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Kinetic Indices of Enzyme Activity used as Sensitive Biomarkers to Monitor Mine Spoil Genesis in Chronosequence Coal Mine Overburden Spoil

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ABSTRACT

Mine spoil genesis mostly focuses on careful consideration of enzyme activities and kinetics parameters to elucidate microbial mediated biotransformation and nutrient cycling including their link with the variation in physicochemical properties, microbial community dynamics and landscape ecology as the functional determinants of ecosystem function. The relative distribution of microbial populations exhibited progressive increase in different age series coal mine overburden spoil over time. The kinetic parameters of amylase, invertase, protease, urease, phosphates and dehydrogenase activities were determined to assess the metabolic response in different age series coal mine spoil. Gradual increase in Vmax and decline in Km of enzyme activities representing the quality and affinity of enzymes correlated well with the shift in microbial community composition over time. Besides, the shift in catalytic efficiency (Vmax/Km) has greater significance as early and sensitive indicators of the changes in soil properties influencing microbial community dynamics across the sites. The enzyme activity and kinetic parameters correlated well the changes in physicochemical properties over time across the sites. PCA and RDA analysis can able to discriminate six different age series coal mine overburden spoil and nearby NF soil into independent clusters based on the variability in physicochemical properties, microbial community structure, enzyme activities and kinetic parameters. The study suggested that the kinetic indices of enzyme activities can be used as reliable ecological biomarkers to monitor the progress of mine spoil genesis.

Key words: *Mine spoil, Microbial community structure, Enzyme activity, Enzyme kinetics, Mine spoil genesis.*

Introduction

Enzymes are primarily derived from soil microbes either as extracellular secretions and/or products from lysed cells. Enzymes are biological entities that catalyses biochemical reactions influencing the rate limiting steps involved in various biotransformation reactions of available nutrients. Enzyme activities provide integrative relationships between biological and biochemical soil attributes and proposed as early and sensitive indicators of the changes mediated by anthropogenic activities. Enzyme activities contributed by proliferating microbes play an important role in the decomposition and mineralization of soil organic matter, which provides insight into microbial community dynamics ad activities. Therefore, the rationale of estimating enzyme activities is prerequisite to establish correlation with soil quality status and microbial activities useful for the periodic assessment of mine spoil genesis.

The biochemical properties are responsive to small changes in microbial activities due to their in-

volvement in mineralization of organic matter and nutrient cycling that influence the ecosystem stability and hence considered as the index of soil quality (Schoenholtz *et al.,* 2000; Zhang *et al.,* 2010). Being their direct involvement in biogeochemical cycling of C, N and P, the enzyme activities quickly respond to environmental changes influencing microbial amelioration and microbial community dynamics (Waldrop *et al.,* 2000; Kizilkaya and Dengiz, 2010). The shift in microbial community composition due to the variation in soil physicochemical properties and their adaptive tolerance in different soil profiles influence the potentiality of soil subsystems through enzyme mediated substrate catalysis reflecting the functional integrity of soil (Nannipieri *et al.,* 1990; Dick, 1997). The enzymatic studies on soil profiles provide information about their origin, existing nature and catalytic efficiency of enzymes (Mateos and Carcedo, 1987) and hence described as the 'biological fingerprints'. Besides, they are sensitive, integrative and indicative of biological equilibrium, soil quality and fertility (Dick, 1997; Bandick and Dick, 1999) and changes in nutritional status of soil (Nannipieri *et al.,* 2002). Relative abundance and distribution of microbial populations in is influenced by soil physicochemical properties (Tangjang and Arunachalam, 2009; Kujur *et al.,* 2012). The changes in enzyme activities correlated well with the variation in soil physicochemical properties (Kujur *et al.,* 2012; Steinweg *et al.,* 2013), vegetational development (Waldrop *et al.,* 2000; Sinsabaugh *et al.,* 2002), anthropogenic disturbances and successional changes in terrestrial ecosystems (Baldrian *et al.,* 2008), microbial community composition (Waldrop *et al.,* 2000; Nancucheo and Johnson, 2005). The potential use of enzymatic studies to monitor ecosystem restoration has been substantiated by several workers (Nannipieri *et al.,* 1990; Mummey *et al.,* 2002; Kujur *et al.,* 2012).

Soil enzyme activity indices appeared to be more informative and highly reliable as it respond quickly and precisely with respect to different soil attributes. However, the selection of enzyme activities as potential biomarkers to monitor the progress of mine spoil genesis is based on their sensitivity to organic matter decomposition, nutrient cycling, anthropogenic disturbances and restoration efforts. Amylases (a-amylase, b-amylase and glucoamylase) are closely interlinked with bacterial populations involved in starch hydrolysis to form glucose or dextrins and small quantity of maltose, which exhibit different mechanism of action with variation in substrate specificities, thermostability, temperature and pH (Dick, 1997; Alariya *et al.,* 2013). Invertases [b-fructo furanosidase (EC.3.2.1.26))] belong to GH32 family of glycoside hydrolases, which hydrolyzes sucrose into a-D-glucose and b-1-fructose and can be served as diagnostics clue to soil functioning. Higher amylase and invertase activity are correlated well with nutrient turnover rate and microbial biomass pool (Bogdevitch and Mikhailouskaya, 2009; Hu *et al.,* 2013). The proteases are involved in proteolysis through hydrolysis of peptide bonds, which play significant role in carbon and nitrogen mineralization and immobilization influencing the overall microbial community structure in soil (Sims and Wander, 2002; Gupta and Lorenz, 2010; Anjaneyulu *et al.,* 2011). Ureases are secreted by urolytic microorganisms and root exudates involved in mineralization and biotransformation of urea to ammonia, which is subsequently nitrified by the nitrifying microorganisms (Gianfreda *et al.,* 2004; Kizilkaya *et al.,* 2004). The change in urease activity with the variation in soil physicochemical properties and organic residues has been reported (De Mora *et al.,* 2005; Kuscu and Karaoz, 2015), which can be useful for developing suitable strategies for ecosystem sustainability. The acid phosphatase (Orthophosphoric monoester phosphohydrolase) hydrolyzes orthophosphoric monoester to alcohol and orthophosphate, which acts as intermediary enzyme involved in the biotransformation of organic phosphates into inorganic form (Harris and Birch, 1989; Kramer and Green, 2000). Dehydrogenases reflects the estimation of overall microbial activities due to its presence in all microbes involved in oxidation-reduction reactions linked with the microbial respiratory processes and nutrients cycling (Bandick and Dick, 1999; Gianfreda *et al.,* 2004). Being intracellular, the dehydrogenase activity is considered as an index of endogenous activity indicating the availability of carbon as energy source for microbes (Masciandaro *et al.,* 2000; Taylor *et al.,* 2002) and efficient indicator of overall microbial activity in terrestrial ecosystem (Pascual *et al.,* 2000; De Mora *et al.,* 2005) to evaluate the degree of restoration in degraded soils (Zhang *et al.,* 2010; Kujur *et al.,* 2012).

Enzyme activity can be used to describe the relative abundance and distribution of microbial communities and their metabolic activities, whereas the kinetic parameters indicate their origin, existing status, substrate affinity and catalytic efficiency of en-

zymes (Marx *et al.,* 2005; Zhang *et al.,* 2010). The Vmax of enzyme catalyzed reaction refers to the splitting velocity of enzyme-substrate complex, which reflects the conjunction affinity between the enzyme and substrate. Km represents the endurance of an enzyme-substrate complex, which is substrate dependent. Soil enzymes catalyzing same biochemical reaction can have different origins and thus exhibit different Km values (Nannipieri *et al.,* 2002). The Vmax and Km value of enzyme revealed the quantity and substrate affinity of an enzyme respectively (Marx *et al.,* 2005). Smaller the Km value, the greater will be the affinity for substrate (Nannipieri *et al.,* 2002). Catalytic efficiency of enzyme activity (Vmax/Km) provides information about enzyme-substrate complex and comparison of dispersion in soil. Higher value of Vmax/Km suggests faster dispersion rate of enzyme-substrate complex than its information (Kizilkaya *et al.,* 2004; Ekberli *et al.,* 2006). The kinetic parameters of enzyme activities were influenced by soil physicochemical properties, substrate availability, microbial community structure, which alter microbial activities (Kizilkaya and Bayrakli, 2005; Ekberli *et al.,* 2006). The shift in microbial community composition produces different enzyme isoforms with variation in their catalytic efficiency leading to the variation in their kinetic parameters.

The assessment of soil quality status with individual soil variable will provide partial evaluation of the state of soil subsystem. The polyphasic approach of soil quality assessment is more reliable to evaluate the drastic consequences of anthropogenic activities in terrestrial ecosystems because each soil attribute has its own limitations. The comparative assessment of enzyme activities provide information about the linkages between physicochemical attributes, available nutrients, microbial community structure, microbial metabolic activity and ecosystem functioning. Keeping in view, the present study was designed to determine the variation in different soil physicochemical properties, microbial community composition and enzyme activities in different age series coal mine overburden spoil over time, which paves the way of greater understanding in the direction of improving soil quality. Besides, the enzymatic activities associated with its kinetic parameters provide insight into the microbial community structure and dynamics reflecting the sign of mine spoil genesis. Further, the efforts were taken to quantify the contribution of different physicochemical properties and microbial community composition influencing the variability in enzyme activities associated with their kinetic properties in chronosequence coal mine overburden spoil over time, which can be used as indices for monitoring the progress of mine spoil genesis.

Materials and Methods

Study site

The present study was carried out in the Basundhara (west) open cast colliery, Ib valley area of Mahanadi Coalfields Limited (MCL), Sundargarh, Odisha (22°03′58"-20°04′11" north latitude and 83°42′46"-83°44′45" east longitude). Topologically, the area is hilly sloppy (244m above sea level) to plateau. The thickness of native top soils in the site varies from 0.15 m to 0.30 m (average: 0.22 m). Climatic condition of the site is considered to be Aw according to the Köppen-Geiger climate classification. The area experiences semi-arid climate with annual rain fall of 1483 mm yr-1, average temperature of 26°C and relative humidity of 58.58%. Tropical dry deciduous forest is considered to be the natural vegetation of the study site and broadly the climate is dry, hot and arid. Because of mining activities and biotic interferences, the forest area is marginally reduced and harbor insufficient organic top soil to support revegetation. Open cast mining activities lead to the formation of different age series coal mine spoil overburdens and grouped according to their inception (Fresh mine spoil: OB0, 3 yr: OB3, 6 yr: OB6, 9 yr: OB9, 12 yr: OB12 and 15 yr: OB15).

Mine spoil sampling

Each overburden was divided into 5 blocks, and from each block, five spoil samples were collected from 0-15 cm depth by digging pits (15×15×15) cm^3 size. Samples collected from each block were referred as 'sub-samples' and were mixed to form 'composite sample' obtained from each overburden site. Similar strategy has been followed for sampling from six different age series of coal mine overburden (OB0 ! OB15) along with the nearby native forest soil (NF), which was used as reference. Composite samples were homogenized, sieved (0.2 mm) and stored at 4°C until analyzed.

Physicochemical characterization

The clay percentage (< 0.002 mm) in different mine

spoil and nearby NF soil was analyzed based on prescribed methodology in 'Tropical soil biology and fertility hand book' (Mishra, 1968; Anderson and Ingram, 1992). The hydrological regimes such as bulk density, water holding capacity and moisture content were also determined. Bulk density (BD) was calculated as [weight of excavated spoil (in g)/ volume of sand (cm^3)] following the TSBF Handbook (Anderson and Ingram, 1992). Water holding capacity (WHC) was determined (Mishra, 1968) and expressed in percentage. Moisture content (MC) was determined by gravimetric method (Mishra, 1968) through oven drying and expressed in percentage. The pH of mine spoil was determined using digital pH meter (Make: Systronics, Model: MK VI).

Organic carbon (OC) content in different age series coal mine overburden spoil and nearby NF soil was estimated following titration method described by Walkley and Black (Mishra 1968) and expressed in mg C/g spoil. Total nitrogen (TN) was determined by Kjeldahl method (Jackson, 1958) and expressed in mg N/g spoil. The extractable phosphorous (EP) was estimated through chlorostannous reduced molybdophosphoric blue colour method in HCl (Olsen and Sommers, 1982) and expressed in µg P/g spoil.

Microbial enumeration

The microbial enumeration was performed by serial dilution technique. Suspension of mine spoil sample (1 g) with 100 ml sterilized distilled water was serially diluted upto 10^{-10} fold. Enumeration of microbial population in different age series coal mine spoil and nearby NF soil were performed through spread plate technique. The azotobacter population (AZB) was enumerated using azotobacter mannitol agar [20 g mannitol; 5 g extract; 1 g K_2PO_4; 0.2 g $MgSO_4$; 0.2 g NaCl; trace of $FeSO_4$ per liter; 1.5% agar, pH 8.3] and incubated at 30ºC for 48 hrs. The arthrobacter population (ARB) was enumerated using arthrobacter medium [4 g trypticase soy agar, 2 g yeast extract, 20 g NaCl, 0.1 g cycloheximide, 150 µg/ml methyl red (Harleco) per liter, 1.5% agar] supplemented with 0.01% cycloheximide and 2% NaCl to inhibit fungal growth. The rhizobial count (RZB) was estimated using yeast extract mannitol agar [10 g mannitol, 4 g $CaCO_3$, 0.5 g K_2HPO_4, 0.4 g yeast extract, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl per liter, 1.5% agar (pH- 6.8)] with congo red dye (Vincent, 1970). The heterotrophic aerobic bacterial population (HAB) in different mine spoil profiles were enumerated using nutrient agar. The sulfate reducing bacterial population (SRB) count was determined using sulfate reducing medium [Part-A: 0.5 g K_2PO_4, 2 g peptic digest of animal tissue, 1 g beef extract, 1.5 g Na_2SO_4, 2 g $MgSO_4.7H_20$, 0.1 g $CaCl_2$; Part-B: 0.392 g $Fe(NH_4)_2SO_4$, 0.1 g sodium ascorbate; Part-C: 3.5 g sodium lactate per liter (pH- 7.5)]. Actinomycetes population (ACT) was determined using starch-casein agar [10 g soluble starch, 0.3 g casein, 0.2 g KNO_3, 2 g NaCl, 2 g K_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.02 g $CaCO_3$, 0.07 g $FeSO_4$ per liter and 1.5% agar (pH 7.2)] (Hunter-Cevera and Eveleigh, 1990) supplemented with streptomycin (40 ìl/ml) and griseofulvin (50ìl/ml) to inhibit bacterial and fungal growth respectively (Alharbi *et al.*, 2012). The yeast count (YES) were estimated using potato sucrose agar [500 ml potato extract, 20 g/l sucrose, 1ml trace metal solution, 500 ml distilled water (pH 6.7)]. The fungal count (FUN) was determined using rose bengal agar [5 g papaic digest of soyabean meal, 10 g dextrose, 1 g K_2PO_4, 0.5 g $MgSO_4$, 0.05 g rose bengal per liter, 1.5% agar (pH-7.2)] supplemented with streptomycin (50ìl/ml) to inhibit bacterial contaminants.

Enzyme activity

The amylase activity was determined by spectrophotometric method (Roberge, 1978) using soluble starch as substrate and expressed in mg glucose/g spoil/hr. Invertase activity was determined by spectrophotometric method (Ross, 1983) using sucrose as substrate and expressed in mg glucose/g spoil/hr. Protease activity was determined by spectrophotometric method (Ladd and Butler, 1972) using tyrosine standard graph and expressed in mg tyrosine/g spoil/hr. Urease activity was determined by titration method (Tabatabai and Bremner, 1971) and expressed in mg NH_4^+/g spoil/hr. Besides, the phosphatase activity was determined by spectrophotometric method using p-nitrophenol as substrate (Tabatabai and Bremner, 1969) and expressed in (µg PNP/g spoil/hr). The dehydrogenase activity was determined by spectrophotometric method (Nannipieri *et al.*, 1990) using triphenyl forrmazone standard graph and expressed in mg TPF/g spoil/ hr.

Enzyme kinetics

Enzymes follow the Michaelis Menten kinetics despite soil being considered as a discontinuous, structured and heterogeneous system (Nannipieri *et al.*, 2002). The Michaelis–Menten equation linearized by

Lineweaver-Burk plot was used to determine Vmax and Km by plotting a graph (1/V vs 1/[S]) and estimated by the intercept and slope respectively; and Vmax/Km as kinetic parameters (Tabatabai and Bremner, 1971). The kinetic parameters of enzyme activity in different age series coal mine spoil and nearby NF soil were determined by taking different substrate concentrations, which ranged from amylase (5mM to 50mM), invertase (10mM to 100mM), protease (1mM to 10mM), urease (5mM to 45mM), phosphatase (10mM to 50mM) and dehydrogenase (10mM to 90mM) respectively. The estimation of kinetic indices of different enzymes was performed in triplicates.

Statistical analysis

Simple correlation analysis was performed to test the level of significance between physicochemical properties, enzyme activities and kinetic parameters in different age series coal mine overburden spoil (OB0 → OB15) and nearby NF soil using SPSS (Version 17.0). The stepwise multiple regression analysis was performed to quantify the contribution of different physicochemical soil variables influencing the variability in enzyme activities over time across the sites using Minitab 16 software. Principal component analysis was performed using Statistrix PC DOS Version-2.0 (NH Analytical software). Redundancy analysis (RDA) was performed using Microsoft Excel XLSTAT-2014 (Version 2.03).

Results

Physicochemical characterization

Wide variation in different physicochemical properties was exhibited by different age series coal mine spoil (OB0 OB15) and nearby NF soil (Table 1). The study indicated that there was gradual increase in clay (5.3 – 11.8%), water holding capacity (26.73 – 45.36%), moisture content (6.913 – 10.238%) with minimum in OB0 and maximum in OB15 over time. However, bulk density exhibited a decline trend, which ranged from OB0 (1.712 g/cm^3) to OB15 (1.268 g/cm^3) across the sites. The pH of different age series mine spoil was found to be within the acidic range (6.12 – 6.68), which progressed towards neutral range over time. The nearby NF soil exhibited relatively higher clay (12.9%), water holding capacity (47.13%) and moisture (11.319%) compared to different age series mine spoil. Besides, the pro-

Table 1. Physicochemical characterization of coal mine spoil collected from different age series overburdens (OB0 → OB15) and NF soil. (Values were mean ± SD; n = 5).

Parameters	Coal mine spoil from different age series overburdens from (0-15) cm soil depth						NF soil
	OB0	OB3	OB6	OB9	OB12	OB15	
Clay (%)	5.3 ± 0.15	7.5 ± 0.21	9.1 ± 0.33	10.3 ± 0.19	11.2 ± 0.42	11.8 ± 0.29	12.9 ± 0.36
BD (g/cm^3)	1.712 ± 0.035	1.584 ± 0.028	1.389 ± 0.032	1.321 ± 0.033	1.293 ± 0.024	1.268 ± 0.022	1.236 ± 0.025
WHC (%)	26.73 ± 1.278	33.29 ± 0.946	39.13 ± 1.214	42.45 ± 1.379	44.67 ± 0.884	45.36 ± 1.169	47.13 ± 1.294
Moisture (%)	6.913 ± 0.195	7.328 ± 0.294	7.967 ± 0.218	8.672 ± 0.254	9.547 ± 0.284	10.238 ± 0.372	11.319 ± 0.343
pH	6.12 ± 0.05	6.21 ± 0.04	6.35 ± 0.06	6.42 ± 0.04	6.59 ± 0.05	6.68 ± 0.04	6.92 ± 0.03
Organic C(mg C.g^{-1} spoil)	nd*	0.358 ± 0.035	1.118 ± 0.051	1.634 ± 0.132	2.118 ± 0.219	2.684 ± 0.227	3.705 ± 0.264
Total N(mg N. g^{-1}spoil)	nd*	32.963 ± 3.315	83.562 ± 13.968	335.523 ± 21.425	915.658 ± 39.559	1267.25 ± 41.136	1733.12 ± 32.576
Extractable P(mg P. g^{-1} spoil)	nd*	6.359 ± 0.638	14.137 ± 1.534	54.522 ± 3.382	108.452 ± 13.658	171.152 ± 11.532	272.531 ± 13.537

nd*: beyond detectable limit.

gressive improvement in organic C (0.358 - 2.684 mg C. g^{-1} spoil), total N (32.963 - 1267.25 mg N. g^{-1} spoil) and extractable P (6.359 - 171.152 mg P. g^{-1} spoil) with the increase in age of mine overburden spoil was observed. The organic C, total N and extractable P in OB0 were found to be beyond the detectable limit. However, the organic C (3.705 mg C. g^{-1} spoil), total N (1733.12 mg N. g^{-1} spoil) and extractable P (272.531 mg P. g^{-1} spoil) in nearby NF soil was found be relatively higher compared to different age series coal mine spoil (Table 1).

Microbial community composition

The relative distribution of microbial populations (log_{10} CFU per g spoil) in different age series coal mine spoil (OB0 → OB15) and nearby NF soil was presented (Figure 1a-h).

The study indicated progressive increase in CFU exhibited by azotobacter (r = 0.980; $p<0.001$), arthrobacter (r = 0.983; $p<0.001$), rhizobia (r = 0.973; $p<0.001$), heterotrophic aerobic bacteria (r = 0.954; $p<0.001$), actinomycetes (r = 0.953; $p<0.001$), yeast (r = 0.988; $p<0.001$) and fungi (r = 0.963; $p<0.001$) respectively with minimum in OB0 and maximum in OB15 was found to be statistically significant. In contrast, the relative distribution of sulfur reducing bacteria exhibited a decline trend with maximum in OB0 and minimum in OB15 with the increase in age of coal mine overburden spoil, which was found to be statistically significant (r = 0.973; $p<0.001$). The study indicated relatively higher abundance and distribution of different microbial populations in nearby NF soil compared to different age series coal mine overburden spoil across the sites (Figure 1a-h).

Enzyme activity

The study revealed wide variation in enzyme activities in different age series coal mine spoil (OB0 → OB15) over time and nearby NF soil across the sites (Table 2).

The amylase, invertase, protease, urease and phosphatase activity in OB0 were found to be beyond detectable limit. The analysis suggested gradual increase in amylase activity (1.564 - 8.671 µg glucose/g/hr), invertase activity (7.139 - 623.472 µg glucose/g/hr), protease activity (4.137 - 88.674 µg tyrosine/g/hr), urease activity (4.532 - 36.784 µg NH_4^+/g /hr), phosphatase activity (5.325 - 62.338 µg PNP/g /hr) and dehydrogenase activity (0.048 - 2.684 µg TPF/g /hr) respectively with the increase in age of mine overburden spoil across the sites

Table 2. Enzyme activities (amylase, invertase, protease, urease, phosphatase and dehydrogenase) exhibited by six different age series coal mine spoil (OB0 → OB15) and nearby NF soil. (Values are mean ± SD; n = 5)

Enzyme activity	Different age series coal mine overburden spoilfrom (0-15) cm soil depth						NF soil
	OB0	OB3	OB6	OB9	OB12	OB15	
Amylase activity (µg glucose/g/hr)	Nd*	1.564 ±0.134	2.259 ±0.167	3.963 ±0.139	5.894 ±0.294	8.671 ±0.283	13.124 ±0.188
Invertase activity(µg glucose/g/hr)	Nd*	7.139 ±0.519	35.361 ±8.647	126.106 ±14.562	361.549 ±16.429	623.472 ±19.668	849.335 ±22.437
Protease activity(µg tyrosine/g/hr)	Nd*	4.137 ±0.113	18.654 ±1.534	31.364 ±3.281	46.357 ±2.965	88.674 ±4.682	215.813 ±8.569
Urease activity (µg NH_4^+/g /hr)	Nd*	4.532 ±0.169	8.667 ±0.154	15.862 ±1.238	22.539 ±2.654	36.784 ±2.997	57.913 ±3.631
Phosphatase activity(µg PNP/g /hr)	Nd*	5.325 ±1.234	21.329 ±2.654	32.467 ±3.246	50.264 ±2.561	62.338 ±3.697	89.175 ±2.893
Dehydrogenase activity (µg TPF/g /hr)	0.048 ± 0.013	0.198 ±0.054	0.635 ±0.096	0.959 ±0.164	2.115 ±0.139	2.684 ±0.206	4.138 ±0.174

nd*: beyond detectable limit.

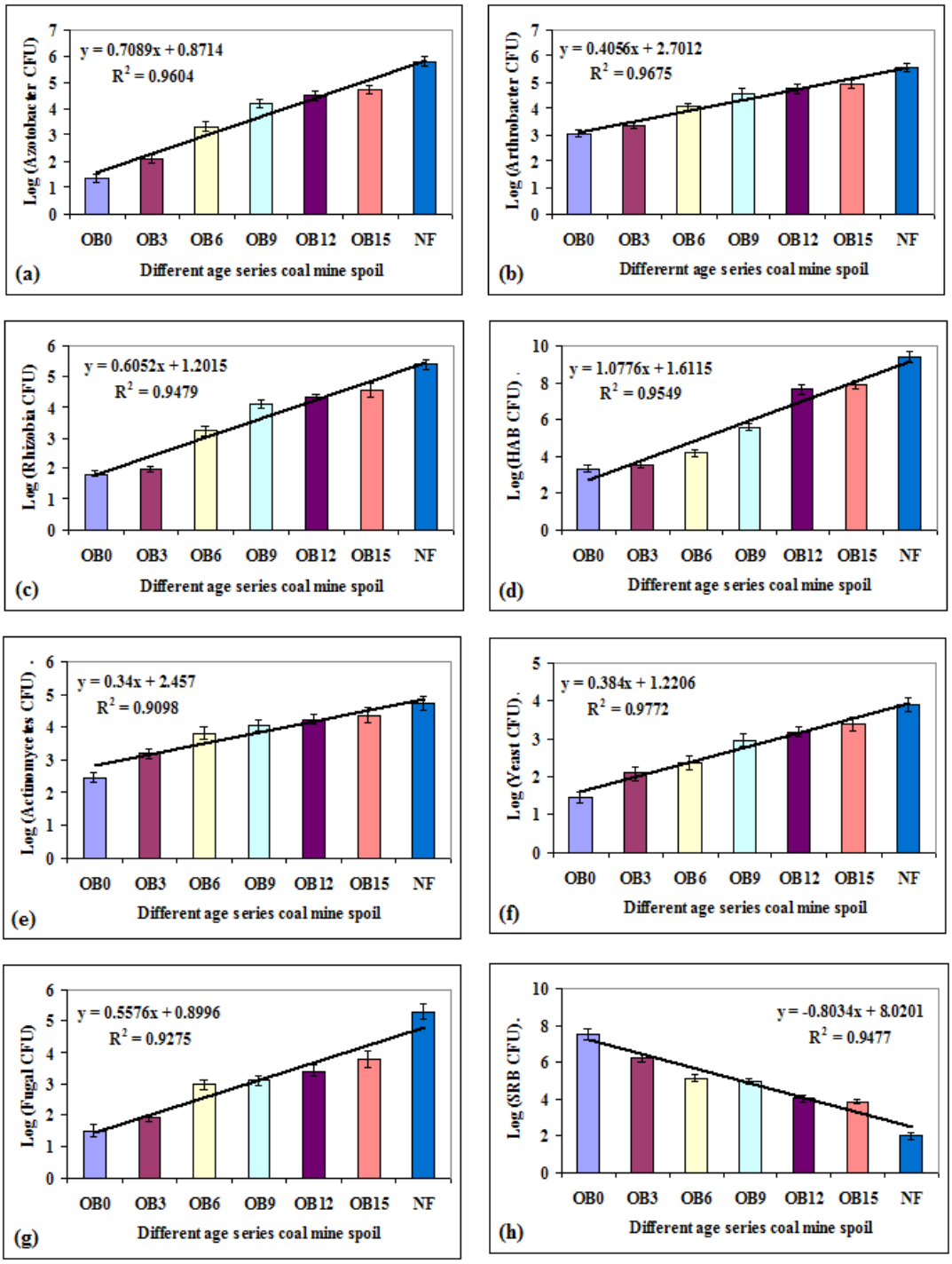

Fig. 1(a-h). Relative distribution of microbial populations (expressed in $\log_{10}$ CFU per g spoil): (a) Azotobacter, (b) Arthrobacter, (c) Rhizobia, (d) Heterotrophic aerobic bacteria, (e) Actinomycetes, (f) Yeast, (g) Fungal counts (h) Sulfate reducing bacteria in different age series coal mine spoil (OB0 → OB15) and nearby NF soil.

(Table 2). However, the enzyme activities exhibited by nearby NF soil was found to be relatively higher compared to different age series coal mine overburden spoil across the sites. The progressive increase in amylase (r = 0.964; $p<0.001$), invertase (r = 0.937; $p<0.001$), protease (r = 0.858; $p<0.05$), urease (r = 0.951; $p<0.001$), phosphatase (r = 0.986; $p<0.001$) and dehydrogenase activity (r = 0.960; $p<0.001$) in different age series coal mine spoil over time was found to be statistically significant.

Enzyme kinetics parameters

The kinetics parameters such as Vmax, Km and Vmax/Km exhibited by soil enzymes in different age series coal mine spoil (OB0 → OB15) and nearby NF soil were presented (Table 3). The Vmax exhibited by amylase (2.105 - 33.953 mg/ g spoil/hr), invertase (7.568 - 636.994 mg/ g spoil/hr), protease (2.864 - 177.558 mg/ g spoil/hr), urease (2.348 - 45.589 g/ g spoil/hr), phosphatase (3.085 - 68.237 mg/ g spoil/hr) and dehydrogenase activity (0.231 - 3.986 mg/ g spoil/hr) exhibited an increasing trend over time (Table 3). Relatively higher Vmax was exhibited by nearby NF soil compared to different age series coal mine spoil.

In contrast, the Km value exhibited a decline trend with maximum on OB0 and minimum in OB15, which ranged from (49.637 - 18.119 mM) for amylase activity, (45.236 - 15.238 mM) for invertase activity, (17.967 - 10.674 mM) for protease activity, (0.085 - 0.035 M) for urease activity, (0.095 - 0.033 M) for phosphatase activity and (0.181 - 0.033 M) for dehydrogenase activity. The Km value exhibited by different enzyme activities in nearby NF soil was found to be minimal compared to different age series coal mine (Table 3). Besides, the study indicated progressive increase in Vmax/Km values for different enzyme activities with the increase in age of mine spoil and was found to be maximum in nearby NF soil.

Discussion

Gradual improvement in clay percentage governed by the progressive establishment of vegetation and litter input from the vegetational compartment in reclaimed coal mine overburden spoil in chronosequence over time influence wide variations in hydrological regimes to promote soil aggregation, available soil nutrients, structural stability and nutrient retention capacity (Jha and Singh, 1991; Dutta and Agrawal, 2002). Improvement in soil pH towards neutrality may be due to restoration by natural succession or vegetational pattern and promotion of organic matter decomposition over time (Sheoran *et al.*, 2010; Kujur *et al.*, 2012). Gradual improvement in organic C, total and extractable P in different age series coal mine overburden spoil re-

Table 3. Enzyme kinetics parameters in different age series coal mine overburden spoil (OB0 → OB15) and nearby NF soil.

Enzymes	Kinetics parameters	Different age series coal mine overburden spoil from (0-15) cm soil depth						NF Soil
		OB0	OB3	OB6	OB9	OB12	OB15	
Amylase	Vmax	2.105	5.263	8.129	14.387	21.148	33.953	54.788
	Km (mM)	49.637	43.528	38.264	32.362	22.927	18.119	13.364
	Vmax/Km	0.042	0.121	0.212	0.444	0.922	1.873	4.099
Invertase	Vmax	7.568	15.428	89.541	168.234	425.694	636.994	913.482
	Km (mM)	45.236	38.569	32.954	27.446	20.697	15.238	11.913
	Vmax/Km	0.167	0.400	2.717	6.129	20.567	41.803	76.679
Protease	Vmax	2.864	8.953	30.258	73.538	115.237	177.558	270.593
	Km (mM)	17.967	15.451	13.015	12.314	11.023	10.674	10.136
	Vmax/Km	0.159	0.579	2.324	5.971	10.454	16.634	26.696
Urease	Vmax	2.348	6.547	15.089	25.426	31.247	45.589	66.276
	Km (M)	0.085	0.071	0.052	0.045	0.039	0.035	0.029
	Vmax/Km	27.623	92.211	290.173	565.022	801.205	1302.54	2285.38
Phosphatase	Vmax	3.085	9.865	27.558	42.138	51.438	68.237	94.568
	Km (M)	0.095	0.072	0.051	0.045	0.038	0.033	0.026
	Vmax/Km	32.473	137.014	540.353	936.40	1353.63	2067.78	3637.23
Dehydrogenase	Vmax	0.231	0.395	0.713	1.565	2.234	3.986	5.764
	Km (M)	0.181	0.136	0.089	0.075	0.048	0.033	0.021
	Vmax/Km	1.276	2.904	8.011	20.866	46.541	120.788	274.476

flect the sign of restoration of coal mine spoil over time (Rajan *et al.,* 2010; Wang *et al.,* 2011). Several studies reported the role of physicochemical properties as key determinants of microbial growth and activity (Banerjee *et al.,* 2000; Tordoff *et al.,* 2000), which in turn can be used as potential biomarkers in microbial ecology studies for the periodic monitoring of mine spoil genesis (Mummey *et al.,* 2002).

The analysis indicated wide variation in microbial community composition in different age series coal mine spoil, which may be due to the variation in microclimatic conditions, nutrient availability and heterogeneity of vegetational patterns over time across the sites. Being obligately aerobic and chemolithotrophic di-hydrogen fixing bacteria, the relative distribution of azotobacter exhibited an increasing trend from the nutrient deficient OB0 to OB15 mine spoil over time. The variability in arthrobacter population among different age series mine spoil is due to nutritional versatility and starvation. Relatively higher rhizobial count was exhibited by OB15 compared to different age series mine spoil due to the gradual establishment of vegetation, because they are highly specific for symbiotic relationship with leguminous plants for nitrogen fixation. Gradual decline in heterotrophic aerobic bacterial population from OB15 to OB0 is due to the environmental extremities caused by mining activities associated with heavy metal toxicity. The relative distribution of actinomycetes is influenced by geographical distribution including temperature, pH, aeration, moisture and organic C level, which may be the possible reason for relatively higher dominance in OB15 compared to different age series mine overburden spoil. Being acid-tolerant, the relative distribution of actinomycetes is minimal in OB0 compared to different age series coal mine spoil. Higher relative dominance of yeast in OB15 compared to different age series mine spoil may be attributed to the gradual increase in different hydrological regimes, pH, organic C, aeration and substrate availability over time across the sites. Being opportunistic (zymogenous), the fungal population exists either as free-living or in mycorrhizal association to promote soil structural stability by forming macroaggregates. Relatively higher fungal dominance in OB15 compared to different age series mine spoil may be due to the prevailing favourable moisture, improvement in soil pH towards neutrality, availability of soil organic matter that enhances microbial colonization over time (Kennedy *et al.,* 2005). Higher relative distribution of sulfur reducing bacteria in OB0 compared to different age series coal mine spoil may be due to their involvement in utilization of sulfur compounds, oxidation of inorganic P compounds and sulfur reduction accounted for organic C mineralization (Schink and Friedrich, 2000).

Wide variation in enzyme activities may be attributed to the variation in available soil nutrients and diversity in microbial community structure. Minimal activity exhibited by OB0 may be due to reduced microbial populations caused by nutrient deficiency and oxidative stress induced by heavy metal toxicity (Brookes, 1995). The study suggested the gradual increase in enzyme activity may be due to progressive accumulation of nutrients and thereby increase in microbial biomass pool and diversity in microbial community structure that causes increased enzyme production and hence higher Vmax (Stone *et al.,* 2011). Besides, the decline in Km value in different age series coal mine overburden spoil may be due to the progressive improvement in different hydrological regimes because of higher organic matter. Stronger the enzyme-substrate affinity, lower the Km value in higher moisture content, which may be caused by higher diffusion rate because of more water solubility and hydrological regimes. Further, relatively lesser Vmax/Km value in nutrient deficient OB0 mine spoil may be due to extreme dryness that limits solubility and restrict the movement of available organic carbon as the energy source and hence inhibit microbial respiration (Stone *et al.,* 2011). Several investigations have been substantiated the concept (Dick, 1994; Sardans and Penulas, 2005: Rajan *et al.,* 2010; Kujur *et al.,* 2012)

Amylases are complex enzymes belong to the glycoside hydrolase group of enzymes (a-amylase, b-amylase and glucoamylase). Gradual increase in amylase activity in different age series coal mine overburden spoil is attributed to the gradual accumulation of available soil nutrients over time (Pascule *et al.,* 1998; Anjeneyulu *et al.,* 2011), which is positively correlated with organic C ($r = 0.980$; $p<0.01$) and total N ($r = 0.982$; $p<0.01$) across the sites (Table 4). Besides, the kinetics parameters of amylase activity showed positive correlation with the progressive increase in physicochemical properties among different age series coal mine spoil over time (Table 4).

Invertase enzyme cleaves the b-glucose bonds resulting sucrose hydrolysis, which reflects the

transformation mechanism of organic C and serves as an indicator of soil maturity and fertility level (Eivazi and Tabatabai, 1990; Pascule *et al.,* 1998). The invertase activity exhibited positive correlation with organic C (r = 0.950; $p<0.01$) and total N (r = 0.995; $p<0.01$) across the sites (Table 5). The kinetic parameters of invertase activity also exhibited positive correlation with different physicochemical properties across the sites (Table 5). The decline in amylase and invertase activity is due to reduction in enzyme synthesis and concentration caused by heavy metal toxicity on microbial communities (Harris and Birch, 1989), changes in active center and structure of enzyme and thereby inhibits decomposition of starch and sucrose respectively (Anjaneyulu *et al.,* 2011).

The gradual increase in protease activity in different age series coal mine overburden spoil was found to be closely related with the progressive increase in organic C, total N and extractable P, NH_4-N accumulation (Sardans *et al.,* 2008), litter input and root exudation facilitated by gradual establishment of vegetation (Stone *et al.,* 2011) and distribution of proteolytic bacteria (Anjaneyulu *et al.,* 2011; Subrahmanyan *et al.,* 2011). Protease activity showed positive correlation with organic C (r = 0.906; $p<0.01$) and total N (r = 0.921; $p<0.01$) and extractable P (r = 0.964; $p<0.01$). The kinetic parameters of protease activity also exhibited positive correlation with different physicochemical variables (Table 6).

The increase in urease activity is due to the establishment of vegetation, successional changes in textural composition and hydrological regimes (Gracia *et al.,* 1993; Juan *et al.,* 2009), accumulation of organic C (Corstanje *et al.,* 2007), and total N (Gianfreda *et al.,* 1995; Kizilkaya and Bayrakli, 2005; Kizilkaya and Ekberli, 2008), Temperature and soil pH, microbial community composition and synthesis of ureases (Dick *et al.,* 1996; Corstanje *et al.,* 2007). Wide variation in urease activity over time exhibited positive correlation with moisture (r = 0.976; $p<0.01$), organic C (r = 0.973; $p<0.01$), total N (r = 0.978; $p<0.01$) and pH (r = 0.978; $p<0.01$). Kinetic parameters of urease activity exhibited positive correlation with all the

Table 4. Simple correlation analysis between physicochemical properties and kinetic parameters of amylase activity in different age series coal mine spoil (OB0 → OB15) and nearby NF soil across the sites.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP
Amylase activity	0.905**	-0.810*	0.842*	0.985**	0.986**	0.980**	0.982**	0.993**
Vmax	0.865*	-0.761*	0.792*	0.968**	0.970**	0.963**	0.977**	0.996**
Km	-0.973**	0.918**	-0.942**	-0.990**	-0.984**	-0.984**	-0.957**	-0.934**
Vmax/Km	0.770*	-0.648	0.682	0.907**	0.917**	0.904**	0.937**	0.974**

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

Table 5. Simple correlation analysis between physicochemical properties and kinetic parameters of invertase activity in different age series coal mine spoil (OB0 → OB15) and nearby NF soil across the sites.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP
Invertase activity	0.850*	-0.742	0.777*	0.967**	0.961**	0.950**	0.995**	0.994**
Vmax	0.871*	-0.770*	0.803*	0.978**	0.975**	0.965**	0.997**	0.996**
Km	-0.984**	0.938**	-0.958**	-0.984**	-0.978**	-0.982**	-0.940**	-0.918**
Vmax/Km	0.792*	-0.671	0.706	0.930**	0.934**	0.921**	0.964**	0.988**

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

Table 6. Simple correlation analysis between physicochemical properties and kinetic parameters of protease activity in different age series coal mine spoil (OB0 → OB15) and nearby NF soil across the sites.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP
Protease activity	0.777*	-0.663	0.693	0.903**	0.916**	0.906**	0.921**	0.964**
Vmax	0.886**	-0.790*	0.819*	0.982**	0.980**	0.976**	0.988**	0.998**
Km	-0.990**	0.992**	-0.998**	-0.911**	-0.913**	-0.924**	-0.817*	-0.791*
Vmax/Km	0.866*	-0.764*	0.795*	0.973**	0.973**	0.966**	0.987**	0.999**

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

tested variables (Table 7). The differences Km value across the sites suggested that the origin of ureases and their binding status are dissimilar (Dick *et al.,* 1996).

Wide variation in phosphatase activity in different age series mine spoil exhibited positive correlation with organic C (r = 0.997; *p*0.01), total N (r = 0.978; *p*<0.01) and extractable P (r = 0.976; *p*<0.01) across the sites, which appeared to be dependent on the metabolic status of soil and hence used as an index of microbial activity (Kramer and Green, 2000). The study suggested gradual increase in Vmax and Vmax/Km of phosphatase activity over time, which exhibited positive correlation with all the tested soil variables (Table 8).

Estimation of dehydrogenase activity is prerequisite as they are integral part of soil microbes involved in organic matter oxidation. The variation in dehydrogenase activity exhibited positive correlation with moisture (r = 0.985; *p*<0.01), pH (r = 0.987; *p*<0.01), organic C (r = 0.976; *p*<0.01), total N (r = 0.993; *p*<0.01) and extractable P (r = 0.993; *p*<0.01) over time across the sites (Table 9). The Vmax and Km of dehydrogenases are used as useful markers representing the quality and affinity to assess the alternations in microbial activity. Wide variation in dehydrogenase activity as well as the kinetic parameters may be due to the shift in microbial community composition with the changes in community of dehydrogenases (Masciandaro *et al.,* 2000), and hence considered as an index of microbial activity (Dick, 1994; Nannipieri *et al.,* 2002) and metabolic status of soil microbes (Pascule *et al.,* 2000; Taylor *et al.,* 2002; Cladwell, 2005).

Further, simple correlation analysis between microbial communities and soil enzyme activities suggested that the variation in enzyme activities in different age series coal mine overburden spoil may be due to the variation in available nutrients and microbial community composition over time (Table 10). The degree of variability in different soil enzyme activities influenced by microbial community composition has been substantiated by several workers (Nannipieri *et al.,* 1990; Mummey *et al.,* 2002).

Table 7. Simple correlation analysis between physicochemical properties and kinetic parameters of urease activity in different age series coal mine spoil (OB0 → OB15) and nearby NF soil across the sites.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP
Urease activity	0.885**	-0.788*	0.818*	0.976**	0.978**	0.973**	0.978**	0.995**
Vmax	0.922**	-0.843*	0.866*	0.990**	0.990**	0.991**	0.975**	0.986**
Km	-0.994**	0.994**	-0.997**	-0.927**	-0.929**	-0.943**	-0.835*	-0.815*
Vmax/Km	0.861*	-0.760*	0.789*	0.964**	0.968**	0.963**	0.970**	0.993**

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

Table 8. Simple correlation analysis between physicochemical properties and kinetic parameters of phosphatase activity in different age series mine spoil (OB0 → OB15) and nearby NF soil across the sites.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP
Phosphatase activity	0.943**	-0.873*	0.895**	0.998**	0.998**	0.997**	0.978**	0.976**
Vmax	0.951**	-0.889**	0.907**	0.995**	0.995**	0.999**	0.964**	0.968**
Km	-0.991**	0.993**	-0.997**	-0.909**	-0.914**	-0.927**	-0.811*	-0.793*
Vmax/Km	0.872*	-0.776*	0.803*	0.969**	0.974**	0.969**	0.970**	0.992**

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

Table 9. Simple correlation analysis between physicochemical properties and kinetic parameters of dehydrogenase activity in different age series mine spoil (OB0 → OB15) and nearby NF soil across the sites.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP
Dehydrogenase activity	0.894**	-0.800*	0.831*	0.985**	0.987**	0.976**	0.993**	0.993**
Vmax	0.867*	-0.766*	0.796*	0.972**	0.968**	0.964**	0.984**	0.997**
Km	-0.995**	0.986**	-0.994**	-0.938**	-0.939**	-0.948**	-0.856*	-0.832*
Vmax/Km	0.745	-0.620	0.654	0.890**	0.900**	0.887**	0.924**	0.966**

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

Table 10. Simple correlation analysis between microbial communities and soil enzyme activities in different age series coal mine spoil (OB0 → OB15) and nearby NF soil.

Microbial community	Enzyme activity					
	Amylase	Invertase	Protease	Urease	Phosphatase	Dehydrogenase
AZB	0.920**	0.865*	0.816*	0.906**	0.959**	0.912**
ARB	0.933**	0.882**	0.838*	0.921**	0.969**	0.926**
RZB	0.911**	0.865*	0.809*	0.900**	0.958**	0.910**
HAB	0.962**	0.958**	0.867*	0.950**	0.984**	0.976**
SRB	-0.955**	-0.902**	-0.888**	-0.944**	-0.972**	-0.948**
ACT	0.869*	0.799*	0.745	0.849*	0.912**	0.853*
YES	0.939**	0.889**	0.828*	0.922**	0.964**	0.925**
FUN	0.963**	0.912**	0.923**	0.960**	0.976**	0.954**

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

Stepwise multiple regression analysis was performed to quantify the contribution of physicochemical properties and microbial community composition influencing the variation in enzyme activities in different age series coal mine spoil over time (Table 11). About 81.9% variability in amylase activity was explained by clay. The 2nd and 3rd variables of importance were HAB (17.7%) and a marginal effect by moisture. The OC explained 95.7% variability in amylase activity, an additional 16% by TN and 2.7% by clay as 2nd and 3rd variables. The clay, OC and moisture explained 72.3%, 19.2% and 8.4% variability in invertase activity. The 1st, 2nd and 3rd variables explaining the variability in invertase activity were OC (98%), HAB (1.9%) and a marginal effect by fungi respectively. About 94.1% variability in protease activity was explained by OC. The 2nd, 3rd, 4th and 5th variables explaining the variability in protease activity were TN, RZB, ACT and FUN. The TN as 1st variable explained 84.7% variability in protease activity, an additional 8.8% (clay), 5.9% (RZB), and a marginal effect by pH. About 94.7% variability in urease activity was explained by OC, TN (3.6%) and an additional 1.7% by ACT. The TN (95.6%), ACT (3.8%) and a marginal effect by clay explained the variability in urease activity (Table 11).

Soil pH explained 94.5% variability in urease activity. The 2nd, 3rd and 4th variables explaining the variability in urease activity were TN (3.4%), clay (1.4%) and a marginal effect by HAB. About 87.9% variability in phosphatase activity was explained by EP (Table 11). The 2nd, 3rd and 4th variables of importance were pH (7.5%), HAB (3.9%) and a marginal effect by AZB. The OC (97.3%), TN (1.8%) and a marginal effect by clay explained the variability in dehydrogenase activity. Besides, 98.6% variability in dehydrogenase activity was explained by EP. The 2nd and 3rd variables were pH (9%) and a marginal effect by HAB. Soil pH (97.3%), ACT (12%) and ARB (1.4%) explained the variability in dehydrogenase activity as 1st, 2nd and 3rd variable of importance.

The principal component analysis was performed (Ludwig and Reynolds, 1988) in order to discriminate six different age series coal mine spoil in chronosequence and nearby NF soil based on the variability in soil physicochemical properties, microbial community composition, enzyme activities and kinetic parameters, which holds the potential criteria for evaluating the progress of mine spoil genesis. Principal component analysis indicated that the Z1 and Z2 components explained maximum variance with their cumulative percentage of variance 99% and different soil profiles well segregated into independent clusters (Figure 2a). Besides, the RDA analysis explained the contribution of soil physicochemical properties and microbial community composition towards the variability in enzyme activities in different soil profiles (Figure 2b), which explained 99.99% variability based on the datasets through canonical sum of eigen values ($p > 0.05$). RDA analysis provides insight into the multifaceted nature of mine spoil variables determining the microbial community composition and activity in different age series coal mine overburden spoil over time (Urbanova *et al.,* 2011).

Conclusion

The heterogeneity in different physicochemical properties within the landscape with the increase in age of mine overburden spoil influence the relative distribution and abundance microbial communities across the sites. Such variation may be attributed to

the difference in microbial community composition over time, which reflects appealing approach prerequisite for monitoring the progress of mine spoil genesis across the sites. The microbial community structure quickly responds to anthropogenic disturbances induced functional changes through enzyme activities and kinetic parameters based on the available soil nutrients. The study suggested gradual ac-

Table 11. Stepwise multiple regression analysis revealed the contribution of different soil physicochemical properties and microbial community composition influencing the variability in enzyme activities over time across the sites.

Enzyme activity	Equation(s)	R^2*
Amylase activity	= -10.20 + 1.57 Clay	0.819
	= -12.35 + 2.36 Clay + 2.69 HAB	0.996
	= -11.09 + 2.14 Clay + 2.45 HAB + 6.14 MC	0.999
	= 0.9932 + 0.00653 OC	0.957
	= 0.1138 + 0.00349 OC + 1.67 TN	0.973
	= 0.5324 + 0.00548 OC + 1.16 TN + 2.71 Clay	0.999
Invertase activity	= -772.7 + 109 Clay	0.723
	= -698.4 + 112 Clay + 321 OC	0.915
	= -768.9 + 142 Clay + 296 OC + 2.045 MC	0.999
	= -18.69 + 0. 488 OC	0.980
	= 207.78 + 0.6901 OC -59.49 HAB	0.997
	= 270.20 + 0.7688 OC – 105.02 HAB + 43.65 FUN	0.999
Protease activity	= -17.40 + 51.1 OC	0.941
	= -36.93 + 17.2 OC + 0.129 TN	0.982
	= -44.25 + 15.7 OC + 0.136 TN + 5.4 RZB	0.991
	= -37.26 + 14.7 OC + 0.258 TN + 3.2 RZB + 1.023 ACT	0.997
	= -21.56 + 11.5 OC + 0.205 TN + 4.1 RZB + 1.114 ACT + 13.1 FUN	0.999
	= -5.432 + 0.101 TN	0.847
	= -159.3 + 0.223 TN + 68 Clay	0.935
	= -148.5 + 0.126 TN + 71 Clay + 11.56 RZB	0.994
	= -381.2 + 0.318 TN + 54 Clay + 19.69 RZB + 1.056 pH	0.999
Urease Activity	= -4.432 + 15.3 OC	0.947
	= -1.6879 + 14.46 OC + 0.0123 TN	0.983
	= -0.6693 + 13.74 OC + 0.0145 TN + 4.5 ACT	0.999
	= 2.785 + 0.0290 TN	0.956
	= -27.760 + 0.199 TN + 4.85 ACT	0.994
	= -31.205 + 0.351 TN + 3.89 ACT - 0.013 Clay	0.999
	= -441.9 + 71.5 pH	0.945
	= -630.7 + 106.6 pH + 3.91 TN	0.979
	= -908.0 + 139.0 pH + 6.84 TN + 4.2 Clay	0.993
	= -713.5 + 106.1 pH + 6.29 TN + 7.0 Clay + 2.06 HAB	0.999
Phosphatase activity	= -21.3 + 0.3156 EP	0.879
	= -32.8 + 0.5364 EP – 0.356 pH	0.954
	= -42.9 + 0.6584 EP – 0.269 pH + 0.1289 HAB	0.993
	= -39.8 + 0.3518 EP – 0.183 pH + 0.2046 HAB + 2.183 AZB	0.999
Dehydrogenase activity	= -32.84 + 5.31 OC	0.973
	= -46.83 + 7.91 OC -0.288 TN	0.991
	= -0.335 + 1.13 OC -0.312 TN + 1.31 Clay	0.999
	= 0.2270 + 0.01464 EP	0.986
	= -13.333 + 0.00887 EP + 2.176 pH	0.995
	= -39.751 + 0.00113 EP + 6.803 pH – 0.7402 HAB	0.999
	= -32.84 + 5.31 pH	0.973
	= -43.3730 + 7.431 pH – 0.8292 ACT	0.985
	= -45.1814 + 7.780 pH – 0.6941 ACT – 0.2238 ARB	0.999

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

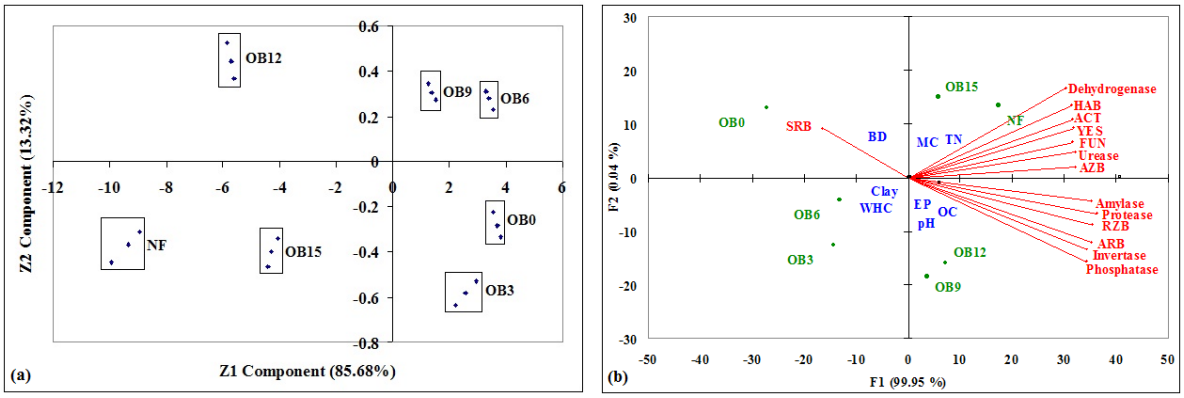

Fig. 2(a). Principal component analysis revealed the segregation of six different age series mine overburden spoil (OB0 → OB15) and the nearby NF soil into independent clusters; (b) RDA analysis based on the variation in physicochemical properties, relative distribution of microbial communities and enzyme activities with site codes for different age series mine spoil (OB0 → OB15) and nearby NF soil.

cumulation of labile carbon inputs by the establishment of vegetation leads to shift in microbial community structure and hence the overall increase in microbial mediated enzyme activities over time. Besides, the kinetic parameters are considered as the sensitive indices of enzyme activities and integrative measure of mine spoil genesis due to the variation in microbial community composition in different age series coal mine overburden spoil over time across the sites.

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References

Alariya, S.S., Sethi, S., Gupta, S. and Gupta, B.L. 2013. Amylase activity of a starch degrading bacteria isolated from soil. *Archives of Applied Science Research.* 5(1) : 15-24.

Alharbi, S.A., Arunachalam, C., Murugan, A.M. and Wainwright, M. 2012. Antibacterial activity of actinomycetes isolated from terrestrial soil of Saudi Arabia. *Journal of Food, Agriculture and Environment.* 10(2) : 1093-1097.

Anderson, J.M. and Ingram, J.S.I. (2nd Edn), 1992. *Tropical Soil Biology and Fertility. A Handbook of Methods*. Oxford University Press, USA.

Anjaneyulu, E., Ramgopal, M., Narasimha, G. and Balaji, M. 2011. Effect of pig iron slag particles on soil physico-chemical, biological and enzyme activities. *Iranica Journal of Energy and Environment.* 2(2): 161-165.

Baldrian, P., Trogl, J., Frouz, J., Snajdr, J., Valaskova, V., Merahutova, V., Cajthaml, T. and Herinkova, J. 2008. Enzyme activities and microbial biomass in topsoil layer during spontaneous succession in spoil heaps after brown coal mining. *Soil Biology and Biochemistry*. 40 : 2107-2115.

Bandick, A.K. and Dick, R.P. 1999. Field management effects on soil enzyme activities. *Soil Biology and Biochemistry*. 31 : 1471-1479.

Banerjee, S.K., Das, P.K. and Mishra, T.K. 2000. Microbial and nutritional characteristics of coal mine overburden spoils in relation to vegetation development. *Journal of Indian Society of Soil Science*. 48 : 63-68.

Bogdevitch, I. and Mikhailouskaya, N. 2009: Relations of enzyme activities with different fractions of soil organic matter. *Polish Journal of Soil Science*. 42 : 175-182.

Brookes, P.C. 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. *Biology and Fertility of Soils*. 19 : 269-279.

Cladwell, B.A. 2005. Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia*. 49: 637-644.

Corstanje, R., Schulin, R. and Lark, R. 2007. Scale-dependent relationships between soil organic matter and urease activity. *European Journal of Soil Science*. 58(5): 1087-1095.

De Mora, A.P., Ortega-Calvo, J.J., Cabrera, F. and Madejon, E. 2005. Changes in enzyme activities and microbial biomass after *in situ* remediation of a heavy metal-contaminated soil. *Applied Soil Ecology*. 28 : 125.

Dick, R.P. (Eds). 1994. Soil enzyme activities as indicators of soil quality. In: Defining soil quality for sustain-

able environment. Doran, J.W., Coleman, D.C., Bezdicek, D.F., and Stewart, B.A.; Special Publication 35. *Soil Science Society of American Journal*, Inc., Madison, WI., pp. 107-124.

Dick, R.P. (Eds). 1997. Soil enzyme activities as integrative indicators of soil health. In: Pankhurst, C.E., Doube, B.M. and Gupta, V.V.S.R.; *Biological Indicators of Soil Health*, CAB International, pp. 121-156.

Dick, R.P., Breakwill, D. and Turco, R. (Eds). 1996. *Soil enzyme activities and biodiversity measurements as integrating biological indicators*. In: Doran, J.W., and Jones, A.J.; Handbook of Methods for Assessment of Soil Quality. *Soil Sci. Soc. of Am.*, Madison, pp. 247-272.

Dutta, R.K. and Agarwal, M. 2002. Effect of tree plantations on the soil characteristics and microbial activity of coal mine spoil land. *Tropical Ecology*. 43(2): 315-324.

Eivazi, F. and Tabatabai, M.A. 1990. Factors affecting glucosidase and galactosidase activities in soils. *Soil Biology and Biochemistry*. 22: 891-897.

Ekberli, I., Kizilkaya, R. and Kars, N. 2006. Urease enzyme and its kinetic and thermodynamic parameters in clay loam soil. *Asian Journal of Chemistry*. 18 : 3097-3105.

Gianfreda, L., Decristofaro, A., Rao, M.A. and Violante, A. 1995. Kinetic behavior of synthetic organo and organo mineral urease complexes. *Journal of Soil Sci. Soc. of America.* 59 : 811-815.

Gianfreda, L., Rao, A.M., Piotrowska, A., Palumbo, G. and Colombo, C. 2004. Soil enzyme activities as affected by anthropogenic alterations: intensive agricultural practices and organic pollution. *Science of the Total Environment*. 341 : 265-279.

Gupta, R. and Lorenz, P. 2010. Bacterial alkaline protease: molecular approaches and industrial applications. *Applied Microbial Biotechnology*. 59: 15-32.

Harris, J.A. and Birch, P. 1989. Soil microbial activity in opencast coal mine restoration. *Soil Use and Management.* 5 : 155-160.

Hu, J.L., Zhu, A.N., Wang, J.H., Dai, J.T., Wang, R.R. and Chen, X.G. 2013. Soil microbial metabolism and invertase activity under crop rotation and no-tillage in North China. *Plant Soil Environment.* 59(11) : 511-516.

Hunter-Cevera, J.C. and Eveleigh, D.E. 1990. *Actinomycetes Soil Biology Guide*, John Wiley and Sons. New York.

Insam, H. and Domsch, K.H. 1988. Relationship between soil organic carbon and microbial biomass on chronosequences of reclamation sites. *Microbial Ecology.* 15: 177-188.

Jackson, M.L. 1958. Soil Chemical Analysis. Prentice-Hall, Englewood, NJ, USA, pp. 485.

Jha, A.K. and Singh, J.S. 1991. Spoil characteristics and vegetation development of an age series of mine spoils in a dry tropical environment. *Vegetation*. 97: 63-76.

Juan, Y.H., Chen, L.J., Wu, Z.J. and Wang, R. 2009. Kinetics of soil urease affected by urease inhibitors at contrasting moisture regimes. *Journal of Soil Science and Plant Nutrition*. 9(2) : 125-133.

Kennedy, N.; Edwards, S. and Clipson, N. 2005. Soil bacterial and fungal community structure across a range of unimproved and semi improved upland grasslands. *Microbial Ecology.* 50 : 463-473.

Kizilkaya, R. and Bayrakli, B. 2005. Effect of N enriched sewage sludge on soil enzyme activities. *Applied Soil Ecology.* 30: 192-202.

Kizilkaya, R. and Dengiz, O. 2010. Variation of land use and land cover effects on some soil physico-chemical characteristics and soil enzyme activity. *Zemdirbyste Agriculture*. 97(2) : 15-24.

Kizilkaya, R. and Ekberli, I. 2008. Determination of the effects of hazelnut husk and tea waste treatments on urease enzyme activity and its kinetics in soil. *Turkey Journal of Agricultural Forest.* 32 : 299-310.

Kizilkaya, R., Askin, T., Bayrakli, B. and Saglam, M. 2004. Microbiological characteristics of soils contaminated with heavy metals. *European Journal of Soil Biology*. 40: 95-102.

Kramer, S. and Green, D.M. 2000. Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in semiarid woodland. *Soil Biology and Biochemistry.* 32 : 179-188.

Kramer, S. and Green, D.M. 2000. Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in semiarid woodland. *Soil Biology and Biochemistry.* 32 : 179-188.

Kujur, M., Gartia, S.K. and Patel, A.K. 2012. Quantifying the contribution of different soil properties on enzyme activities in dry tropical ecosystems. *ARPN Journal of Agriculture and Biological Science.* 7: 763-772.

Kuscu, I.S.K. and Karaoz, M.O. 2015. Soil enzyme and characteristics. *International Journal of Engineering Sciences and Research Technology.* 4(1): 34-38.

Ladd, J.N. and Butler, J.H.A. 1972. Short term assay of soil proteolytic enzymes activities using proteins and dipeptide derivatives as substrates. *Soil Biology and Biochemistry*. 4 : 19-30.

Ludwig, J.A. and Reynolds, J.F. 1988. *Statistical Ecology: A Primer in Method and Computing,* John Wiley and Sons, pp. 337.

Marx, M.C., Kandeler, E., Wood, M., Wermbter, N. and Jarvis, S.C. 2005. Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle size fractions. *Soil Biology and Biochemistry*. 37 : 35-48.

Masciandaro, G., Ceccanti, B. and Ronchi, V. 2000. Kinetics parameters of dehydrogenase in the assessment of the response of soil to vermicompost and inorganic fertilizers. *Biology Fertility and Soils.* 32(6) : 479-483.

Mateos, M.P. and Carcedo, J.G. 1987. Effect of fractionation

on the enzymatic state and behavior of enzyme activities in different structural soil units. *Biology Fertility and Soils*. 4 : 151-154.

Mishra, R. 1968. *Ecology Work Book.* Oxford IBH, New Delhi.

Mummey, D., Stahl, P.D. and Buyer, J. 2002. Microbial markers as an indicator of ecosystem recovery following mine reclamation. *Applied Soil and Ecology Journal*. 21 : 251-259.

Nancucheo, I. and Johnson, D.B. 2005. Significance of microbial communities and interactions in safeguarding reactive mine tailings by ecological engineering. *Applied and Environmental Microbiology*. 77(23) : 8201-8208.

Nannipieri, P., Grego, S. and Ceccanti, B. 1990. Ecological significance of the biological activity in soil. *Soil Biochemistry*. 6 : 293-355.

Nannipieri, P.B., Kandler, E. and Ruggiero, P. (Eds). 2002. Enzyme activity and microbial and biochemical processes in soil. In: Burns, R.G. and Dick, R.P.; *Enzymes in the Environment: Activity, Ecology and Applications.* Marcel Dekker Inc, New York, pp. 1-33.

Olsen, S.R. and Sommers, L.E. (Eds.). 1982. Phosphorous. In: *Methods of Soil Analysis,* P-II, Miller, R.H. and Keeney, D.R.; American Society of Agronomy, Inc, Madison, WI.

Pascual, J.A., Garcia, C., Hernandez, T., Moreno, J.L. and Ros, M. 2000. Soil microbial activity as a biomarker of degradation and remediation processes. *Soil Biology and Biochemistry*. 32 : 1877-1883.

Pascule, J.A., Hernanzed, T., Garcia, C. and Ayuso, M. 1998. Enzymatic activities in an arid soil amended with urban organic wastes: laboratory experiment. *Bioresource Technology*. 64 : 131-138.

Rajan, K., Natarajan, A., Anil, K.S., Badrinath, M.S. and Gowda, R.C. 2010. Soil organic carbon: the most reliable indicator for monitoring land degradation by soil erosion. *Current Science*. 99 : 823-827.

Roberge, M.R. (Eds). 1978. *Methodology of Soil Enzyme Measurement and Extraction*. In: Soil Enzymes, Burns, R.G., London, Academic Press, pp. 341-369.

Ross, D.J. 1983. Invertase and amylase activities as influenced by clay minerals, soil clay fractions and topsoil under grassland. *Soil Biology and Biochemistry*. 15: 287-293.

Sardans, J. and Penuelas, J. 2005. Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. *Soil Biology and Biochemistry*. 37 : 455-461.

Sardans, J. ; Penuelas, J. and Estiarte, M. 2008. Changes in soil enzymes related to C and N cycle and in soil C and N content under prolonged warming and drought in a mediterranean shrubland. *Applied Soil Ecology*. 39 : 223-235.

Schink, B. and Friedrich, M. 2000. Phosphite oxidation by sulfate reduction. *Nature*. 406 : 37-42.

Schoenholtz, S.H., Van Miegroet, H. and Burger, J.A. 2000. A review of chemical and physical properties as indicators of forest soil quality: Challenges and opportunities. *Forest Ecology and Management*. 138 : 335-356.

Sheoran, V., Sheoran, A.S. and Poonia, P. 2010. Soil reclamation of abandoned mine land by revegetation: a review. *International Journal of Soil, Sediment and Water*. 3(2) : 1-20.

Sims, G.K. and Wander, M.M. 2002. Proteolytic activity under nitrogen or sulfur limitation. *Applied Soil Ecology*. 19 : 217-221.

Sinsabaugh, R.L., Carreiro, M.M. and Repert, D.A. 2002. Allocation of extracellular enzymatic activity in relation to litter composition, N deposition and mass loss. *Biogeochemistry*. 60 : 1-24.

Steinweg, J.M., Dukes, J.S., Paul, E.A. and Wallenstein, M.D. 2013. Microbial responses to multi-factor climate change: effects on soil enzymes. *Frontiers in Microbiology*. 4(146) : 1-11.

Stone, M.M., Weiss, M.S., Goodale, C.L., Adams, M.B., Fernandez, I.J., German, D.P. and Allisons, S.D. 2011. Temperature sensitivity of soil enzyme kinetics under N- fertilization in two temperate forests. *Global Change Biology*. 18(3) : 1173-1184.

Subrahmanyan, Z., Archana, G. and Chamyal, L.S. 2011. Soil microbial activity and its relation to soil indigenous properties in semi arid alluvial and estuarine soils of Mahi river basin, Western India. *International Journal of Soil Science*. 6(4) : 224-237.

Tabatabai, M.A. and Bremner, J.M. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry*. 1 : 301-307.

Tabatabai, M.A. and Bremner, J.M. 1971. Michaelis constants of soil enzymes. *Soil Biology and Biochemistry*. 3(4): 317-323.

Tangjang, S. and Arunachalam, K. 2009. Microbial population dynamics of soil under traditional agroforestry systems in Northeast India. *Research Journal of Soil Biology*. 1 : 1-7.

Taylor, J.P., Wilson, B., Mills, M.S. and Burns, R.G. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and sub-soils using various techniques. *Soil Biology and Biochemistry*. 34 : 387-401.

Tordoff, G.M.; Baker, A.J.M. and Willis, A.J. 2000. Current approaches to the revegetation and reclamation of metalliferous mine wastes. *Chemosphere*. 41 : 219-228.

Urbanova, M., Kopecky, J., Valaskova, V., Mareckova, M.S., Elhottova, D., Kyselkova, M., Loccoz, Y.M. and Baldrian, P. 2011. Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. *FEMS Microbiology Ecology*. 78: 59-69.

Vincent, J.M. 1970. A manual for the practical study of root nodule bacteria. IBP Handbook of methods. No.15. Blackwell Scientific Publication, Oxford, pp. 181.

Waldrop, M.P., Balser, T.C. and Firestone, M.K. 2000. Link-

ing microbial community composition to function in a tropical soil. *Soil Biology and Biochemistry*. 32 : 1837-1846.

Wang, B., Liu, G.B., Xue, S. and Zhu, B.B. 2011. Changes in soil physico-chemical and microbiological properties during natural succession on abandoned farmland in the Loess Plateau. *Environ Earth Sciences*. 62 : 915-925.

Zhang, Y.L., Chen, L.J., Sun, C.X., Wu, Z.J., Chen, Z.H. and Dong, G.H. 2010. Soil hydrolase activities and kinetic properties as affected by wheat cropping systems of northeastern china. *Plant Soil and Environment*. 56(11) : 526-532.