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Research Article

SOIL ENZYME ACTIVITIES AND IT'S KINETICS USED AS INDEX OF MINE SPOIL GENESIS IN CHRONOSEQUENCE IRON MINE OVERBURDEN SPOIL

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ABSTRACT

Iron mining activities disrupt the original landscape resulting huge mine spoil dumped in the form of overburden, which alter ecosystem function. Monitoring of mine spoil is pre-requisite to predict soil quality with appreciable potential in contributing towards sustainable soil management. The study addressed the assessment of enzyme activities that reflect soil quality with concomitant ecosystem functioning and provide empirical evidences in support of mine spoil genesis explaining the variability in microbial community composition among different age series mine overburden spoil over time. The study represents holistic approach using quantitative biomarkers such as enzyme activities (amylase, invertase, protease, urease and dehydrogenase) and their kinetic parameters (Vmax, Km and Vmax/Km), which established linkages between the fluxes driving nutrient pool for its worth in sustainable soil management. Comparative assessment revealed gradual improvement in enzyme activities from fresh mine spoil to 25yr old mine spoil. The study indicated consistent increase in Vmax with concomitant decrease in Km with increase in age of mine spoil reflecting mine spoil genesis. The shift in Vmax/Km revealed the variation in microbial community composition with changes in enzyme activities. Stepwise multiple regression analysis was performed to quantify the contribution of physico-chemical properties influencing enzyme activities. Principal component analysis can able to discriminate seven different mine spoil and NF soil into independent clusters based on their enzyme activities and kinetic parameters. The study clearly revealed that enzyme activities and kinetic parameters can be considered as soil quality descriptors for the assessment of mine spoil genesis promoting the progress of restoration.

INTRODUCTION

Soil is a heterogeneous microhabitat regulating plant productivity, maintenance of biogeochemical cycles and degradation of organic xenobiotic compounds by the microbial activity (Avidano *et al.,* 2005). The microbiological and biochemical aspect of soil status has been considered as the pristine and sensitive indicator of soil restoration processes in different ecosystems (Dick and Tabatabai, 1992; Dick, 1994). Several bioindicators of soil health and quality have been reviewed (Trasar-Cepeda *et al.,* 2000; Anderson, 2003). Among them, the microorganisms are the efficient bioindicators, which play an important role in decomposition and mineralization of organic matter by producing various enzymes (Burns, 1982) and also respond quickly to the environmental changes. Therefore, the modifications in soil enzyme activities reflect the alterations in microbial activity, community structure and environmental conditions (Avidano *et al.,* 2005). Thus, it is pertinent to study enzymatic activities, which provide information about the release of nutrients in soil by the degradation of organic matter and microbial activity in mediating various bio-transformations in soil.

Enzymes are macromolecular biological entities, which catalyses various biochemical reactions in terrestrial ecosystem (Tabatabai and Dick, 2002; Kuscu and Karaoz, 2015). Soil enzymes are derived primarily from microbial sources and resulting soil biological activity. Besides, the soil enzyme activity is used to determine soil microbiological characteristics, which is pre-requisite for the assessment of soil quality and fertility (Askin and Kizilkaya, 2005). Hence, the soil enzyme activities can be considered as index of soil fertility due to its involvement in mineralization process of organic matters (Schoenholtz *et al.,* 2000; Sajjad *et al.,* 2002; Tabatabai and Dick, 2002; Cladwell, 2005). In addition, the enzymatic study provides valuable information about their origin, existing nature and catalytic properties of soil enzymes (Perez-Mateos and Gonzales Carcedo, 1985). Several investigations have been suggested that the soil enzyme activities were significantly correlated with soil physico-chemical properties (Dodor and Tabatabai, 2002; Taylor *et al.,*

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2002; Kujur *et al.,* 2012); microbial community structure (Waldrop *et al.,* 2000; Kourtev *et al.,* 2002), vegetational pattern (Waldrop *et al.,* 2000; Sinsabaugh *et al.,* 2002), disturbances (Garcia and Hernandez, 1997; Kujur *et al.,* 2012) and succession (Baldrian *et al.,* 2008). The periodic assessment of the shift in soil enzyme activities provides useful index of changes in soil quality and fertility (Dick, 1997; Bucket and Dick, 1998; Antonious, 2003), microbial activity (Visser and Parkinson, 1992; Kuscu and Karaoz, 2015). Therefore, it can be potentially used to monitor and assess soil restoration in perturbed ecosystem (Nannipieri *et al.*, 1990).

Soil enzyme activities can be used to describe the relative abundance and metabolic activities of soil microbes, whereas the kinetic parameters indicate their origin, existing status, substrate affinity of the enzymes and other catalytic activities (Garcia-Gil *et al.,* 2000; Zhang *et al.,* 2009). Kinetic parameters (Vmax and Km) are used to characterize free enzymes in solution (Marx *et al.,* 2005). Maximum reaction rate (Vmax) of an enzyme catalyzed reaction implies the splitting velocity or rate of dispersion of the enzyme-substrate complex into enzyme and reaction products, which reflects the conjunction affinity between the enzyme and substrate. The higher and lower Vmax value can be used as an indicator to designate the faster or slower enzyme mediated catalysis respectively. Besides, the Vmax and Km of an enzyme express the quantity of an enzyme and substrate affinity respectively (Marx *et al.,* 2005; Davidson *et al.,* 2006). However, the Michaelis constant (Km) represents the endurance of an enzyme-substrate complex, which is associated with substrate. The Km is independent of enzyme concentration and kinetically reflects the apparent affinity of enzyme for the substrate. In addition, the Km influences enzyme activity at low concentration (Davidson and Janssens, 2006; Davidson *et al.,* 2006). Hence, smaller the Km value, the greater will be the affinity for substrate (Masciandaro *et al.,* 2000). Many investigations have dealt with the kinetic properties of enzymes (Masciandaro *et al.,* 2000; Zhang *et al.,* 2009; 2010; Juan *et al.,* 2010). Further, the Vmax/Km provides information of about the enzyme-substrate complex and the comparisons of dispersion of this complex in soil. The higher value of Vmax/Km suggests faster rate dispersion of enzyme-substrate complex than its information (Ekberli *et al.,* 2006; Kizilkaya *et al.,* 2007; Kujur and Patel, 2014). The Vmax/Km value of enzyme was reported to be influenced by soil physico-chemical properties (Garcia *et al.,* 1993; Maharana and Patel, 2013; Kujur and Patel, 2014) and source of substrate availability (Kizilkaya and Bayrakli, 2005), which indirectly alter microbial activity. Any change in microbial indices in terms of enzyme activities and its kinetic parameters shows rapid response to both natural and anthropogenic disturbances.

Soil enzymes catalyses various important reactions prerequisite for the life processes of soil microorganisms and soil structure stabilization. The enzymes mediate the conversion of soil organic matter to maintain the balance in soil environment and influence ecosystem functioning (Dinesh *et al.,* 2004; Caldwell, 2005). In the present study, five different enzymes were selected such as amylase, invertase, protease, urease and dehydrogenase. The bacterial populations are considered as the predominant producer of amylases (Pandey *et al.,* 2000; Taylor *et al.,* 2002), which are starch degrading enzymes (Khajeh *et al.,* 2006). Soil invertase hydrolyzes glucose into α-D glucose and β-1 fructose and serves as an important diagnostic clue to soil functioning (Tabatabai, 1982; Shi *et al.,* 2008). The proteases are proteolytic enzymes that hydrolyze the peptide bonds of long polypeptide chain (Anjaneyulu *et al.,* 2011; Subrahmanyan *et al.,* 2011). Proteases play the significant role in C and N mineralization (Ladd and Jackson, 1982) and thereby influencing the overall microbial community structure in soil (Sims and Wander, 2002; Sims, 2006). Soil urease is secreted by the urolytic microorganisms as well as root exudates (Dkhar and Mishra, 1985; Palma and Conti, 1990), which play an important role in mineralization (Gianfreda *et al.,* 2005) and transformation of urea to ammonia that is subsequently nitrified by the nitrifying microorganisms (Palma and Conti, 1990; Xiaobin *et al.,* 1995; Sajjad *et al.,* 2002; Salazar *et al.,* 2011). Soil dehydrogenases produced by all microbes are involved in oxidation-reduction reactions linked with the microbial respiratory process (Garcia *et. al.,* 1993; Bandick and Dick, 1999; Cladwell, 2005) and C cycle (Gianfreda *et al.,* 2005). Being intracellular, the soil dehydrogenase activity is considered as an index of endogenous activity (Garcia *et al.,* 1993; Taylor *et al.,* 2002) and efficient indicator of overall microbial activity in terrestrial ecosystems (Pascule *et al.,* 1998; Taylor *et al.,* 2002; Kujur *et al.,* 2012).

Soil enzymes have been reported as the biological fingerprints due to their relationship with soil processes, being operationally practical, sensitive and integrative ease to measure. They are indicative of biological equilibrium, resource availability, microbial community structure, soil fertility and other changes in the biological status of soil. Therefore, the comparative assessment of enzyme activities represents the direct expression of soil microbial community to metabolic requirements. Further, the kinetic characteristics have attracted considerable attention and provide information about the relationship between soil management practices and enzyme activities through enzyme substrate affinity. Therefore, the present study was designed to quantify the variation in soil enzyme activities associated with their kinetic properties in chronosequence iron mine overburden spoil over time, which can be used as indices for the assessment of mine spoil genesis involved in reclamation process.

MATERIALS AND METHODS

Study Site

The present study was carried out in the Thakurani iron mining area at Noamundi (geographical location: 85° 28' 02.61" east longitude and 22° 8' 33.93" north latitude), maintained by M/s. Sri Padam Kumar Jain sponge mines Private Ltd. located in the revenue district of West Singhbhum, Jharkhand, India (Fig 1). The study site is surrounded by a number of new, old and abandoned mine of iron ore overburdens, which were classified according to the time elapsed since inception such as fresh iron mine spoil (IB_0), 2yr (IB_2), 4 yr (IB_4), 6 yr (IB_6), 8 yr (IB_8), 15 yr (IB_{15}) and 25 yr (IB_{25}) respectively. Besides, the nearby forest soil (NF) was selected adjacent to the core iron mining area for comparison. The district experiences semi-arid climate with annual average rainfall estimated to be 1250.43 mm as

compared to the state average of 1340 mm. The mean annual temperature and humidity is around 19.67°C and 20% respectively. The study site is situated away from the mean sea level of about 581m altitude.

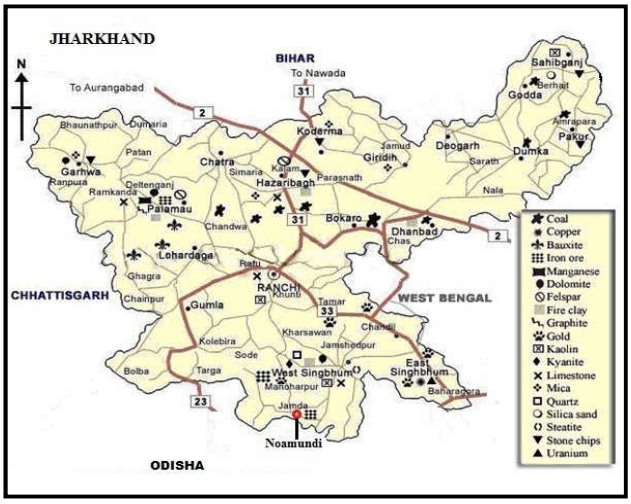

Figure 1 Geographical location and mineral map of the study site at Noamundi, Jharkhand, India.

Mine Spoil Sampling

Sampling was done from seven iron mine overburdens (IB_0, IB_2, IB_4, IB_6, IB_8, IB_{15} and IB_{25}) and nearby forest within a peripheral distance of 10 km from the core iron mining area. During sampling, each site was divided into 3 blocks and five mine overburden spoil samples were collected randomly from 0-15cm soil depth by digging pits of (15x15x 15) cm3 size. The samples collected from each block were referred as 'sub-samples', which were thoroughly mixed to form one 'composite sample' obtained from each overburden. Similar strategies have been followed to obtain three composite samples from each site. The samples were subjected to sieving (0.2 mm mesh size) and stored at 4ºC until analyzed.

Soil Enzyme Activities

Amylase activity of different iron mine overburden spoil as well as nearby NF soil samples were determined in adaptation to the procedures described by Somogyi (1952) and Roberge (1978) by taking absorbance at 540 nm with starch as substrate and incubated at 30°C for 24hr. The invertase activity was determined by spectrophotometric method by taking absorbance at 540 nm (Ross, 1983) by using sucrose as substrate and incubated at 37°C for 24hr. Protease activity was determined by spectrophotometric method by taking absorbance at 700 nm (Ladd and Butler, 1972) with sodium caseinate as substrate. Urease activity of different age series iron mine overburden spoil as well as NF soil were determined by titration method using 0.005N NH_2SO_4 with boric acid indicator (Tabatabai and Bremner, 1972) using urea as substrate. Dehydrogenase activity was estimated through the reduction of 2, 3, 5-triphenylotetrazolium chloride (TTC) as an electron acceptor to red-coloured triphenyl formazon (TPF), which was determined by spectrophotometric method by taking absorbance at 485 nm (Nannipieri *et al*., 1990; Alef and Nannipieri, 1995).

Kinetic Parameters

Kinetic parameters (Km and Vmax) of soil enzyme activities were determined by taking five different substrate concentrations individually. The substrate concentrations used for amylase, invertase, protease, urease and dehydrogenase activities were ranged from 5 to 50 mM, 10 to 100 mM, 1 to 10 mM, 5 to 45 mM and 10 to 90 mM respectively. For the estimation of different kinetic parameters, triplicates were taken for each substrate concentration for individual enzyme assay. Soil enzymes follow Michaelis-Menten kinetics despite soil being considered as a discontinuous, structures and heterogeneous system (Nannipieri *et al.,* 2002). The Michaelis-Menten equation linearized by Lineweaver-Burk was used to determine Vmax (maximum velocity), Km (substrate concentration at ½ Vmax) by plotting a graph *i.e.* 1/V against 1/S and estimated by the intercept and slope respectively, and Vmax/Km as kinetic parameters.

$$\frac{1}{V} = \frac{Km}{Vmax} \cdot \frac{1}{[S]} + \frac{1}{Vmax}$$

Statistical Analysis

The data obtained from soil analyses were subjected to simple correlation analysis to test the level of significance of soil physico-chemical properties and soil enzyme activities (amylase, invertase, protease, urease and dehydrogenase) among seven different age series of iron mine overburden spoil as well as nearby forest soil across the sites using SPSS (Version 17.0). Stepwise multiple regression analysis was employed to model the quantitative relationship between different enzyme activities and soil physico-chemical properties in different soil profiles using Minitab 16 software. Principal components analysis (PCA) was performed using Statistrix PC DOS Version-2.0 (NH Analytical software). Redundancy analysis (RDA) was performed to determine the relationship between different soil physico-chemical properties influencing enzyme activities in seven different age series iron mine overburden spoil using Microsoft Excel XLSTAT-2014 (Version 2.03).

RESULTS AND DISCUSSION

Soil Enzyme Activities

Soil enzyme activity is considered as the direct expression of microbial community to metabolic requirements and available soil nutrients. Soil microbial activity is an important constituent for ecosystem functioning as well as resource management in which the interpretation of biological and biochemical trait can be favorable for identifying the impacted ecosystem of iron mine overburden spoil (Harries, 2003). Soil enzyme activity can be used to indicate the intensity of biochemical processes because it is considered to be the major contributor to overall microbial activity (Nannipieri *et al.,* 1990; Bentham *et al.,* 1992; Badiane *et al.,* 2001). Soil enzymes also have the potential to provide a unique integrative biological assessment due to their relationship with soil processes and their rapid response to changes in soil management (Dick, 1997; Badiane *et al.,* 2001; Ndiaye *et al.,* 2000). Hence, it can be considered as important indicator of mine spoil genesis over time (Roberts *et al.,* 1988; Machulla *et al.,* 2005). Several soil processes affect soil quality directly or indirectly, which are related with enzymatic degradation and biosynthesis (Chen, 2003). Therefore, the approaches used to assess soil enzyme activity with their kinetic parameters will provide insights into the linkages between resource availability, microbial community

structure and function with the ecosystem processes (Tabatabai and Dick, 2002; Caldwell, 2005; Kujur and Patel, 2012).

Comparative assessment of soil enzyme activities (amylase, invertase, protease, urease and dehydrogenase) indicated minimal activity in IB_0, which may be due to the reduced microbial population caused by the toxic effect and oxidative stress of mine spoil metal impurities (Kandeler *et al.,* 2000), there interference in osmotic balance and nutrition deficiency (Brookes, 1995). Amylase (α-amylase; E.C.: 3.2.1.1) is starch hydrolyzing enzyme, which hydrolyzes α-1,4-glucan links in polysaccharides containing α-1,4-linked D-glucose units. The study revealed that the amylase activity showed a range of 0.525 µg glucose g^{-1} spoil hr^{-1} to 25.625 µg glucose g^{-1} spoil hr^{-1} with minimum in IB_0 and maximum in IB_{25} (Table 1). However, the amylase activity is quite higher in NF soil (36.333 µg glucose g^{-1} soil hr^{-1}) as compared to different mine spoil. Such variation in amylase activity with respect to different mine overburden spoil in chronosequence may be due to the variation in available soil nutrients (Maharana and Patel, 2013) and diverse microbial community structure (Waldrop *et al.,* 2000; Anjaneyulu *et al.,* 2011). Similarly, the invertase (β-fructofuraanosidase; E.C.: 3.2.1.5) showed progressive increase from 3.641 µg sucrose g^{-1} spoil hr^{-1} (IB_0) to 927.083 µg sucrose g^{-1} spoil hr^{-1} (IB_{25}) across the sites (Table 2). The decrease in amylase and invertase activity is attributable mainly to the declination of enzyme synthesis due to the accumulation of heavy metals and the associated toxic influence on soil microbes inhibiting microbial growth (Lee *et al.,* 2002; Kizilkzya *et al.,* 2004; Gao *et al.,* 2009) thus reducing the synthesis and secretion of enzymes.

Proteases catalyze proteolysis and help in nitrogen mineralization (Gupta and Lorenz, 2010). The protease activity was found to be relatively higher in nearby NF soil (217.512 µg tyrosine g^{-1} soil hr^{-1}) as compared to different age series iron mine overburden spoil (Table 1). Gradual improvement in protease activity from IB_0 (2.515 µg tyrosine g^{-1} spoil hr^{-1}) to IB_{25} (173.755 µg tyrosine g^{-1} spoil hr^{-1}) was due to the progressive improvement in available soil nutrients (Kujur and Patel, 2012) and the distribution of proteolytic bacteria (Sardans *et al.,* 2008; Anjaneyulu *et al.,* 2011; Subhrahmanyam *et al.,* 2011) across the sites over time, which regulates the amount of plant available nitrogen and plant growth.

Besides, the ureases (Urea amidohydrolase; E.C.: 3.5.1.5) belong to soil hydrolases, which act as extracellular enzymes held by the organic and inorganic soil colloids (Dkhar and Mishra, 1985). It helps in the transformation of urea into CO_2 and NH_3. Hence, the emphasis on urease activity has been given in order to evaluate the N supply to plants, because large N loss to atmosphere by volatilization process is controlled by these enzymes. It is evident from the study that higher urease activity was exhibited by nearby NF soil (54.502 µg NH_4 g^{-1} soil hr^{-1}). The urease activity showed an increasing trend from IB_0 (2.322 µg NH_4 g^{-1} spoil hr^{-1}) to IB_{25} (43.752 µg NH_4 g^{-1} spoil hr^{-1}) across the sites (Table 1), which may be due to the gradual accumulation of N (Garcia *et al.,* 1993; Kizilkaya and Ekberli, 2008) and the synthesis of urease enzyme by increased microbial population over time (Bandick and Dick, 1999). Besides, the variations in soil physico-chemical properties (soil textural composition, pH, moisture and organic C) were suggested to influence urease stabilization in soil (Kizilkaya and Ekberli, 2008).

Dehydrogenase is an intracellular oxido-reductase group of enzymes, which is considered to be an indicator of oxidative metabolism in soil and consequently affect the overall microbial community (Pascule *et al.,* 1998; Taylor *et al.,* 2002; Cladwell, 2005) with their microbiological activities (Alef and Nannipieri, 1995; Dick, 1997; Kizilkaya and Hepsen, 2007). Therefore, the dehydrogenase activity can be used as the important parameter to assess soil quality to evaluate the degree of regeneration in degraded soil (Gil-Sotres *et al.,* 2005). The dehydrogenase activity was found to be relatively higher in nearby NF soil (4.583 µg TPF g^{-1} soil hr^{-1}) as compared to different iron mine spoil (Table 1). The study indicated gradual improvement in dehydrogenase activity from 0.125 µg TPF g^{-1} spoil hr^{-1} (IB_0) to 3.658 µg TPF g^{-1} spoil hr^{-1} (IB_{25}), which may be due to the gradual deposition of organic matter that support increased microbial activity and biomass in due course of time (Margesin *et al.,* 2000; Nannipieri *et al.,* 2002; Mukhopadhyay and Maiti, 2011).

Kinetics Parameters

Kinetics study of soil amylase activity indicated an increasing trend in Vmax from IB_0 (4.583 µg g^{-1} spoil hr^{-1}) to IB_{25} (45.662 µg g^{-1} spoil hr^{-1}). Higher Vmax value was estimated in NF soil (53.475 µg g^{-1} soil hr^{-1}) as compared to different iron mine spoil (Table 2).

Table 1 Enzyme activities in different age series iron mine overburden spoil in chronosequence ($IB_0 \rightarrow IB_{25}$) as well as nearby forest (NF) soil across the sites.

Enzyme activity	Mine spoil collected from different age series iron mine overburdens							NF soil
	IB_0	IB_2	IB_4	IB_6	IB_8	IB_{15}	IB_{25}	
Amylase	0.525 ±	2.417 ±	4.583 ±	9.917 ±	12.042 ±	18.011 ±	25.625 ±	36.333 ±
(µg glucose/g/hr)	0.131	0. 224	0.336	0.451	0.599	0.605	1.223	1.288
Invertase	3.641±	12.654 ±	36.257 ±	98.339 ±	324.832 ±	670.83 ±	927.083 ±	1106.25 ±
(µg sucrose/g/hr)	0.125	1.167	1.625	5.333	11.083	14.083	15.708	21.625
Protease	2.515 ±	5.163 ±	31.253 ±	53.753 ±	81.253 ±	145.754 ±	173.755 ±	217.512 ±
(µg tyrosine/g/hr)	0.252	0.642	3.825	4.375	6.025	6.215	6.315	7.252
Urease	2.322 ±	4.965 ±	7.501 ±	9.144 ±	12.787 ±	28.323 ±	43.752 ±	54.502 ±
(µg NH_4^+/g /hr)	0.026	0.147	0.385	0.504	1.129	1.143	2.113	2.064
Dehydrogenase	0.125 ±	0.313 ±	0.542 ±	0.708 ±	1.563 ±	2.813 ±	3.658 ±	4.583 ±
(µg TPF/g /hr)	0.012	0.035	0.044	0.042	0.066	0.081	0.093	0.103

Values are expressed in mean ± SD; n = 3.

Similarly, the Vmax value of invertase activity exhibited an increasing trend from IB_0 (8.246 µg g^{-1} spoil hr^{-1}) to IB_{25} (941.272 µg g^{-1} spoil hr^{-1}). However, relatively higher Vmax was observed in NF soil (1143.251 µg g^{-1} soil hr^{-1}). The Km value of amylase activity exhibited a decline trend from IB_0 (63.148 mM) to IB_{25} (18.554 mM), which may be due to the gradual increase in moisture content in IB_{25} (Kujur and Patel, 2012; Maharana and Patel, 2013). Similar trend was exhibited in case of invertase activity, where Km value ranges from 48.675 mM (IB_0) to 15.429 mM (IB_{25}). Further, Vmax/Km value of amylase was estimated to be lowest in IB_0 (0.0725) as compared to IB_{25} (2.4610). Similar trend was also exhibited in soil invertase *i.e.* minimum in IB_0 (0.1694) and maximum in IB_{25} (61.0066).

It is evident from the data that the amylase activity and its Vmax value were positively correlated with clay, WHC, MC, OC TN and EP, but negatively correlated with BD (Table 3). Similarly, the invertase activity and its Vmax value were positively correlated with WHC, MC, OC, TN and EP, but negatively correlated with BD (Table 4). Such variation in soil amylase and invertase activity with respect to different iron mine overburden spoils may be due to the variation in available soil nutrients and gradual accumulation of organic C over time (Kujur *et al.,* 2012; Maharana and Patel, 2013; Kujur and Patel, 2014). Gradual accumulation of soil nutrients promote microbial diversity as well as their biomass (Singh *et al.,* 2007; Kujur and Patel, 2012), which lead to increased microbial enzyme production and hence higher Vmax (Allison and Martiny, 2008; Nemergut *et al.,* 2008; Stone *et al.,* 2011).

In contrast, the relatively lower Vmax value exhibited by IB_0 may be due to the accumulation of toxic heavy metals, which inhibit the growth and proliferation of soil microorganisms (Yang *et al.,* 2006; Gao *et al.,* 2009) thus reducing the synthesis, secretion of enzymes and finally leading to the decrease in soil amylase and invertase activity (He *et al.,* 2002). Besides, the type of organic matter was shown to influence the amylase and invertase activity more than the quantity of organic matter. Further, the substrate diffusion rate influences Km value of enzyme activities in heterogeneous soil system. Stronger is the enzyme-substrate affinity lower is the Km value, which is affected by higher moisture content and water solubility rate (Zhang *et al.,* 2009). Therefore, the potential reason of lower Km value exhibited by nearby NF soil as compared to different age series mine overburden spoil may be due to higher water holding capacity and organic matter content (Zhang *et al.,* 2009). However, the lowest Vmax/Km exhibited by IB_0 may be due to the nutrient deficient situation with extreme dryness in fresh iron mine overburden spoil that limits the solubility and restrict the movement of available organic C as energy source.

The Vmax of protease was found to be minimum in IB_0 (5.594 µg g^{-1} spoil hr^{-1}) to maximum in IB_{25} (194.626 µg g^{-1} spoil hr^{-1}). The Km and Vmax/Km value of soil protease varies from 20.587 mM (IB_0) to 11.262 mM (IB_{25}) and 0.2717 (IB_0) to 17.2816 (IB_{25}) respectively (Table 2). The variation in protease activity among different age series iron mine overburden spoil may be due to the progressive improvement in OC, TN and EP over time (Sardans and Penuelas, 2005; Tischer, 2005; Kujur and Patel, 2012) and the distribution of proteolytic bacteria

Table 2 Kinetic parameters (Vmax, Km and Vmax/Km) of different enzyme activities in seven different age series iron mine overburden spoil as well as nearby forest (NF) soil across the sites.

Enzymes	Kinetic parameters	IB_0	IB_2	IB_4	IB_6	IB_8	IB_{15}	IB_{25}	NF
Amylase	Vmax	4.583	7.947	9.518	12.371	15.666	33.735	45.662	53.475
	Km (mM)	63.148	58.574	52.128	45.354	39.187	28.547	18.554	13.159
	Vmax/Km	0.0725	0.1356	0.1825	0.2727	0.3997	1.1817	2.4610	4.0637
	R^2	0.985**	0.879**	0.961**	0.950**	0.974**	0.962**	0.989**	0.906**
Invertase	Vmax	8.246	16.983	41.965	105.033	338.519	685.845	941.272	1143.251
	Km (mM)	48.675	42.587	38.051	33.548	28.667	21.458	15.429	12.257
	Vmax/Km	0.1694	0.3987	1.1028	3.1308	11.8086	31.9622	61.0066	93.2733
	R^2	0.892**	0.919**	0.911**	0.892**	0.981**	0.957**	0.853*	0.971**
Protease	Vmax	5.594	8.224	35.006	58.062	91.671	151.784	194.626	248.875
	Km (mM)	20.587	18.253	17.059	16.954	16.023	13.524	11.262	10.118
	Vmax/Km	0.2717	0.4505	2.0520	3.4246	5.7212	11.2233	17.2816	24.5972
	R^2	0.894**	0.886**	0.879**	0.943**	0.966**	0.892**	0.934**	0.798*
Urease	Vmax	4.129	7.986	11.778	15.749	21.761	35.587	58.102	71.258
	Km (M)	0.112	0.105	0.094	0.085	0.077	0.051	0.037	0.028
	Vmax/Km	36.8660	76.0571	125.2978	185.2823	282.6103	697.7843	1570.3243	2544.928
	R^2	0.986**	0.953**	0.919**	0.891**	0.887**	0.964**	0.922**	0.959**
Dehydrogenase	Vmax	0.238	0.579	0.784	0.998	1.674	3.152	4.016	5.134
	Km (M)	0.184	0.175	0.162	0.149	0.131	0.089	0.053	0.021
	Vmax/Km	1.2934	3.3085	4.8395	6.6979	12.7786	35.4157	75.7735	244.4761
	R^2	0.995**	0.965**	0.996**	0.988**	0.983**	0.985**	0.970**	0.988**

** Correlation is significant $p < 0.01$, and * correlation is significant $p < 0.05$.

Table 3 Simple correlation between soil physico-chemical properties and amylase activity

Parameter	Clay	BD	WHC	MC	pH	OC	TN	EP	Amylase activity	Vmax	Km	Vmax /Km
Amylase activity	0.992**	-0.970**	0.962**	0.969**	0.939**	0.966**	0.978**	0.947**	1			
Vmax	0.975**	-0.977**	0.949**	0.990**	0.907**	0.994**	0.994**	0.989**	0.978**	1		
Km	-0.970**	0.996**	-0.993**	-0.983**	-0.980**	-0.971**	-0.954**	-0.927**	-0.976**	-0.970**	1	
Vmax/Km	0.964**	-0.892**	0.863**	0.917**	0.824*	0.934**	0.972**	0.955**	0.963**	0.955**	-0.893**	1

**Correlation is significant $p < 0.01$, * Correlation is significant $p < 0.05$ (2- tailed test).

Table 4 Simple correlation between soil physico-chemical properties and invertase activity.

Parameter	Clay	BD	WHC	MC	pH	OC	TN	EP	Invertase activity	Vmax	Km	Vmax/Km
Invertase activity	0.968**	-0.977**	0.962**	0.996**	0.910**	0.999**	0.994**	0.979**	1			
Vmax	0.970**	-0.977**	0.962**	0.995**	0.910**	0.998**	0.995**	0.979**	0.999**	1		
Km	-0.958**	0.993**	-0.992**	-0.972**	-0.988**	-0.955**	-0.932**	-0.902**	-0.954**	-0.954**	1	
Vmax/Km	0.973**	-0.919**	0.895**	0.946**	0.848**	0.960**	0.989**	0.971**	0.968**	0.970**	-0.891**	1

**Correlation is significant $p < 0.01$ (2- tailed test).

(Sardans *et al.,* 2008; Subrahmanyam *et al.,* 2011; Anjaneyulu *et al.,* 2011). The gradual accumulation of pertinacious substrate across the sites was facilitated by the differences in quantity and quality of plant litter inputs and root exudation (Stone *et al.,* 2011) that influence the microbial community structure and alter enzyme activity (Horwath, 2007). Overall process contributed to increased Vmax value in NF soil (Stone *et al.,* 2011). The protease activity and its Vmax showed positive correlation with WHC, MC, pH, OC, TN, EP, where as its Km value showed negative correlation with all tested soil properties except BD (Table 5).

Table 5 Simple correlation between soil physico-chemical properties and protease activity.

Parameter	Clay	BD	WHC	MC	pH	OC	TN	EP	Protease activity	Vmax	Km	Vmax/Km
Protease activity	0.970**	-0.995**	0.981**	0.991**	0.950**	0.986**	0.977**	0.955**	1			
Vmax	0.980**	-0.990**	0.979**	0.990**	0.948**	0.987**	0.984**	0.958**	0.998**	1		
Km	-0.977**	0.987**	-0.967**	-0.974**	-0.964**	-0.966**	-0.954**	-0.937**	-0.977**	-0.977**	1	
Vmax/Km	0.987**	-0.959**	0.940**	0.972**	0.903**	0.978**	0.994**	0.973**	0.979**	0.987**	-0.959**	1

**Correlation is significant p < 0.01 (2- tailed test).

Table 6 Simple correlation between soil physico-chemical properties and urease activity

Parameter	Clay	BD	WHC	MC	pH	OC	TN	EP	Urease activity	Vmax	Km	Vmax/Km
Urease activity	0.983**	-0.963**	0.936**	0.979**	0.898**	0.987**	0.996**	0.988**	1			
Vmax	0.992**	-0.965**	0.948**	0.978**	0.918**	0.983**	0.992**	0.975**	0.997**	1		
Km	-0.966**	0.999**	-0.985**	-0.990**	-0.965**	-0.981**	-0.963**	-0.946**	-0.969**	-0.971**	1	
Vmax/Km	0.967**	-0.892**	0.865**	0.916**	0.829*	0.932**	0.971**	0.952**	0.975**	0.974**	-0.900**	1

**Correlation is significant $p < 0.01$, * Correlation is significant $p < 0.05$ (2- tailed test).

The comparisons of Vmax of urease activity showed similar trend like protease activity *i.e.* progressive increase from 4.129 µg g^{-1} spoil hr^{-1} (IB_0) to 58.102 µg g^{-1} spoil hr^{-1} (IB_{25}) across the sites (Table 2), which may be due to the gradual establishment of vegetation cover over time that check the problem of runoff of the residual soil nutrients (Bandick and Dick, 1999; Maharana and Patel, 2013). The Km value of urease activity varied from 0.112 M (IB_0) to 0.037 M (IB_{25}), which suggested that the binding status and the origin of soil urease are dissimilar (Table 2). The catalytic efficiency Vmax/Km of urease in IB_0 (36.8660) was estimated to be comparatively lower than IB_{25} (1570.3243), which may be due to the variation in soil organic matter content and successional changes in soil textural composition over time (Bery *et al.,* 1978; Garcia *et al.,* 1993). Further, the shift in kinetic parameters of urease activity may be due to the variation in soil physico-chemical properties such as soil moisture, heavy metal contamination, temperature, pH, microbial community and gradual accumulation of N content (Garcia *et al.,* 1993; Sarkar *et al.,* 2003; Tischer, 2005; Corstanje *et al.,* 2007; Kizilkaya and Ekberli, 2008; Kujur *et al.,* 2012). Besides, the supplement of urea to soil with high organic matter content can enhance urease activity significantly and thereby its catalytic efficiency (Blagodatsky *et al.,* 1998). Thus, the better understanding of urease activity dynamics could indicate more effective way of managing soil quality. The urease activity exhibited positive correlation with all tested soil properties. The Vmax of urease activity was positively correlated with all the tested soil properties except BD. However, the Km showed negative correlation with all the soil physico-chemical properties except BD. Further, it is evident from data that the catalytic efficiency (Vmax/Km) value of soil urease exhibited positive correlation with all the tested soil properties except Km and BD, which were negatively correlated (Table 6). Dehydrogenases are intracellular enzymes mainly associated with the living cells (Garcia-Gil *et al.,* 2000; Masciandaro *et al.,* 2000; Taylor *et al.,* 2002; Zhang *et al.,* 2009). The dehydrogenase activity is considered as the measure of the intensity of microbial metabolism in soil and thus reflects the overall microbial activity (Tabatabai, 1982). Besides, it can also indicate the type and significance of pollution in soil (Pascual *et al.,* 2000). Their kinetic parameters are used to describe the catalytic activity, origin and enzymes-substrate affinity of soil (Garcia-Gil *et al.,* 2000; Zhang *et al.,* 2009). The study showed relatively higher Vmax value in NF soil (5.134 µg g^{-1} soil hr^{-1}) as compared to different age series iron mine overburden spoil across the sites (Table 2). The Vmax of dehydrogenase activity exhibited an increasing trend from IB_0 (0.238 µg g^{-1} spoil hr^{-1}) to IB_{25} (4.016 µg g^{-1} spoil hr^{-1}), which may be due to the gradual accumulation of soil organic matter in IB_{25} over time that support enhanced microbial activity and microbial biomass, consequently the concentration of soil dehydrogenase (Copper and Warman, 1997; Pascual *et al.,* 2000; De Mora *et al.,* 2005; Tan *et al.,*

2008). The variation in Km of dehydrogenase activity from 0.184 M (IB_0) to 0.053 M (IB_{25}) with the increase in age of iron mine overburden spoil can be explained on the basis of the capability of enzyme catalyzing the same reaction can have different sources in soil and thus different Km values (Nannipiei *et al.,* 1990). Besides, the Km value of dehydrogenase activity was found to be minimal in nearby NF soil (0.021 M). The Vmax/Km value of dehydrogenase activity was found to be maximum in IB_{25} (75.7735) and minimum in IB_0 (1.2934), which may be due to the changes in microbial community composition with changes in the community of dehydrogenases (Masciandaro *et al.,* 2000). However, the Vmax/Km of dehydrogenase activity exhibited by nearby NF soil was found to be relatively higher (244.4761) as compared to different age series iron mine overburden spoil across the sites (Table 2).

The dehydrogenase activity and its Vmax value were positively correlated with all the tested soil properties except BD (Table 7). However, the Km value of dehydrogenase showed negative correlation with all the soil variables except BD. Similarly, the catalytic efficiency (Vmax/Km) of dehydrogenase activity was found to be positively correlated with all the soil variables except BD and Km, which were negatively correlated (Table 7). Further, the increased detritus inputs lead to the gradual accumulation of available labile carbon substrate in soil and thereby enhancing soil dehydrogenase activities, which is used as indicator of soil health (Nannipieri *et al.,* 2002). The study suggested that the shift in kinetic parameters of dehydrogenase activity seem to indicate the change in microbial community composition and activity in different soil profiles, which can be used to monitor mine spoil genesis in chronosequence iron mine overburden spoil over time.

Table 7 Simple correlation between soil physico-chemical properties and dehydrogenase activity

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	DHase activity	Vmax	Km	Vmax /Km
DHase activity	0.977**	-0.983**	0.968**	0.995**	0.925**	0.996**	0.993**	0.973**	1			
Vmax	0.981**	-0.982**	0.961**	0.992**	0.921**	0.993**	0.994**	0.977**	0.999**	1		
Km	-0.987**	0.988**	-0.972**	-0.991**	-0.941**	-0.990**	-0.989**	-0.969**	-0.997**	-0.998**	1	
Vmax/Km	0.889**	-0.776*	0.748*	0.795*	0.717*	0.814*	0.883**	0.845**	0.849**	0.862**	-0.853**	1

**Correlation is significant $p < 0.01$, * Correlation is significant $p < 0.05$ (2- tailed test).

Stepwise Multiple Regression Analysis

Stepwise multiple regression analysis was performed to quantify the contribution of soil physico-chemical properties influencing the changes in enzyme activities in seven different age series iron mine overburden spoil across the sites. The analysis revealed that the clay fraction explained 96.6% of the variability in amylase activity (Table 8). Besides, 93% variability in amylase activity was explained by OC as 1st variable and an additional 6.2% by slit as 2nd variable ($p < 0.001$). Stepwise multiple regression analysis revealed the relationship between invertase activity and clay, which explained 90.6% variability (Table 8). The 2nd and 3rd variables of importance in explaining the variability in invertase activity were OC and WHC ($p < 0.001$). Besides, the OC explained about 99.7% of the variability in invertase activity and a marginal change by EP as 2nd variable ($p < 0.001$). The study suggested that about 90.2% of the variability in invertase activity was explained by WHC. The 2nd and 3rd variables of importance explaining the variability in invertase activity were OC and pH ($p < 0.001$). In addition, the MC explained about 99.3% of the variability in invertase activity and a marginal change was accounted by OC as 2nd variable ($p < 0.001$).

It is evident from the stepwise multiple regression analysis that about 91.6% of the variability in protease activity was explained by clay and an additional 7.5% by BD as 2nd variable (Table 8). In addition, the WHC explained about 96% of the variability in protease activity and a marginal change by BD and pH as 2nd and 3rd variables respectively ($p < 0.001$). Besides, the pH explained about 87.6% variability in protease activity and an additional 1.6% was contributed by BD as 2nd variable ($p < 0.001$). About 93.1% of the variability in protease activity was explained by TN as 1st variable and an additional 6.3% variability was explained by BD as 2nd variable ($p < 0.001$). Similarly, the clay explained about 9.5% of the variability in urease activity and an additional 6.4% was accounted by EP as 2nd variable (Table 8). Besides, the anlysis revealed the relationship between urease activity and MC, which explained about 96.5% of the variability ($p < 0.001$). The 2nd, 3rd, 4th and 5th variables of importance explaining the variability in urease activity were EP, sand, OC and TN respectively. In addition, the pH explained about 75.3% of the variability in urease activity and an additional 24.3% was accounted by EP as 2nd variable ($p < 0.001$). Besides, the TN explained about 98.5% of the variability in urease activity as 1st variable ($p < 0.001$).

Further, the stepwise multiple regression analysis revealed that the clay explained about 92.6% of the variability in dehydrogenase activity (Table 8).

The 2nd variable of importance explaining the variability in dehydrogenase activity was accounted by MC as 2nd variable ($p < 0.001$). About 92.9% of the variability in dehydrogenase activity was explained by WHC as 1st variable. The 2nd and 3rd variables of importance explaining the variability in dehydrogenase activity were OC and TN ($p < 0.001$). The pH explained 81.3% of the variability in dehydrogenase activity and an additional 17.6% variability was accounted by MC as 2nd variable of importance ($p < 0.001$). In addition, the OC explained 99.3% of the variability in dehydrogenase activity and a marginal change was contributed by MC as 2nd variable ($p < 0.001$). About 98% of the variability in dehydrogenase activity was explained by TN as 1st variable and a marginal (1.9%) effect was explained by MC as 2nd variable ($p < 0.001$). Further, in order to discriminate seven different age series iron mine overburden spoil ($IB_0 \rightarrow IB_{25}$) in chronosequence as well as nearby NF soil, the principal component analysis was performed (Ludwig and Reynolds, 1988) on the basis of their enzyme activities and kinetic parameters.

Table 8 Stepwise multiple regression analysis of enzyme activities on physico-chemical properties

Enzyme activity	Equation(s)	R^2*
Amylase activity	= -18.28 + 4.01 Clay	0.966
	= 1.779 + 10.7 OC	0.930
	= -29.694 + 1.7 OC + 3.7 Slit	0.992
	= -838.58 + 158.2 Clay	0.906
	= -68.13 – 0.03 Clay + 451 OC	0.997
	= -162.99 -16.5 Clay + 434 OC + 6.7 WHC	0.999
	= -69.9 + 450.4 OC	0.997
	= -77.73 + 509.8 OC – 0.14 EP	0.998
Invertase activity	= -1240.3 + 46.041 WHC	0.902
	= -175.8 + 4.035 WHC + 415.1 OC	0.998
	= 1216.8 + 14.529 WHC + 385.88 OC – 267.48 pH	0.999
	= -1549.5 + 225.3 MC	0.993
	= -404.7 + 50.9 MC + 349 OC	0.998
	= -138.1 + 29.1 Clay	0.916
	= 662.4 – 1.2 Clay – 359 BD	0.991
	= -219.4 + 8.69 WHC	0.960
	= 533.6 + 1.03 WHC – 307 BD	0.992
Protease activity	= 1015.8 + 3.45 WHC – 307 BD – 87 pH	0.996
	= -1778.5 + 286 pH	0.876
	= -938.1 – 37 pH – 386 BD	0.992
	= 18.39 + 907 TN	0.931
	= 524.79 + 170 TN – 286 BD	0.994
	= -31.61 + 6.58 Clay	0.935
	= -12.98 + 2.98 Clay + 0.0247 EP	0.999
	= -58.87 + 9.08 MC	0.965
	= -29.22 + 4.73 MC + 0.0208 EP	0.987
Urease activity	= 162.87 – 1.06 MC + 0.0272 EP – 1.774 Sand	0.998
	= 22.49 – 8.14 MC + 0.0214 EP – 2.009 Sand + 22.1 OC – 77.1 TN	0.999
	= -368 + 59.3 pH	0.753
	= -118.3 + 19.3 pH + 0.0323 EP	0.996
	= 210.5 – 6 pH + 0.0234 EP – 1.97 Sand	0.999
	= 3.552 + 209 TN	0.985
	= -2.862 + 0.593 Clay	0.926
	= -5.474 – 0.002 Clay + 0.839 MC	0.999
	= -4.3878 + 0.1731 WHC	0.929
	= -1.0294 + 0.0406 WHC + 1.31 OC	0.997
	= -0.8395 + 0.0282 WHC + 2.279 OC – 9.8 TN	0.999
Dehydrogenase activity	= -34.696 + 5.58 pH	0.813
	= -5.534 + 0.01 pH + 0.835 MC	0.999
	= 0.3556 + 1.665 OC	0.993
	= -4.7248 + 0.227 OC + 0.72 MC	0.999
	= 0.3059 + 18.9 TN	0.980
	= -5.0565 + 1.4 TN + 0.777 MC	0.999

* All R^2 values are significant at $p < 0.001$.

Based on the eigen values, the Z1 and Z2 components explained the maximum variance with 99% cumulative percentage of variance, which can be able to segregate seven different iron mine overburden spoil and nearby NF soil profiles into independent clusters (Fig 2).

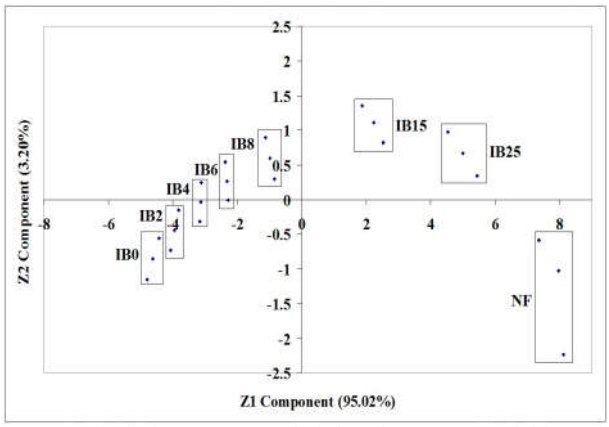

Figure 2 Principal component analysis based on enzyme activities and its kinetic parameters in chronosequence iron mine overburden spoil as well as nearby NF soil across the sites.

It is evident from the study that soil enzyme activity and its associated kinetic parameters can serve as an integrative measure of mine spoil genesis reflecting soil quality assessment in chronosequence iron mine overburden spoil over time.

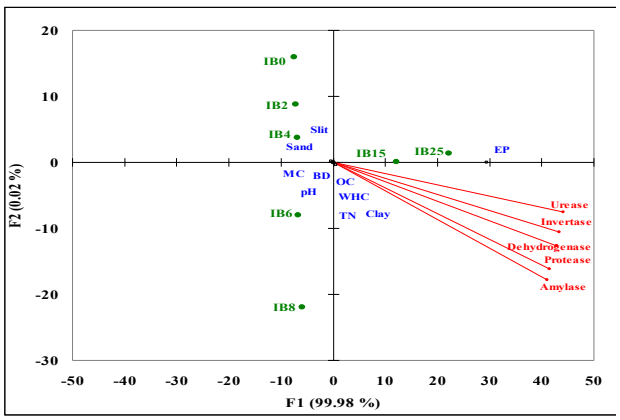

Figure 3 Redundancy analysis illustrated the relationship between soil physico-chemical properties influencing enzyme activities in different age series iron mine overburden spoil across the sites.

Besides, redundancy analysis was performed in order to explain the relationship between different age series iron mine overburden ($IB_0 \rightarrow IB_{25}$) and soil physico-chemical properties gradients altogether in order to quantify its contribution towards the shift in enzyme activities (Fig 3).

CONCLUSION

The assessment of soil enzyme activity appeared to be more informative to characterize soil quality status due to it involvement in organic matter decomposition, biotransformation and mineralization. The shift in microbial community structure associated with their activities is quickly monitored through enzyme activities, which can be integrated to provide information about the microbial status and the physico-chemical condition of soil. Thus, the periodic monitoring of enzyme activities in chronosequence iron mine overburden spoil can be used as bioindicator of mine spoil genesis reflecting the shift in microbial community composition over time and could be used to guide the selection of appropriate reclamation strategies in terrestrial ecosystem. Besides, the study indicated that the kinetic parameters of different soil enzyme activities were sensitive towards the variation in available soil nutrients, microbial community structure as well as soil physico-chemical properties, which can be served as an integrative measure of soil quality. Keeping in view, the estimation of enzyme activity and its kinetic parameters in chronosequence iron mine overburden spoil over time could significantly provide insights into the linkages between microbial community compositions with ecosystem processes. Comparative assessment of the kinetic parameters of different soil enzyme activities revealed consistent increase in Vmax and decline in Km value showed the sign of mine spoil genesis over time. Besides, the gradual improvement in catalytic efficiency (Vmax/Km) of different soil enzyme activities in chronosequence iron mine overburden spoil reflecting the progress of mine spoil restoration. Principal component analysis can able to segregate the seven different age series mine overburden spoil and nearby NF soil into independent clusters based on the soil enzyme activities and kinetic parameters. Therefore, the study evident that the microbial characterization including enzyme activity and kinetic parameters can be used as potential indicator of soil quality and useful for the assessment of successful rehabilitation of the ecologically disturbed habitat as they provide early and sensitive indicators of the changes involved with mine spoil genesis over time.

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