



## **Role of biomarkers to elucidate red mud induced shift in microbial community structure and its implications in soil quality assessment**

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### **Abstract**

Red mud is the by-product of bauxite refining process, which poses significant environmental challenges due to high alkalinity and heavy metal toxicity. The review dealt with the influence of red mud on microbial community dynamics reflecting the relative distribution of microbial population and its impacts on soil quality status. Several soil quality biomarkers have been used for the exploration of microbial community structure that provide insights into ecological impacts of red mud on soil quality. The physico-chemical, microbiological and biochemical indicators are considered as sensitive biomarkers essential for soil quality assessment and elucidate functional status of red mud spoil. The shift in microbial community structure in red mud spoil has been addressed using high-throughput sequencing and metagenomic approaches. The red mud induced shift in microbial community structure with relative distribution of metal-tolerant alkaliphiles followed by gradual amelioration of other microbial communities with notable decline in extremophiles, which reflect sign of red mud spoil genesis promoting ecological restoration. Overall, the review provided an insight into ecological consequences of red mud on microbial community dynamics, which pave the way of greater understanding in the direction of improving soil quality for sustainable development.

**Keywords:** Microbial community dynamics, red mud, ecological restoration, soil quality.

## 1. Introduction

Aluminium being the most abundant metal in the Earth's crust is liable for energy generation due to its low density, high electrical and thermal conductivity, corrosion resistance and high strength to weight ratio (Rai et al., 2020). Despite its abundance, aluminium does not exist in its pure form rather it occurs as metal oxide due to its high reactivity. Bauxite is the principal ore of aluminium consisting mixture of aluminium containing minerals such as gibbsite, boehmite and diaspore (Kannan et al., 2021). Globally, the bauxite reserves constitute about 55-75 billion tones. Australia is the world's top bauxite producing country that contributes nearly 110 million metric tons followed by China, Brazil and India. India ranks seventh globally comprising about 7.5% of the total bauxite reserves accounting to 4.87 billion tonnes (Yadav et al., 2022). Over the past 25 years, aluminium production has witnessed steady growth, with an average 2.5% annual increase (Sverdrup et al., 2015), which highlights its importance in global industrial and economic context.

The major bauxite reserves in India are distributed mostly in Odisha (49%), Jharkhand (9%), Maharashtra (8%), Gujarat (23%), Chhattisgarh (8%) and Madhya Pradesh (3%) (Figure 1) (Sutar et al., 2014; Patel and Pal, 2015; Rai et al., 2020a). This distribution influences the regional

industrial growth, environmental policies related to mining and resource management. The principal bauxite producing districts include Koraput and Sundargarh in Odisha, Lohardaga and Gumla in Jharkhand, Ratnagiri in Maharashtra, Kachchh and Jamnagar in Gujarat, Bastar, Surguja, Shahdol, Bilaspur and Jabalpur in Madhya Pradesh and Chhattisgarh (Samal et al., 2013). Koraput with mining area of 4647 hectares is the largest bauxite producing district in Odisha that contributed about 98% of the state's total reserve followed by Rayagada and Sundargarh (Department of Steel and Mines, Government of Odisha). Raw materials extracted from bauxite ore have limited applications as compared to aluminium, which drives its frequent extraction from bauxite (Komlóssy et al., 2022). About 90% of the world's bauxite deposits are utilized in aluminium production (Rai et al., 2020a). The extraction process involves two key steps such as the alumina refining followed by aluminium smelting. Alumina refining involves processing of bauxite to extract alumina applying Bayer process. Aluminium smelting involves processing of the alumina to extract aluminium using Hall-Heroult electrolytic process (Sutar et al., 2014). Huge quantity of waste residues generated as by-products have distinctive properties which pose significant environmental challenges (Agrawal and Dhawan, 2022; Chao et al., 2022).

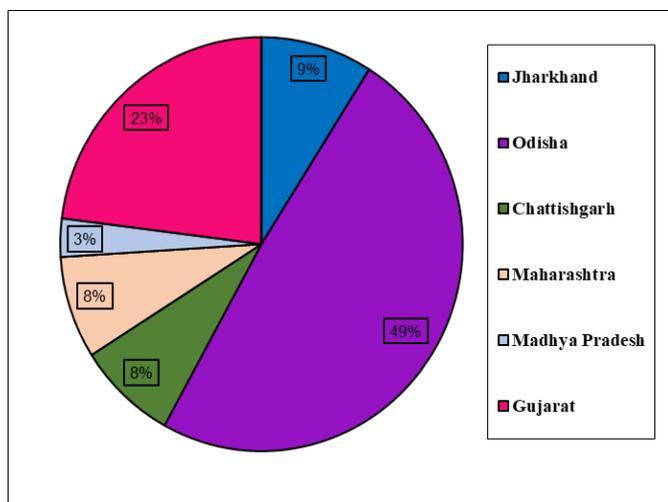


Figure 1 State-wise distributions of bauxite reserves (in percentage) in India.

Red mud known as ‘bauxite residue’ is an insoluble waste product generated during Bayer process, which is primarily used to extract alumina from bauxite ore. It is named so for its reddish-brown colour due to high concentration of iron oxide and similar appearance to red soil (Liu et al., 2021a). Under Hazardous and other wastes Management and Transboundary Movement Rules, 2016 (HOWM Rules, 2016), the red mud is categorized as ‘high volume, low effect waste’ that is generated in huge quantities but exhibit low environmental risk compared to other hazardous wastes. At the end of twentieth century, the utilization of red mud became universal problem (Evan, 2016). Amount of bauxite residues generated per tonne of alumina using Bayer process that is employed in 95% of alumina plants ranges from 1 to 1.5 tonnes worldwide (Klauber et al., 2011). However, the actual amount of red mud generated by different plants may vary based on the type of bauxite ore used as well as the operation process employed whose neutralization and handling becomes major environmental concern.

Global red mud disposal accounted 120-150 million tons per year, which has continued to rise

(Zampieri et al., 2019; Silveira et al., 2021). About 4.6 billion tons of bauxite residue was disposed globally in red mud ponds (Khanna et al., 2022; Kar et al., 2023). India generated about 13.73 million metric tons of bauxite residue annually, which represented 5-7.5% of the world's total production (Panda et al., 2021; Patil and Thorat, 2022). With the existence of six major bauxite ore refineries, around 50% of total bauxite residue was generated in Odisha, India. Amongst the Indian aluminium refineries, NALCO, Damanjodi, Odisha is the major producer of red mud spoil that generated around 4 million tons annually (Figure 2) followed by Vedanta, Lanjigarh that generated around 2.5 million tonnes per year (Evans et al., 2011; Rai et al., 2020b). Untreated bauxite residue is difficult to utilize due to its complex mineral composition, fine particle size and high corrosivity (Lyu et al., 2021). Therefore, it is primarily disposed into long-term storage (Archanbo and Kawatra, 2021a). The extensive storage not only takes up huge land area but also incurs significant cost for its maintenance following acquisition of specialized equipment leading to increased storage cost accounting to 2% of the total alumina production cost (Wang et al., 2019).

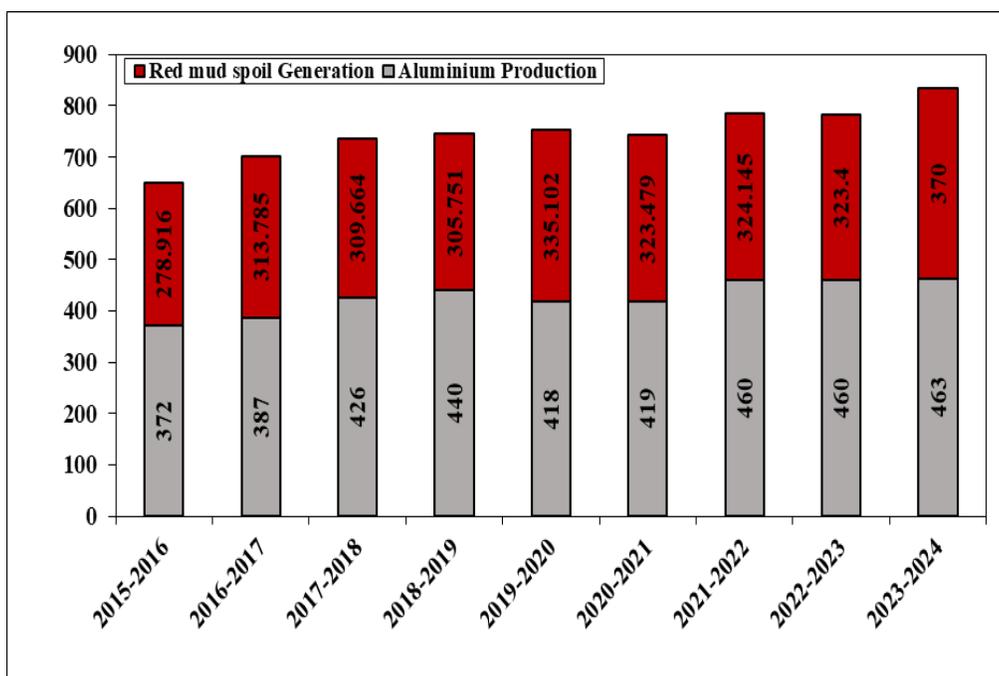


Figure 2 Year-wise annual aluminium production (metric tonnes) and red mud spoil generation (million cubic meter) from NALCO, Damanjodi, Koraput, Odisha.

Long-term storage of red mud in tailing ponds pose severe environmental risks including lack of vegetation cover, increased vulnerability to wind, soil erosion and leaching of heavy metal and rare earth elements into ground water pose serious effects on ecosystem (Renforth et al.,2012; Pradhan et al., 2024). Despite its potential risks, red mud is rich in valuable metals such as iron, aluminium, silicon, titanium and vanadium. Several studies have highlighted novel methods for treating red mud and metal extraction (Archambo and Kawatra, 2021b) as well as reutilization of red mud (Wang et al., 2021b; Mi et al., 2022). Despite multiple initiatives to utilize red mud in mining, construction and agro-economic industries, only 2-3% of the total residue has been reused while the rest remain unprocessed (Ujaczki et al., 2018). Therefore, it is crucial to develop implementation strategies focusing on their utilization in various applications and restoration to minimize environmental risks associated with disposal of red mud (Xue et al., 2016b). However, ecological restoration through microbial amelioration is the most effective and economic way to counterbalance the adverse impacts of red mud spoil (Chauhan and Silori, 2010; Patil and Thorat, 2022).

### 1.1 Red mud and its composition

Bauxite residues in form of red mud is extremely alkaline by-product of alumina refinery, classified as non-ferrous metallurgical solid waste (Xue et

al., 2022; Chao et al., 2022). Nature of red mud varies depending on the mining site, grade of bauxite and implementation of alumina refinery process (Liu et al., 2021a). Production methods have been classified into (a) Bayer process, (b) Sintering process and (c) combined Bayer-Sintering process (Pattajoshi and Dash, 2024). Currently, bauxite ore is processed primarily through Bayer’s process that accounted 90% of total aluminium production, involving caustic digestion of alumina from bauxite ore using concentrated sodium hydroxide at 150-250°C and 400 ppm pressure result in the production of substantial amounts of red mud (Tabereaux and Paterson, 2024). Owing to its extreme alkalinity, heavy metal toxicity, high exchangeable sodium percentage and electrical conductivity, red mud is disposed in large artificial containment areas referred as ‘Bauxite residue disposal areas’ (BRDAs) or ‘Red mud ponds’ (Sedlackova et al., 2024). Further, bauxite residue (red sludge) is an artificial ore supplemented with aluminium, iron, silicon, rare earth elements and radioactive elements (Klauber et al., 2011; Li et al., 2023). Red mud includes minerals along with several major and minor elements during bauxite processing (Table 1). Besides, the source material of bauxite, local climatic conditions, age of the deposit and topography play crucial role in determining the composition of red mud. Moreover, the concentration and elemental content of red mud vary based on the location of bauxite deposits (Swain et al., 2022).

Table 1. Composition of red mud generated from alumina refinery in India.

Major elements		Minor elements	
Composition	Weight (%)	Composition	ppm
Fe <sub>2</sub> O <sub>3</sub>	4.5-57	V	23.52
Al <sub>2</sub> O <sub>3</sub>	33.2-76.9	Cr	497-848
SiO <sub>2</sub>	2.16-19.2	Ni	31-329
Na <sub>2</sub> O	2-10	Co	24-59
TiO <sub>2</sub>	0.98-19	Zn	20-105
CaO	2-8	Nd	48-341
P <sub>2</sub> O <sub>5</sub>	1-2	Ce	430-842
K <sub>2</sub> O	0.66	Zr	279-2070
MgO	0.13	La	112-416
MnO <sub>2</sub>	0.15	Sc	54-368

## 2. Biomarkers for restoration assessment

Soil serves as complex microhabitat that governs ecosystem functioning owing to (i) dynamic changes in physico-chemical and biological characteristics over time and space, (ii) differential degree of soil to adsorb biomolecules, (iii) influence of soil mineral composition on enzyme mediated catalysis under different conditions, (iv) diverse microbial community structure across different soil profiles, (v) structured, heterogeneous and discontinuous nature of soil leading to variation in microbial community composition in discrete microhabitats.

Alumina refinery activities alter the geomorphic system, causes imbalance between landforms and microbial mediated soil processes which leads to alternations in physico-chemical and microbiological properties influencing microbial colonization and vegetational development (Courtney, 2015; Qaidi et al., 2022). Besides, the large-scale generation of red mud spoil associated with heavy metal toxicity buffer the flow of contaminants that influence soil productivity (Mishra et al., 2017). Though the fresh bauxite residue is not fertile, it is not completely sterile (Gadd, 2010). As microbial communities establish, it gradually restores soil quality such as soil texture, availability of soil nutrients through red mud spoil genesis (Gautam et al., 2018). The process emphasizes critical role of soil quality biomarkers and microbial activity in assessing the progress of bauxite residue restoration (Monokrousos et al., 2014; Naykodi et al., 2024). Hence, their periodic evaluation in response to the dynamic changes in microbial community structure associated with diversity in time scale can provide valuable information about the contribution of various soil variables towards red mud spoil restoration (Berta et al., 2021; Naykodi et al., 2023). Quantitative assessment involves the selection of suitable soil variables, which can be used as sensitive biomarkers of red mud spoil genesis that reflect the sign of improvement (Santini et al., 2015; Zhu et al., 2016b). The sensitive biomarkers are used to explore the site-specific ecology and promote effective residue

management strategies. Exploring the dynamics of red mud spoil genesis through sensitive biomarkers is crucial for evaluating restoration strategies that contribute to long-term agro-ecosystem sustainability (Zhu et al., 2016a; Avery et al., 2022; Chao et al., 2022).

### 2.1 Physico-chemical properties

Environmental degradation by alumina refinery operations is not an irreversible process but the recovery becomes slow because of the initial recovery of physico-chemical attributes followed by functional biological attributes (Krishna *et al.*, 2014). The physical characterization involves the textural composition, which indicates size distribution of soil subsystem and is a crucial factor affecting accumulation of organic matter (Colemon *et al.*, 2024). The chemical characterization involves the interactions between various chemical constituents in soil. The physico-chemical characterization includes soil textural composition, hydrological regimes (bulk density, water holding capacity, moisture content), pH, available soil nutrients (organic carbon, total nitrogen, extractable phosphorous) (Grandy *et al.*, 2009).

Soil texture is a qualitative classification tool used in both the field and laboratory to determine the classes of soil based on their physical texture (Veum et al., 2021). Textural composition influences organic matter content in soil, water retention capacity, soil available nutrients, nutrients retention capacity and microbial activities (Khairul et al., 2019). Several studies reported that red mud spoil consisted of about 25-80% clay (< 0.002 mm), 0.5-30% sand (0.02-2 mm) and 18% silt (0.002-0.02 mm) respectively (Xue et al., 2016a; Zhu et al., 2018). Bulk density serves as an indicator of soil structure providing insights into porosity, soil compaction and water content (Wang et al., 2021b). These factors directly impact root growth, infiltration, nutrient availability and microbial activities that influence key soil processes and used as an indicator of soil

eration status (Guo et al., 2019; Xue et al., 2016a). Soil water holding capacity is controlled by textural composition and organic matter, which influence biochemical reactions by regulating the available soil nutrients to microbes and plants for their microbial activities (Maurya et al., 2020). It is positively regulated by moisture content which is characterized by the infiltration rate, accumulation rate and field capacity (Di Carlo et al., 2019). Soil pH reflects the potential of hydrogen describing the relative acidity or alkalinity of soil which regulates the chemical and biological status of soil by influencing soil structure, nutrient availability, microbial community structure and nutrient leaching that impact the vegetational growth in terrestrial ecosystems (Neina, 2019). Red mud being highly alkaline is characterized with pH range of 10-12.8 due to excess of lime and NaOH employed in Bayer process, which inhibits solute export to xylem, root development and respiration (Grafe et al., 2011). Several studies reported the effect of vegetation on different physico-chemical soil attributes (Haynes and Zhou, 2019; Wu et al., 2020) as well as microbiological soil attributes (Santini and Fey, 2018; Berta et al., 2021).

## **2.2 Soil organic matter (SOM)**

Restoring soil quality and establishment of basic soil functionality in red mud deposits is essential for the sustainable management, where soil quality serves as the key indicator to evaluate the progress of red mud spoil genesis. Soil organic matter serve as source of nutrient and energy (Powlson et al., 2011; Xue et al., 2016b) and play vital role in determining the soil health and ecosystem services. SOM promotes soil aggregate stability, soil resistance, water holding capacity and moisture content (Dong et al., 2019; Xue et al., 2022). SOM determines the physico-chemical and biological properties of soil. Ecological restoration includes addition of organic amendments to bauxite residue that improve soil quality through the recovery of the pre-disturbed organic matter level by lowering bulk density, enhancing nutrient availability and water holding capacity (Tian et al., 2020; Guo et al., 2024).

Soil represents the largest organic carbon sink that influence the carbon and nutrient flow in terrestrial ecosystems (Zhu et al., 2016b; Tian et al., 2023). Soil organic carbon (SOC) pool holds more than twice the amount of carbon found in terrestrial vegetation and nearly thrice that of atmospheric carbon pool therefore any variation in SOC can significantly impact the global carbon cycling (Zhu et al., 2024). Besides, total nitrogen is an important parameter that indicates soil fertility and regulate plant growth (Guo et al., 2019). Studies revealed gradual increase in soil organic nitrogen with age series of red mud spoil (Goloran et al., 2017; Krishna et al., 2014). Besides, phosphorus is the most limiting nutrient for plant growth followed by nitrogen and considered as crucial parameter for ecosystem restoration (Courtney et al., 2014; Goloran et al., 2014; Cheng et al., 2020). Thus, Soil organic C, total N and extractable P are the key drivers which limit soil functioning in various nutrient deprived ecosystems (Di Carlo et al., 2019).

## **2.3 Microbial biomass pool**

Unlike the physico-chemical properties, the microbiological characteristics are extremely responsive to even minor alteration in soil variables (Feigl et al., 2017). Microbial biomass is defined as living component of organic matter that constitutes living organisms smaller than 5-10  $\mu\text{m}^3$  (Banning et al., 2011). Soil microbial biomass serves as the labile reservoir of accessible nutrients and plays vital role for the survival of plants in nutrient deficient ecosystems (Singh and Gupta, 2018). It also governs pathways involved in nutrient transformation (Dey, 2022), microbial turnover, cycling of soil organic matter, aeration and biological stability (Jones et al., 2009; Wu et al., 2020; Wu et al., 2021). Besides, soil microbial biomass influences the interactions between the soil organic matter and microbial community, thereby act as key biological driver to the ecosystem functioning (You et al., 2019; Prasad et al., 2021). Lower ratio of microbial biomass to organic nutrients in soil indicates progress towards ecological restoration (Mishra et al., 2017; Dong et al., 2022).

Soil microbes require essential elements for optimal functioning and store them in their microbial biomass such as carbon, nitrogen, phosphorus, sulphur as well as other secondary and trace elements (Bandyopadhyay and Maiti, 2019). Microbial biomass carbon is considered as the living portion of soil organic carbon that plays vital role in controlling nutrient dynamics, soil fertility and carbon sequestration which impact primary productivity in terrestrial ecosystem (Kaschuk et al., 2010; Dong et al., 2019). Microbial biomass nitrogen acts as labile reservoir for the uptake of plant available nitrogen (Prasad et al., 2021). The increased microbial biomass nitrogen contributes to plant growth by promoting their nutrient source and sink function (Krishna et al., 2014). Microbial biomass phosphorus is sensitive to size and structure of soil. Besides, soil microbes are integral to phosphorus cycle mediating P availability to plants (Cheng et al., 2020; Henriksen, 2023). Assessment of ecological restoration has been studied by several workers through the estimation of microbial biomass in different land use practices (Lewis et al., 2010; Teixeira et al., 2019), which can be used as the effective ecological markers for soil management and perturbation studies in terrestrial ecosystems (Courtney et al., 2014). The contribution of microbial biomass pool towards organic matter turnover, nutrients flow (Dey, 2022) and structural stability (Dey and Paul, 2021), marked it as sensitive indicators for ecological restoration (Dong et al., 2022).

#### **2.4 Microbial basal soil respiration**

Basal soil respiration is defined as the steady rate of microbial respiration resulting from the mineralization of soil organic matter and measured by either O<sub>2</sub> uptake or CO<sub>2</sub> evolution (Jones et al., 2012; Weng et al., 2024). Both soil microbial respiration and mineralization of organic matter are considered as sensitive indicators for monitoring changes in soil quality (Di Carlo et al., 2019; Phillips and Courtney, 2022). Basal soil respiration mainly depends on the microbial community composition and available substrates for the microbial

communities, which is determined by their biomass and activity (NRM, 2008; Xue et al., 2022). Microbial soil respiration is regulated both directly and indirectly through root-microbe-soil interactions, release of substrates from root exudates, litter quality or quantity, CO<sub>2</sub> concentration and edaphic properties such as soil porosity and nutrient abundance (Gautam et al., 2018; Charan and Bhattacharyya, 2023). Besides, the basal soil respiration is influenced directly by temperature and precipitation effects and indirectly by climate change, abiotic and biotic disturbances (Jones et al., 2012; Banning et al., 2014; Ke et al., 2021).

Further, the insight into the factors controlling the basal soil respiration rate per unit microbial biomass is crucial for understanding the terrestrial carbon cycle. Quantifying soil CO<sub>2</sub> efflux is one of the key components of ecosystem carbon balance. Microbial respiration per unit of microbial biomass carbon is defined as 'microbial metabolic quotient' (qCO<sub>2</sub>), which is used as the measure of eco-physiological status of soil microbes (Ashraf et al. 2022). Besides, it reflects the quantum of organic matter decomposition and mineralization, microbial activity and hence can be used for microbial biomass assessment (Jones and Haynes, 2011). The microbial metabolic quotient is affected by microbial community structure, microbial biomass, microbial metabolic processes associated with their relative abundance and distribution, and basal respiration (NRM, 2008), which in turn regulated by several factors such as nutrient availability and quality (Banning et al., 2011), soil texture and different hydrological regimes (NRM, 2008), temperature (Ke et al., 2021), soil physico-chemical properties and the existence of heavy metal pollutants (Jones et al., 2009). High qCO<sub>2</sub> value indicates rapid turnover of microbial biomass in disturbed red mud pond. Several studies reported that due to natural weathering and plant colonization, the qCO<sub>2</sub> value tends to decline, which suggested the efficient carbon utilization and establishment of stable microbial communities (Santini et al., 2015; Li et al., 2016).

## 2.5 Enzyme activities

The biochemical reactions brought about by the catalytic contribution of different enzymes from microbial origin decompose soil organic matter, humification, nutrient cycling, nutrient turnover, decomposition of xenobiotics and release of nutrients through complex and interacting mechanisms (Meena et al., 2022), which respond rapidly to environmental changes (Daunoras et al., 2024) and often used as suitable indicators to predict soil health (Xue et al., 2016b). Enzyme catalyzed reaction may be intracellular or extracellular, which is stabilized by interactions with organo-mineral complexes, allowing them to remain active for long period. The persistence of carbon pool is primarily controlled by extracellular enzymes released by soil microbes, which influence soil properties, mineralization (Jiang et al., 2024), microbial metabolism and ecosystem functioning (da Silva et al., 2022).

The enzyme activity is influenced by organic carbon and microbial biomass pool, which are often used for the comparison of different land use with varying soil organic matter content (Bisswanger, 2017). The proliferating microbes associated with enzyme activities play significant role in microbial mediated soil processes and delivery of essential ecosystem services (Ke et al., 2021) attributed to human-induced variation in abiotic and biotic factors in soil (Courtney et al., 2014). Assessment of enzyme activities in nutrient cycling is widely recognized as a potential indicator for determining the impacts of land use conversions and management practices on soil health (Guo et al., 2024). Nevertheless, soil pH has direct impact on biochemical reactions involving different soil enzymes (Jones et al., 2012). Therefore, the coexistence of multiple plant species with progressive vegetational development and their diversity over time supported by litter decomposition and root activity can increase the complexity of microbial community structure and activity in red mud spoil (Wu *et al.*, 2020; Dong et al., 2024), which in turn stimulate enzyme activities due to the increasing heterogeneity in available soil nutrients. Further, the changes in microbial community structure are

likely to impact the functional integrity of soil, as microbial communities influence the potentiality of soil subsystems through the enzyme mediated substrate catalysis.

Amylases are involved in cellular metabolism of microbes by hydrolyzing starch, glycogen and polysaccharides to produce oligosaccharides, polymers of glucose units (Paul et al., 2021). Similarly, invertase hydrolyses sucrose into glucose and fructose (Gracida-Rodriguez et al., 2006). Invertase enzyme is important because it releases sugar units that are used by microorganisms. The hydrolase activity is directly involved in the mineralization of soil organic matter influencing nutrient cycling (Dey, 2022). Soil proteases produced by bacteria are extracellular enzymes, which degrade proteins and release  $\text{NH}_4\text{-N}$  prerequisite for maintaining nitrogen cycle (Courtney et al., 2014). Protease activity involves N-mediated biochemical processes in soil including nitrogen mineralization that regulates the plant available nitrogen and plant growth. Protease activity plays key role in microbial ecology studies, because the enzymatic conversion of substrate is independent of the extent of microbial activity (Dey and Paul, 2021). Protease activity significantly decreases with low moisture content (Fourrier et al., 2021).

Soil enzymes especially urease, phosphatase and dehydrogenase offer comprehensive insights into soil function as they catalyze a wide range of reactions essential for various biological processes (Dey, 2022). Soil ureases are extracellular enzymes of microbial origin, which act on the (C-N) bonds other than peptide linkages (Neemisha and Sharma, 2022) and hydrolyze urea to form  $\text{NH}_3$  and  $\text{CO}_2$ , which regulate nitrogen cycle (Zhang et al., 2020). Soil urease activity accounts 63% of the total enzyme activity and hence can be used as sensitive biological indicator reflecting the variation in available N in different soil profiles induced by external factors (Martinez-Salgado et al., 2010).

Phosphatases are diverse group of enzymes that catalyse the hydrolysis of esters and anhydrides of phosphoric acid. Production of phosphatases is

mostly induced under limited conditions, which causes the release of phosphate group from monoesters for microbial uptake. Two phosphatases are usually estimated such as (i) monoesterases that are highly active in acidic soils and (ii) alkaline phosphatases and diesterases that include bis-phosphatases, which are active in alkaline soils. Apart from being a suitable biomarker of soil quality assessment, the phosphatase activity correlated well with soil P stress for plant growth (Wu et al., 2024). Secretion of acid phosphatase from plant roots increases phosphate availability (Wu et al., 2021). The negative correlation between phosphorus availability and phosphatase activity is due to negative feedback effect of phosphorus concentration on enzyme activity (Di Carlo et al., 2019). The variation in phosphatase and urease activity is correlated with microbial community structure (Jones et al., 2009).

Dehydrogenases belong to oxidoreductase group of enzymes, which catalyze the oxidation of organic matter by separating two H-atoms into either nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate. Dehydrogenase activity is used as the indicator of overall microbial activity (Garau et al., 2007; Mishra et al., 2017) and oxidative index of cell (Kujur and Patel, 2014), because it occurs intracellularly in all the living microbes that is linked with the microbial respiratory processes. Dehydrogenases typically function as intracellular enzymes in soil and offer valuable insights into the biochemical processes crucial for maintaining soil quality. The dehydrogenase activity exhibits closer relationship with the diversity of microbial community structure in different soil profiles (Paul et al., 2021).

## **2.6 Enzyme kinetic indices**

Enzyme activities are influenced by multiple factors that can directly or indirectly affect their kinetics, thermodynamics and expression beyond the intrinsic traits of the microbiome and the enzymes they encode (Paul and Frey, 2023). These activities are not only influenced by inherent properties of enzymes and microbiome

but also altered by external variables such as heavy metal content, pH and nutrient availability (Gabarrón et al., 2019). The microbe-plant interactions, soil properties and environmental conditions influence the enzyme expression, turnover, mobility and substrate accessibility. The rate-limiting steps in soil organic matter decomposition are primarily catalyzed by microbial exoenzymes, which depolymerize the residues into lower molecular weight compounds that assimilated by both plants and microbes (Daunoras et al., 2024). Thus, the kinetic properties of exoenzymes are fundamental determinants of soil organic matter turnover, nutrient availability, soil stability and their responses to environmental changes (Purich, 2010; Wang and Allison, 2019).

Kinetic studies offer valuable insights into the soil condition, behaviour, origin, existing status and catalytic efficiency of enzymes (Davidson et al., 2006). Kinetic indices ( $V_{max}$  and  $K_m$ ) are found to be constant for an enzyme under defined experimental condition, but may vary independently, which are used for the characterization of free enzymes. Besides, the kinetic indices ( $V_{max}$  and  $K_m$ ) are used as sensitive biomarkers for determining the catalytic efficiency in terms of microbial activities representing the quantity and affinity of different enzymes respectively (Reddy, 2007).  $V_{max}$  of enzyme catalysed reaction implies splitting velocity representing the rate of dispersion of enzyme-substrate complex into enzyme and resulting products reflecting conjunction affinity between enzyme and substrate (Kuby, 2019). Besides, the  $K_m$  value provides useful information regarding the endurance of enzyme-substrate complex in soil. However,  $K_m$  is a measure of the apparent affinity of an enzyme for its substrate and is independent of enzyme concentration. Smaller  $K_m$  value indicates higher affinity for the substrate (Karaca et al., 2011). The efficiency of the enzymes to decompose substrate at low concentration is directly related to their  $K_m$  value (Bisswanger, 2017). When the  $K_m$  is having close proximity to substrate concentration, the affinity for substrate binding provides

valuable information regarding its adsorption level or enzyme accessibility (Mbira et al., 2024)

The ratio of  $V_{max}/K_m$  is a key indicator of the formation and dispersion of enzyme-substrate complex that serves as the measure of an enzyme's catalytic efficiency. Higher  $V_{max}/K_m$  value suggests that the dispersion of enzyme-substrate complex occurs faster compared to its formation (Kujur and Patel, 2014). These properties represent microbial evolutionary adaptations and trade-offs related to supply and/or demand of resources associated with distinct ecological niches (Naykodi et al., 2024). Consequently, the effective kinetics of mixed enzyme pools in complex environment are emergent properties, which reflect not only the summation of traits from distinct isozymes, but also the direct and indirect interactions between enzymes and environment (Gianfreda and Rao, 2019). Soil enzyme activities and its kinetic indices are key regulators of ecosystem functioning and have been studied as sensitive biomarkers of soil quality. These studies primarily focused on organic matter decomposition, nutrient availability and microbial activity (Peacock and White, 2017).

### **3. Microbial community structure**

#### **3.1 Microbial community composition**

Microbial communities are crucial for biogeochemical processes by virtue of their number, relative abundance, distribution, and metabolic activities (Sahu et al., 2017; Dong et al., 2022). The phylogenetic diversity and microbial metabolic activities within any environmental sample reveal their diverse existence (Bier et al., 2015) and varied metabolic states (growing, active, dormant, deceased), which suggested that 'who is present' is not a proxy for 'who is active' (Bier, 2016). However, terrestrial ecosystem is highly disturbed by human interventions leading to decline in biodiversity. Ecological restoration primarily focuses on the aboveground plant community undermining the role of soil parameters (Schmalenberger et al.,

2013). Neglecting the relationship between aboveground and belowground communities deteriorate the effective restoration of soil quality (Ponge, 2013). Therefore, the attention should be given to explore the role of microbial communities influencing soil quality and ecosystem functionality (McKinley, 2019).

Ecological restoration in red mud spoil is a complex process which involve restoring microbial community and transforming the hostile environment into more stable and productive ecosystem (Chao et al., 2022). It is a long-term process to reestablish pre-disturbance complexity and diversity of microbial communities including recovery of species richness and ecological functions (Moreno-Mateos et al., 2020; Wu et al., 2021; Pedrinho et al., 2024). Being ubiquitous in nature and sensitive to environmental changes, the microbial communities are gaining recognition as sensitive biomarkers for tracking disturbances and restoration progress (Lewis et al., 2010; Dong et al., 2023). The contribution of microbial communities to the metabolic processes performed about 90% of soil functions including the decomposition of organic matter, nitrogen fixation, increasing the bioavailability of nutrients to plants, humification, active or passive release of  $CO_2$ , biogeochemical cycling and plant growth (Condron et al., 2010; Banning et al., 2011; Nannipieri et al., 2017; Wu et al., 2021).

Understanding the microbial community dynamics and their interactions with biotic and abiotic factors is crucial for effective monitoring of red mud spoil restoration (Qu et al., 2024). Microbial community structure is shaped not only by extreme environmental factors such as alkalinity and heavy metal toxicity but also by the interactions among microbial taxa (Xu et al., 2022). Such interactions play crucial role in stabilizing the microbial community structure that enhance ecosystem resilience and mitigate the impact of both natural and induced disturbances towards ecosystem functioning (Krause et al., 2014; Pradhan et al., 2024).

Microbial community composition varies in accordance with the physiological and nutritional status of the soil profiles along with environmental factors such as textural composition (Kujur and Patel, 2014), aggregate size (Naylor et al., 2022), temperature (Zhou et al., 2014), moisture and soil type (Nkongolo and Narendrula-Kotha, 2020), salt stress (Rath and Rousk, 2015), soil pH (Rousk et al., 2010; Zhalnina et al., 2015), heavy metal toxicity (Garau et al., 2011) and the level of organic C, total N and extractable P (Dey and Paul, 2021). Besides, the relative distribution of microbial communities in degraded environments is governed by the interactions between plant, climate and soil management practices (Bray et al., 2018), soil depth contributes to community assemblages and biomass (Courtney et al., 2014), plant inputs from litter and root exudates, vegetation development and enzyme activities in soil profiles (Zhu et al., 2024). The distribution and activity of microbial communities in degraded red mud spoil and establishment of functional ecosystem are also influenced by climatic and edaphic conditions (Bian et al., 2022; Avery et al., 2022).

Further, soil with higher microbial diversity is the characteristic features of fertile ecosystems, whereas the degraded soil is characterized with low microbial diversity that hardly responds to environmental changes (Krause et al., 2014). Such disturbed environments limit the overall microbial diversity enabling the growth of alkaliphiles, halophiles and metal-tolerant microbes that thrive to survive under extreme conditions (Naykodi et al., 2024). However, healthy soil with well-balanced microbial community is beneficial for supporting plant growth and prevention of plant diseases (Huang et al., 2022). Organic amendments shift the microbial community composition and functional capacity through the changes in taxonomic richness (Xue et al., 2024), microbial biomass activity, microbial community composition and increases in active copiotrophic taxa (Miura et al., 2023). Several studies highlighted the relationships between physico-chemical properties and microbial community structure demonstrating the role of microbial

community in ecological restoration of red mud spoil (Lockwood et al., 2015; Ke et al., 2021). High-throughput DNA sequencing and metagenomics studies have been proven valuable for monitoring the shifts in microbial community structure (Luo et al., 2023; Wang et al., 2024). Therefore, the assessment of microbial community structure has attracted considerable research attention, which is prerequisite to determine the progress of red mud spoil genesis and ecosystem functioning (Santini et al., 2021).

### **3.2 Phospholipid fatty acid (PLFA)**

Microorganisms owing to their cellular abundance and chemical diversity contain lipids and nucleic acids that are considered as the promising constituents for investigating microbial community structure. Being a major cellular component constituting varieties of structurally diverse and functionally distinct compounds, the variation in fatty acid composition between microbes can be exploited, which reveal the shift in microbial community structure among soil profiles (Hou et al., 2018). The useful biomarkers are membrane lipids and their related fatty acids as they are essential components of all living cells exhibiting greater structural diversity coupled with relatively higher biological specificity. Changes in the specific microbial population are known to occur even when the total microbial community size remains unchanged (Di Carlo et al., 2019), which suggested its usefulness as highly sensitive biomarker of disturbances and assessment of ecological restoration over time and space.

Phospholipid fatty acids (PLFA) are the major cell membrane constituents in which their polar head groups and ester-linked side chains vary in composition among the microbial groups of different origin. These compounds undergo rapid degradation upon cell death and not found in storage lipid (Condrón et al., 2010) and hence the total amount of PLFAs is an important indicator of the biomass of living microorganisms (Peacock and White, 2017). Phospholipid fatty acids (PLFAs) are widely used in microbial ecology studies as the chemotaxonomic markers of soil

microbes. Phospholipid fatty acid (PLFA) analysis is used to determine the viable microbial biomass and the nutritional/ physiological status of soil profiles (Kujur and Patel, 2014).

PLFA analysis provides an effective culture-independent approach for fingerprinting microbial communities. The assessment of PLFAs has been widely used to characterize microbial community structure, investigate the changes in microbial communities in different land uses (Li et al., 2019) and heavy metal-contaminated soil (Schmalenberger et al., 2013). Some PLFAs are specific to a single microbial taxon and therefore can be used as taxonomic biomarkers albeit with low taxonomic resolution (Lewe et al., 2021). Membrane lipids are affected by either changes in PLFA composition of cell membranes (phenotypic plasticity) or alternation in PLFA profiles due to the shift in microbial community structure. In addition to the differentiation of taxonomic groups, certain fatty acids and ratios of fatty acids are used to assess the physiological state of microbial communities (Willers et al., 2015a). The composition of PLFAs can be compared with the PLFA pattern of known microbes, which can be used for identification of microbes. Though PLFA profiling does not give actual species composition but instead gives an overall picture of the microbial community structure. PLFA analysis provides a broad-spectrum diversity index for the assessment of microbial community structure at phenotypic level and robust tool to discriminate microbial communities based on their origin and management practices (Bhattacharjya et al., 2024).

Several appealing features of PLFA profiling reinforce their use in microbial ecology studies suited for ecological assessment, because of their quick response to environmental disturbances and anthropogenic activities reflected through the shift in microbial community structure and PLFA composition in microbial membrane (Frostegård et al., 2011). The advantage of PLFA analysis compared to molecular methods is that it produces quantitative outputs of the PLFAs, which can be applied as proxies for the biomass contributed by

microbial groups affiliated with their respective biomarkers (Willers et al., 2015b), and the ratios of microbial groups with specific biomarkers can be used to evaluate the shift in microbial community structure. In addition, the fungal-to-bacterial biomass ratio serves as the critical indicator of microbial community status across different soil profiles, which offer an efficient tool to differentiate disturbed and undisturbed ecosystems by illustrating management practices and their impacts on microbial dynamics, which provide insights into soil health and ecosystem functionality. An increase in fungal-to-bacterial ratio indicated the improvement in nutrient cycling that is useful in promoting ecological restoration of red mud spoil (Paul et al., 2021). Due to the sensitivity of PLFA biomarkers, they respond to relatively rapid shift in the environment (Willers et al., 2015a; Morris et al., 2023).

### 3.3 Community level physiological profiling (CLPP)

Soil microbes characterized by metabolic heterogeneity encompass wide range of bacterial mediated pathways and diverse microbial processes (da Silva et al., 2022). Therefore, from an ecological perspective, it is important to determine the microbial metabolic profile in disturbed ecosystems such as red mud ponds (Dong et al., 2022). One of the tools appropriate for the analysis of the functional diversity of microbial communities is the community level physiological profile (CLPP), which is also known as the BIOLOG system (Feigl et al., 2017). Occurrence of higher microbial diversity and difficulties in culturing microorganisms necessitate the use of culture-independent approach through community level physiological profiling to differentiate microbial communities. The term community level physiological profiling (CLPP) was first coined by Lehman *et al.* (1995) to characterize heterotrophic microbial communities based on sole carbon source utilization patterns (CSUPs) (Garland et al., 2007), which demonstrated application of BIOLOG™ EcoPlates to differentiate microbial communities. BIOLOG™ EcoPlate features 96

wells containing 31 different carbon substrates that support microbial growth and blank control with no carbon source for endogenous respiration. BIOLOG™ method compares functional diversity of microbial communities in undisturbed and disturbed soil profiles rather than describing the microbial community structure (Wu et al., 2024).

CLPP involves direct inoculation of samples and the resulting responses provide information about mixed microbial communities and their functional adaptations (Insam and Goberna, 2004). Inoculation of sample lead to production of NADH *via* cellular respiration that reduces tetrazolium dye (colourless) to formazan (purple), which is used as indicator of respiration using carbon source and cell viability (Braissant et al., 2020). The formation of coloured formazan due to microbial growth provides the overall microbial community fingerprints based on substrate utilisation profiles (Pretson-Mafham et al., 2002). BIOLOG™ microplate detects the utilisation of specific carbon source by the existing microbial communities by average well colour development (AWCD) (Fourrier et al., 2020). Additionally, Shannon diversity index (H') evaluates the physiological diversity of the bacterial communities. Microbial diversity is influenced by both richness (number of positive responses) and evenness (variation in colour development among wells) of responses (Feigl et al., 2017) which are sensitive enough to determine the shift in microbial community structure and functional diversity (Ujaczki, 2017).

Thus, CLPP reflects (i) overall rate of colour development, (ii) microbial diversity, and the pattern or relative rate of substrate utilisation (Lewis et al., 2010). CLPP of environmental samples based on the pattern of substrate utilisation provides rich data set to determine soil functional status and associated diversity among the microbial communities over time (Jones et al., 2012). The functional diversity can be correlated with taxonomic diversity as it is unlikely that a single genotype can express so much plasticity in carbon source utilisation (Papp, 2016). Microbes are primarily responsible for the degradation and detoxification of environmental contaminants and

hence the changes in microbial community composition or activity might have immediate or long-lasting effects on ecosystem functioning. Microbial populations are much less abundant in contaminated soil compared to non-contaminated soil (Tobor-Kaplon et al., 2005). Level of heavy metal contaminants influences not only population size, but also the physiological activity of soil microbial communities (Wang et al., 2007). The long-term metal stress within soil microbial communities can lead to changes of the genetic structure of microbiome and decrease its diversity (Ma et al., 2022). Several studies revealed the effective use of CLPP technique to explore the physiologically active microorganisms in heavy metal contaminated soil (Garau et al., 2007; Feigl et al., 2017). Application of BIOLOG system is an indirect technique for profiling the changes in carbon metabolism of microbial communities during ecological restoration (NRM, 2008).

The community level physiological profiling is a rapid, sensitive, reproducible, ease to use and relatively inexpensive community level approach to correlate microbial functional diversity with various environmental factors following catabolic traits (Wallis, 2011). Additionally, the CLPP is advantageous over the isolate based culture techniques in which it eliminates the need for sampling few isolates and basing inferences about the whole community structure (Weber and Legge, 2010). Community level approach provides sensitive and ecologically meaningful measure of heterotrophic microbial communities rather than relying on the determination of changes in individuals that may not equate to meaningful shift in microbial community function (Santini et al., 2015). Besides, the CLPP approach has the ability to detect the synergistic relationship based on their interactions among microbial communities over time, which can not be shared by any other molecular techniques including the genetic analysis and fatty acid profiling.

### 3.4 Metagenomic approach

Microbial community structure influence soil quality and sustainability. The diverse and complex nature of microbial communities within soil ecosystems present significant challenges for soil microbiology research. The traditional culture-dependent strategies are insufficient for capturing the full extent of microbial diversity, as many of the soil microbes are difficult to cultivate under laboratory conditions. Metagenomic tools have emerged as powerful solutions to allow precise identification of microbial taxa. The key approach used for assessing microbial community composition, population dynamics and diversity in disturbed red mud ponds is based on the high-throughput 16S rRNA gene amplicon sequencing (Kumaishi et al., 2022). Metagenomic studies based on the Next-generation sequencing (NGS) is highly valued for its ability to analyze microbial composition and diversity comprehensively due to their reduced costs, faster sequencing speeds, multiplexing capabilities and generation of extensive data that enable deeper understanding of microbial diversity (Maljkovic et al., 2020; Garg et al., 2024). It has successfully resulted in the identification of few most uncommon microbes in a disturbed ecosystem (Maza-Márquez et al., 2021). Metagenomic approaches offer a complete view of microbial diversity, taxonomic structure, functions and their interactions with soil processes that revealed structural and functional shifts in microbial communities in red mud spoil over time (Dong et al., 2021; Maza-Márquez et al., 2021; Feigl et al., 2024). Several studies revealed the changes in microbial diversity with notable reduction in the beneficial microorganisms and relatively higher relative distribution of the metal-tolerant species (Peng et al., 2024; Deng et al., 2024).

Metagenomic techniques provide insights into the activity, function and diversity of soil microbes in red mud spoil that is useful in identifying new microorganisms and genes involved in

establishing stable ecosystem (Krishna et al., 2014; Dey and Paul, 2021). It provides insights into the role of microbes in nutrient cycling, plant-microbe interactions and their responses to different environmental stressors. DNA-based studies of specific environments offer deeper understanding of microbial communities and their potential in transforming hostile environment into sustainable ecosystem. Metagenomic studies are helpful in assessing the impacts of pollution, climatic change and land-use alterations on soil microbiota, which are essential for developing sustainable soil management strategies.

Marker gene-based experiments and whole genome shotgun sequencing are the two major approaches in high-throughput metagenomic studies (Bansal et al., 2018). In marker gene-based experiments, each genome found in the sample as 16s rRNA for bacteria and archaea or 18s rRNA for fungus is amplified using PCR followed by sequencing. Operational taxonomic units are generated from the sequences, which are then compared among spoil samples of different age series. Several studies revealed the presence of alkaliphilic microbial communities in red mud spoil using 16s rRNA sequencing (Jones et al., 2009; Miura et al., 2023; Wu et al., 2024). Whole genome sequencing involves the sequencing of entire genome of isolated microbial communities. It is useful to assess metabolic processes and functional gene processing of microbes in presence of different environmental stressors. Whole genome sequencing involves soil DNA extraction, construction of clone library and screening. Together, the metagenomic studies provide insight into diversity, functional capabilities and microbial adaptation strategies in distinct ecological niches (Tiwari et al., 2021). Several metagenomic studies revealed the shift in microbial community structure from alkaliphilic to neutrophilic (Wu et al., 2020; Paul et al., 2021) indicated the establishment of stable functional ecosystem.

## 4. Conclusion

Huge quantity of red mud spoil being disposed in BRDAs pose serious environmental challenges, which require proper management and reclamation. This review highlighted the multifaceted issues associated with red mud including its disposal, utilisation and spoil genesis due to variation in spoil properties with increasing age of bauxite residue. Variation in physico-chemical, microbiological and biochemical properties over time induce shift in microbial community structure promoting red mud spoil genesis. Emphasis on microbial mediated ecological restoration can further improve soil quality and promote biodiversity in areas affected by red mud disposal. The specific microbial species tolerant to extreme alkalinity and metal tolerance dominate red mud spoil over time facilitating toward ecological processes. Advances in recycling technologies and soil amendment applications offer promising pathways for reducing the environmental footprint of red mud. Therefore, by leveraging innovative technologies and sustainable practices, prospects for red mud spoil can be significantly improved. With advance technology, red mud posing several environmental risks could be transformed into valuable resource for various applications contributing towards sustainable development and environmental stewardship.

## 5. Conflict of Interest

None

## 6. Acknowledgments

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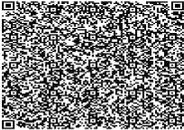
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