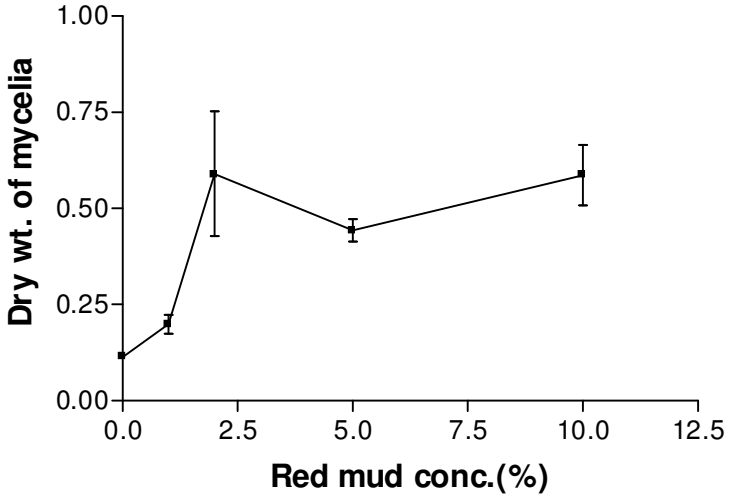

Fig 20. Dry wt. of *A. tubingensis* at different red mud concentrations

was observed in control than red mud treated mycelium (**Fig. 22**), the Pb levels varied at lower concentration where as it was increased in the mycelia when grown in higher concentration of red mud (**Fig. 23**). The Fe content of the mycelium increased as the concentration of the red mud increased in the growth medium (**Fig. 24**). The other metals such as S, Ca, Mg and K were decreased in the mycelium as the concentration of red mud increased in growth medium (**Table 21,Fig. 25,26,27,28 and 29**).

Fungi are good in the accumulation of heavy metals. The ability of fungi to transform a wide variety of hazardous chemicals has aroused interest in using them in bioremediation. Red mud consists of different toxic metals such as Al, Na, Fe, Cd, Cu, Pb, Hg, Ni and other toxic metals. Fungi plays important role in accumulation of these metals in their extramatrical hyphae. In the present study *A. tubingensis* is able to accumulate higher levels of these metals when grown in presence of the red mud. Many extracellular fungal products can complex and precipitate heavy metals. Citric acid can be an efficient metal ion chelators and oxalic acid can interact with metal ions to form insoluble oxalate crystals around cell walls and in the external medium (**Murphy and Levy**, 1983).

Table 21. Intracellular accumulation of various metal (mg/gm of dry mycelia) in *A. tubingensis* grown in different red mud concentration (%)

Concn.	Al		Na		Pb		Fe		S	
0.0	0.385	0.6374	1.5511	1.5588	0.0168	0.0190	3.0989	1.0168	2.0147	1.9177
1.0	84.470	155.3075	0.8677	1.2038	0.1683	0.4909	16.2771	26.5459	0.8551	1.2756
2.0	289.240	92.3936	1.2608	0. 0.0579	0.1837	32.6109	13.3809	0.6062	0.2785	0.606230
5.0	160.664	149.1313	0.7284	0.6886	0.3258	0.30640	28.5193	27.2372	0.42286	0.390773
10.0	170.406	179.2618	0.72291	0.79425	0.3430	0.36176	27.6751	26.8661	0.33847	0.313848

Table 21. Intracellular accumulation of various metal (mg/gm of dry mycelia) in *Aspergillus tubingensis* grown in different red mud concentration (%)

Conc.	**Ca**		**K**		**Mg**		**P**	
0.00	5.419950	6.751680	2.32810	7.030250	0.638980	0.856840	2.520370	3.817050
1.00	2.710310	2.889655	1.29970	2.303680	0.307460	0.355080	1.515850	2.094480
2.00	1.086000	0.487406	0.93883	0.429220	0.1750817	0.199647	1.628970	0.927120
5.00	1.259780	0.894703	1.145603	0.893177	0.221328	0.197224	1.441666	1.835160
10.00	1.377970	0.7102756	0.69339	0.687244	0.205740	0.200098	1.771200	1.933858

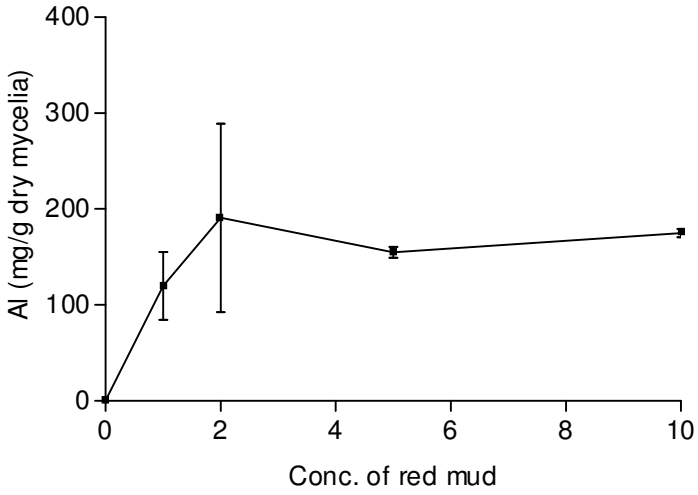

Fig. 21 – Al uptake in *A. tubingensis* from different red mud Concentration

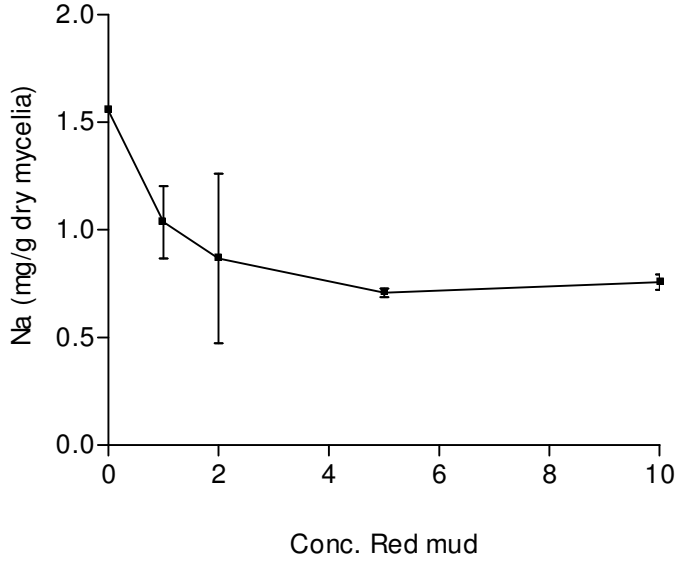

Fig. 22 – Na uptake in *A. tubingensis* from different red mud concentration

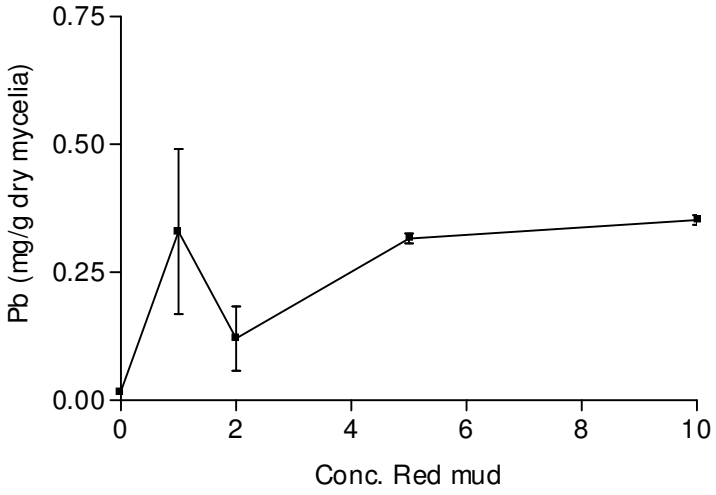

Fig. 23 – Pb uptake in *A. tubingensis* from different red mud concentration

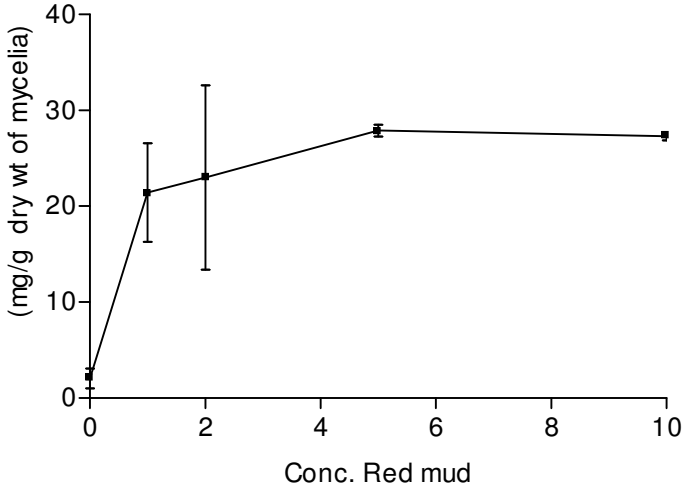

Fig. 24 – Fe uptake in *A. tubingensis* from different red mud concentration

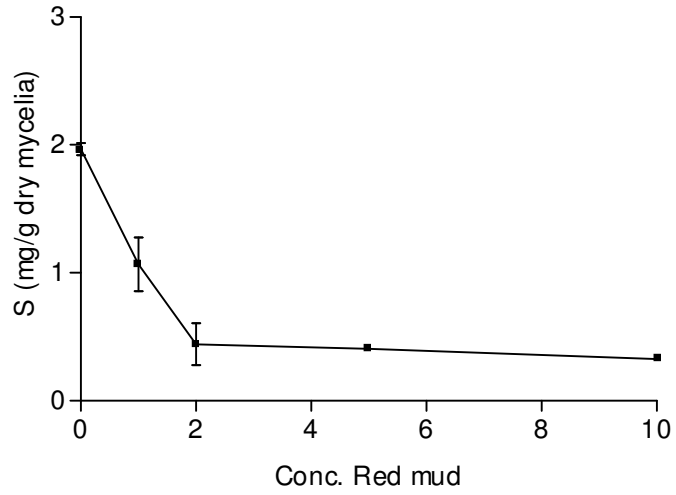

Fig. 25 – S uptake in *A. tubingensis* from different red mud concentration

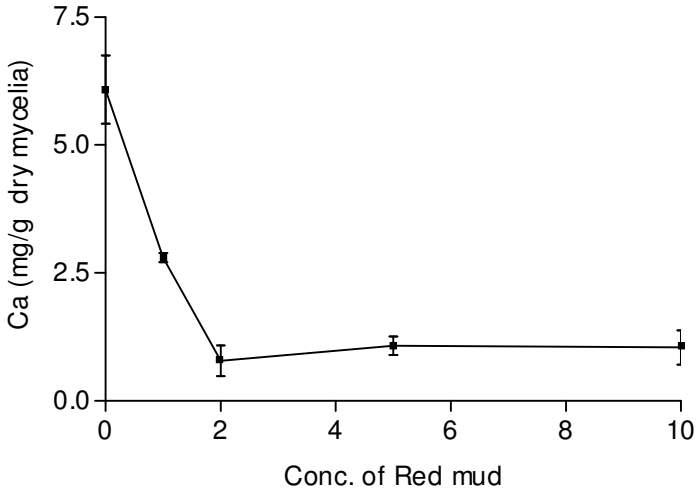

Fig. 26 – Ca uptake in *A. tubingensis* from different red mud concentration

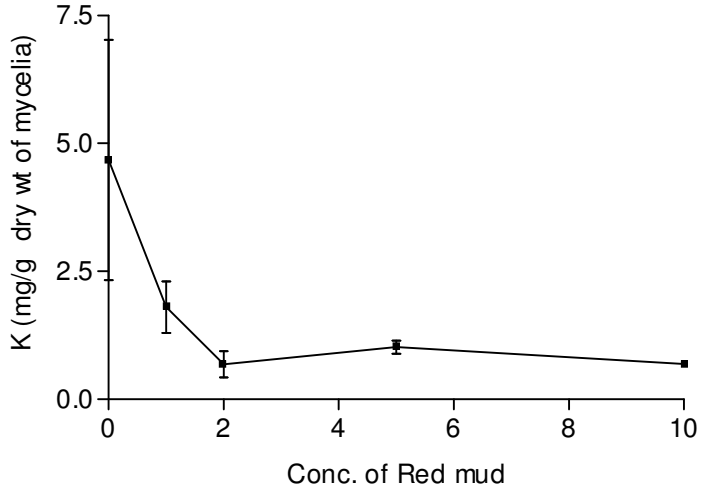

Fig. 27 – K uptake in *A. tubingensis* from different red mud concentration

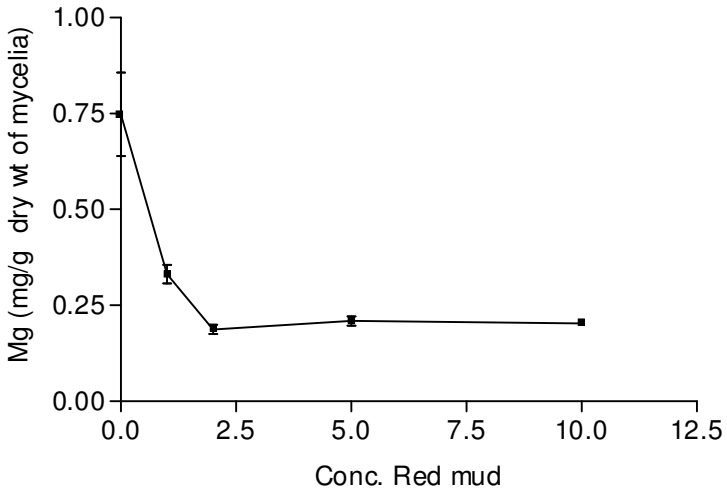

Fig. 28 – Mg uptake in *A. tubingensis* from different red mud concentration

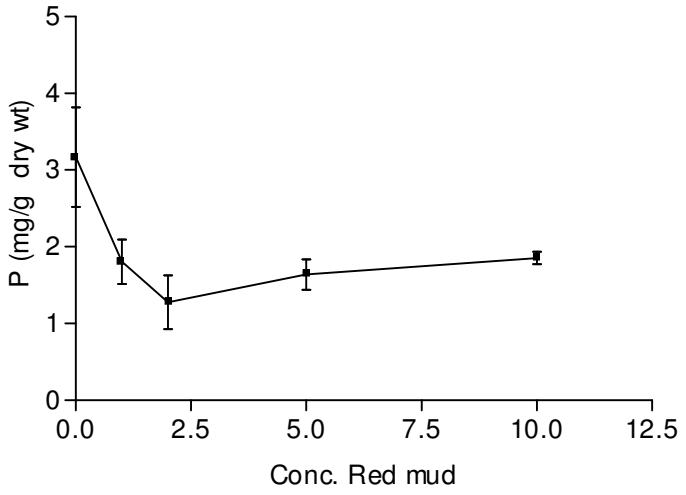

Fig. 29 – P uptake in *A. tubingensis* from different red mud concentration

Nursery experiment

A nursery experiment was carried out to see whether the bacteria isolated from the red mud and also *A. tubingensis* used in this study can reduce the pH of the soil and help the plants to grow in the red mud amended soils. Different concentrations of red mud (0, 25, 50 and 75%) were mixed with native soil. The consortia of the three bacterial isolates (MSR-P, E & D) and *A. tubingensis* were inoculated separately. The seeds of maize were grown and harvested after one month. Destructive samplings were made and various growth parameters of plants and the physico-chemical properties of the soil were recorded.

The shoot height of the maize plants were higher in control seedlings when compared the red mud amended soils. In consortia inoculated seedlings and also in *A. tubingensis* inoculated seedlings. There was no significant difference in shoot height was observed (**Table 22 & 23, Fig.30**). The shoot height was less in case of 75% red mud soils in both the treatments. The shoots dry weight was almost same in all the treatments when inoculated with bacterial consortia, whereas in case of *A. tubingensis* inoculated seedlings higher biomass was in control seedlings. There was no significant difference between the red mud concentrations in the shoot dry weight, the root dry weight was observed (**Table 22 &23**).

The pH of the soil was reduced more than two units in both bacterial consortia and *A. tubingensis* inoculated soils. In case of control there was only a slight decrease in the

Table 22. Effect of bacterial consortia (MSR-P/E/D) on the growth of maize seedlings grown in different concentration of red mud

Red mud Concentration	Shoot height	Shoot Dry Wt.	Root Dry Wt.	Shoot/root (dry wt.)
0%	11.08	0.462	0.44	1.05
25%	906	0.4	0.322	1.36
50%	9.44	0.444	0.32	1.38
75%	8.58	0.404	0.42	0.961

Table 23. Effect of *A. tubingensis* on the growth of maize seedlings grown in different concentration of red mud

Red mud Conc.	Shoot height	Shoot Dry Wt.	Root Dry Wt.	Shoot/root (dry wt.)
0%	11.82	0.482	0.574	0.839
25%	9.62	0.370	0.554	0.667
50%	9.22	0.352	0.528	0.615
75%	7.22	0.32	0.52	0.615

Table 24. The pH change of different conc. of red mud in nursery trials

Red mud Cons.	Initial pH	Final pH (Consortia)	Final pH (A. tubingensis)
0%	8.03	7.74	7.73
25%	9.37	7.03	7.17
50%	9.8	7.17	7.13
75%	10.01	7.09	7.16

Table 25. Effect of red mud isolates consortia (MSR-P/E/D) and growth of maize seedlings on different concentration of red mud for chemical changes in the soil

Red mud concentration	Organic % C before inoculum	Organic matter before inoculum	Organic C after inoculum	Organic matter after inoculum
0%	13.28	22.89	19.24	33.16
255	11.497	19.82	18.27	31.16
50%	10.785	18.59	18.96	31.49
75%	10.424	17.97	13.14	32.68

Table 26. Effect of *A. tubingensis* and growth of maize seedlings on different concentration of red mud for chemical changes in the soil

Red mud concentration	Organic % C before inoculum	Organic matter before inoculum	Organic C after inoculum	Organic matter after inoculum
0%	13.28	22.89	46.1814	33.49
255	11.497	19.82	45.96	33.32
50%	10.785	18.59	45.334	32.88
75%	10.424	17.97	30.93	21.975

final pH i.e., the pH was reduced from 8.03 initial pH to 7.7 of final pH (**Table 24**).

The organic carbon and organic matter was also increased in the soil when compared to the initial soil, which was used in this study (**Table 25 & 26**). These results suggest that the microbes play an important role in reducing the pH of the soil and also help the plants to survive in red mud soil.

Hamdy and Williums (2001) reported that the treated bauxite residue supported growth of several plants and earthworms that survived for 300 days. In a test plot bioremediation on a bauxite deposits at Alcoa point comfort, TX, the *Bermuda* grass has used effective mulch material and encouraged water filtration, leading to establishment and growth of the salt tolerant vegetative species. They also reported that various plants (popular, pampas and cactus) grew in alfalfa hay treated bauxite residue for 6 to 12 months. **Valarie** (1999) reported the reduction in red mud pH by bacterial action. She also reported a strong positive correlation between the acidification of sterile glucose amended red mud and growth of these isolates. Her experiments were also showed that, oxygen was necessary for the pH reduction under the chosen conditions and that pH reduction was related to the bacterial growth. This suggests that it would be possible to reduce the pH of red mud by bacterial metabolism in the presence of aeration by inoculating the red mud with pure cultures of indigenous bacteria and incorporating nutrients to encourage bacterial growth. From these results it was concluded that the bacterial isolates used in this study plus the fungus *A. tubingensis* play an important role in reducing the pH of the red mud and also promoting the growth of the plants.

Further studies are required to inoculate these microbes in red mud ponds and monitor their growth and survival.

Summary

To meet the growing demand of materials natural resources are exploited to the greater extent. As a result of which there is depletion of various resources as well as accumulation of different generated wastes. Red mud (bauxite residue) is one such waste produced during the aluminium extraction from bauxite ore with concentrated NaOH at elevated temperature. The use of NaOH in Bayer's process results in bauxite residue being extreme saline, sodic and alkaline. In addition to these properties the bauxite residues are generated in very large quantities in each year. The major components in the red mud are iron oxide, silica, unreacted alumina residual NaOH as Na_2CO_3 as well as alkali bound in the form of sodalite and ferrite etc. The residual alkali contents make the red mud alkaline with a pH from 9–13. These alkaline materials have to be safely dispersed off by putting them to the red mud ponds. Reclamation of red mud ponds is difficult because of high pH and high concentration of soluble ions and toxic compounds, which competitively inhibit the nutrient uptake in plants and microbes. Hence, it is necessary to reclaim such sites that are depleted due to industrial waste. Because of the extreme alkalinity and salinity the microbial growth is also limiting factor in red mud soil. Present study investigated the microbiology of the red mud and ascertains possibility of using these microbes to reduce the alkalinity of red mud.

The red mud samples were collected from National Aluminium Company, Damanjodi, Orissa and microbes were isolated by serial dilution. Three morphologically different microbes were isolated from red mud and subjected to the Gram staining. Two of the isolates (MSR–D and E) are G+ve and

one isolate (MSR-P) is Gram-ve. MSR-E is whitish and pleomrphic. MSR-P is reddish and pleomorphic and MSR-D is yellowish and cocci. The microbes were tested for the tolerance of different pH and the results showed that all the microbes tolerated up to pH 11 where as MSR-P grow at pH 12. The growth kinetics of these isolates were studied. MSR-P had a short lag phase of 4 hrs. whereas MSR-E and D had longer lag phase of 8-10 hrs. These isolates were tested for salinity for growing them in different concentration of NaCl. MSR-P survived the higher concentration of NaCl i.e.10% whereas the other two isolates (MSR-E/D) grew only up to 2%.

As red mud contains different toxic compounds, these bacteria were tested for their ability to survive in presence of Al, Na and Fe. When grown in different concentration of Al, the MSR-E accumulated higher level of Al in the cells compared to MSR-P. The accumulation level of the Al in both the bacterial isolates increased as the concentration of the Al increases in the growth medium. MSR-D was able to grow up to 50ppm of Al in the media. When tested for different concentrations of Na, MSR-P was grown in 10% of Na where as the other two isolate did not grow beyond 2%. The maximum uptake of Na in MSR-P was observed when grown in 5% of NaCl. These bacteria were grown in different concentrations of Fe to see their tolerance levels. The result showed that as the concentrations of Fe in growth media increased; there was decrease in the growth of bacterial isolates. When tested for uptake of iron MSR-P accumulated higher levels of Fe in the cells compared to other bacteria. These results suggest that bacterial

isolate can play an important role in bioremediation of red mud.

A. tubingensis a phosphate solubililizing fungus was also used to explore the possibility to reduce the pH of the red mud soil. This fungus was able to solubilize the insoluble phosphate through the process of acidification. It is worth tasting to use this fungus to reduce the pH of the red mud soil. This fungus was tested for its tolerance the different pH and from that it is possible to grow up to pH 11. To check the tolerance level of Al, Na and Fe. This fungus was grown in different concentration of these metals. When grown in presence of Al, it did not grow beyond 200ppm of Al concentration. When mycelium was digested for the elemental analysis P and Al increases in the mycelia as the concentration of Al incraeses in the growth media. This fungus was able to accumulate higher level of Fe in the mycelium when grown at 600ppm. The maximum uptake of Na was also observed when grown at 200ppm.

The fungus was grown in different concentration of red mud to see whether it survives in red mud or not. The results showed that this fungus was able to grow up to 10% of the red mud amended media. In fact, the mycelia growth increases in red mud amended media compared to control. The pH of the cultural filtrate was significantly decreased from the initial pH. The Inductively Coupled Plasma Emission spectroscopy analysis showed that the different element levels varied in the mycelium when grown in presence of different red mud concentrations. This results

suggests that *A. tubingensis* is able to tolerate the higher concentrations of different metals present in the red mud. This fungus would also play an important role in bioremediation of red mud ponds, which can help the detoxification of metals, and solubilization of insoluble phosphate.

Nursery experiment was also carried out to see these microbes (MSR-P/E/D and *A. tubngensis*) could reduce the pH of the red mud amended soil and also help the plants to grow. Different concentrations of red mud were amended with native soil and consortia of these three bacterial isolate and *A. tubingensis* were inoculated separately and maize seeds were sown. The destructive samplings were done after one month and various growth parameters were studied. The shoot height was not significant in both the cases up to 50%. But in 75% there was decrease in shoot height. The root and shoot biomass ratio was almost similar in all the cases.

The pH of the soil reduced more than two units in *A. tubingensis* and consortia in amended soils where as in control there is slight decrease in the pH. Organic mater and organic carbon was also increased in microbial inoculated soil.

The above result suggest that inoculation of these microbes in red mud would reduce the pH of the soil and also increases the organic matter of red mud ponds. Further, studies are required to inoculate these microbes in the red mud ponds and monitor their growth and survival also their role in improving the plant growth.

ANNEXURE–I

Nutrient media broth

Yeast extract 2.8 g/l
Dextrose 1 g/l
Peptone from meat 7.8 g/l
Sodium chloride 5.6 g/l
Peptone from casein 7.8 g/l
Final pH (37 °) 7.5

Potato dextrose agar

Potato infusion 4.0 (infusion from 200 g potatoes)
D(+)glucose 20.0
agar–agar 15.0.
Preparation: Suspended 39 g/litre (for solid plates)
autoclaved (15 min at 121 °C).
pH: 5.6 ± 0.2 at 25 °C.
The plates are clear and yellowish–brown.

Czapek broth (modified)

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
Preparation: Suspend 48 g/litre (solid plates)
autoclave (15 min at 121 °C) pour plates.
pH: 7.3 ± 0.2 at 25 °C.
The plates are turbid and whitish.

ANNEXURE–II

Table 1. Physico-chemical characteristics of red mud collected from Damanjodi, Orissa.

Table 2. Morphological identification and Gram staining of the bacterial isolates.

Table 3. Alkali tolerance of different red mud bacterial isolates.

Table 4. Time Vs absorbance of red mud bacterial isolate MSR–P.

Table 5. Time Vs absorbance of red mud bacterial isolate MSR–D.

Table 6. Time Vs absorbance of bacterial isolate MSR–E.

Table 7. Effect of different concentration of Fe on the growth of the bacterial isolates from red mud (Fe Vs OD).

Table 8. Red mud isolates (MSR–P/E/D) grown in different concentration of Fe and their intracellular Fe uptake in μg/mg of fresh wt. of cells.

Table 9. Effect of different NaCl(%) concentration on the growth of different red mud isolates (MSR–P/E/D)(Na Vs OD).

Table 10. Intracellular uptake of Na in isolate MSR–P.

Table 11. Intracellular accumulation of Al, Fe, Na, Ca & P in μg/mg of fresh wt. of red mud isolate MSR–E grown in different Al conc.

Table 12. Concentration of intracellular Al, Fe, Na, S & P in μg/mg of fresh wt. of red mud isolate MSR–P grown in various concentration of Al.

Table 13. Dry wt. of *A. tubingensis* at various pH.

Table 16. various metal uptakes by *A. tubingensis* when grown in different concentration of aluminium sulfate.

Table: 17 Fe uptake by *A. tubingensis* grown in different iron concentration.

Table 18. Fe uptake by *A. tubingensis* grown with different concentration of ferric chloride

Table 19. Uptake of Na by *A. tubingensis* grown in different Na conc.

Table 20.1. Change in pH of different red mud concentrations by *A. tubingensis.*

Table 20.2. Dry wt. of *A. tubingensis* at different red mud concentrations.

Table 21. Intracellular accumulation of various metal (mg/gm of dry mycelia) in *A. tubingensis* grown in different red mud concentration (%).

Table 22. Effect of bacterial consortia (MSR-P/E/D) on the growth of maize seedlings grown in different concentration of red mud.

Table 23. Effect of *A. tubingensis* on the growth of maize seedlings grown in different concentration of red mud.

Table 24. The pH change of different conc. of red mud in nursery trials.

Table 25. Effect of red mud isolates consortia (MSR-P/E/D) and growth of maize seedlings on different concentration of red mud for chemical changes in the soil.

Table 26. Effect of *A. tubingensis* and growth of maize seedlings on different concentration of red mud for chemical changes in the soil.

Fig. 1 – Compound microscopic view (40X) of red mud isolate MSR-P.

Fig. 2 – Compound microscopic view (40X) of red mud isolate MSR-E.

Fig. 3 – Compound microscopic view (40X) of red mud isolate MSR-D.

Fig. 4 – Growth curve of red mud bacterial isolate MSR-P.

Fig. 5 – Growth curve of red mud bacterial isolate MSR-D.

Fig. 6 – Growth curve of red mud bacterial isolate MSR-E.

Fig. 7 – Effect of different concentration of Fe on the growth of the bacterial isolates from red mud.

Fig. 8 – Red mud isolates (MSR-P/E/D) grown in different concentration of Fe and their intracellular Fe uptake in (μg/mg of fresh wt. of cells).

Fig. 9 – Effect of concentration of different NaCl (%)on the growth of different red mud isolates MSR-P/E/D

Fig. 10 – Intracellular uptake of Na in isolate MSR-P grown in different Na conc.

Fig. 11 – Concentration of intracellular Al, Na & P (μg/mg) of fresh wt. of red mud isolate MSR-E grown in various Al concentrations.

Fig. 12 – Concentration of intracellular Fe & Ca (μg/mg) of fresh wt. of red mud isolate MSR-E grown in various Al concentrations.

Fig. 13 – Concentration of intracellular Al, Na & P in μg/mg of fresh wt. of red mud isolate MSR-P grown in various concentration of Al.

Fig. 14 – Concentration of intracellular accumulation of Fe & S in μg/mg of fresh wt. of red mud isolate MSR-P grown in various concentration of Al.

Fig. 15 – Dry wt. of *A. tubingensis* at various pH.

Fig. 16 – Al & P metal uptake by *A. tubingensis* when grown with different concentration of aluminium sulfate.

Fig. 17 – Fe, Na Ca and S metals uptake by *A. tubingensis* when grown with different concentration of aluminium sulfate.

Fig. 18 – Fe uptake by *A. tubingensis* grown with different concentration of ferric chloride

Fig. 19 – Uptake of Na by *A. tubingensis* grown in different Na conc.

Fig 20. Dry wt. of A. *tubingensis* at different red mud concentrations.

Fig. 21 – Al uptake in *A. tubingensis* from different red mud Concentrations.

Fig. 22 – Na uptake in *A. tubingensis* from different red mud concentrations.

Fig. 23 – Pb uptake in *A. tubingensis* from different red mud concentrations.

Fig. 24 – Fe uptake in *A. tubingensis* from different red mud concentrations.

Fig. 25 – S uptake in *A. tubingensis* from different red mud concentrations.

Fig. 26 – Ca uptake in *A. tubingensis* from different red mud concentrations.

Fig. 27 – K uptake in *A. tubingensis* from different red mud concentrations.

Fig. 28 – Mg uptake in *A. tubingensis* from different red mud concentrations.

Fig. 29 – Maize seedlings grown in different concentrations of red mud inoculated with red mud isolates bacteria in consortium and *A. tubingensis*.

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