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MICROBIAL COMMUNITIES AND ENZYME KINETICS USED AS INDEX OF RECLAMATION IN A CHRONOSEQUENCE COAL MINE OVERBURDEN SPOIL

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ABSTRACT

Restoration ecology is required to access the role(s) of microorganisms in facilitating mine spoil genesis, activity, biodiversity, ecosystem function, and metabolic response as a measure of reclamation in a chronosequence coal mine overburden spoil. Differentiation between six mine spoil and native forest soil was determined based on microbial enumeration and enzyme activity. The relationship between microbial populations and enzyme activity was analyzed. Kinetic parameters (Vmax and Km) have greater significance as early and sensitive indicators to assess changes in microbial activity representing quantity and affinity of soil enzymes (amylase: 2.013-12.629; invertase: 8.193-411.528; protease: 2.949-75.942; urease: 2.544-29.307; phophatase; 3.106-53.608 and dehydrogenase: 0.225-2.129). The catalytic efficiency (Vmax/Km) was estimated to be higher in NF as compared to mine spoil, which reflects an impression on microbial community with a change in soil enzymes. Principal component analysis was performed to discriminate soil profiles, which holds potential as complementary criteria for evaluating the progress of reclamation.

KEYWORDS: **Enzyme activity, enzyme kinetics, microbial communities, reclamation.**

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INTRODUCTION

Open cast coal mining activities lead to pit scarred landscape in the form of mine overburden spoil with adverse changes in soil textural and structural attributes. Being deficient in plant nutrients due to lack of biologically rich top soil, mine overburden spoil represents a disequilibriated geomorphic system and poses problem for the process of pedogenesis[1,2], revegetation[3,4] and restoration[5,6]. In view of increasing mining activities and decreasing soil fertility, it is of utmost concern to access the process of soil reclamation in a chronosequence coal mine overburden spoil over time. Soil microbial community composition regulates the soil[7] and play a fundamental role in biogeochemical cycles[8] by producing various enzymes,[9,10] which are constantly being synthesized, accumulated, inactivated/decomposed in soil[11]. Soil enzymes are necessary for the stabilization of soil structure and function such as nutrient mineralization and cycling, transformation and energy metabolism, decomposition and formation of organic matter,[12,13] decomposition of xenobiotics. However, the microbial population dynamics is influenced by soil physico-chemical and biological properties[14], land use, elemental composition and available soil nutrients, which determine the characteristics of microbial community by providing habitat and nutrients[15,16]. Thus, soil enzyme activities have the potential to be used as indicator of soil quality, sustainability and changes in biogeochemical functions due to management or perturbations, and hence can be used as a useful tool for accessing functional diversity of microbial communities[17].

Soil enzyme activities can be used to characterize the abundance and metabolic activity of soil microorganisms where as kinetic parameters are used to describe the catalytic activity, origin and substrate affinity of enzymes[10]. Soil enzyme activity provides an indication of its amount and overall contribution in soil[11], while enzyme kinetics study can provide useful information regarding their origin, existing status and catalytic properties, state and behavior of soil enzymes[18]. Kinetic parameters are useful markers for assessing rapid changes due to soil management practices[19].

Kinetic parameters (Vmax, Km) are considered to be constant for a specific enzyme under defined experimental condition, but may vary independently[20,21]. Kinetic parameter implies the splitting velocity of enzyme substrate complex and therefore reflecting the conjugation affinity between the enzyme and substrate[22]. Vmax and Km express the quantity of enzyme and substrate affinity respectively[20]. The Vmax refers to the rate at which enzyme substrate complex is dissociated into enzyme and product, and hence represents the potential capacity index of soil. The Km represents the endurance of an enzyme-substrate complex in soil, which indicates how firmly the soil enzyme combines with the substrate. The efficiency of enzymes to decompose the substrate at low concentration is directly related to their Km value[20,23]. Higher is the endurance of an enzyme-substrate complex, lower will be the Km value. Enzymes catalyzing the same reaction, but derived from different sources have different Km values[23]. Besides, Km is independent of enzyme concentration and kinetically reflects the apparent affinity of enzyme for substrate. In other words, smaller the Km value, the greater will be the affinity for substrate[19]. Moreover, enzymes may operate under non-saturating conditions in soil, which supplements Km an important parameter that merits increased attention[24]. Further, catalytic efficiency *i.e.* Vmax/Km represents the formation of an enzyme-substrate complex[25] and the comparison of dispersion of this complex. Higher Vmax/Km value suggested faster dispersion rate of enzyme-substrate complex than its formation[26]. Vmax/Km value of soil enzymes was affected by soil physico-chemical properties[27], available substrate, organic matter[28], stimulation of microbial activity by N, P and K containing compounds, and microbial populations[29].In the present study, six enzymes (amylase, invertase, protease, urease, phosphatase and dehydrogenase) were selected. Amylase is a starch hydrolyzing enzyme that hydrolyzes starch to form low molecular weight reducing

sugar and small quantity of maltose, which are the important energy source of soil microbes[30]. Invertase hydrolyzes sucrose to form a low molecular weight sugar *i.e* glucose and fructose, and hence used as an index for nutritive transformation, energy metabolism and pollutant degradation[23]. Soil proteases are extracellular enzymes, which degrade proteins and release NH_4-N important in N cycle[31]. Estimation of protease activity provides information about the N-mediated biochemical process in soil[32,33,34]. Soil urease activity is extracellular[35], which is responsible for the breakdown of urea into CO_2 and ammonia. Urease activity used as soil quality indicator[36] beneficial for developing strategies for efficient N management[26,37,38]. Phosphatase activity hydrolyzes both esters and anhydrides of phosphoric acid[39]. Dehydrogenase activity can be used as an index of overall microbial activity[23], and is linked with microbial respiratory processes[57]. Thus, it can provide information about the key reactions involved in rate limiting steps of microbial oxidoreduction processes in soil[40].

The biological and biochemical parameters are responsive to small changes in soil due to the microbial activity that directly influences the ecosystem stability and fertility. Differential microbial community may produce distinct enzyme isoforms, which could differ in their catalytic properties. Thus, the knowledge about microbial community, enzyme activities, kinetic properties and their variation in a chronosequence mine overburden spoil over time has considerable biological significance, which paves the way of greater understanding the direction of mine spoil reclamation. Further, the kinetic parameter is most attractive from the standpoint of forensic comparison of mine spoil, since it is independent of sample size and its potential usefulness is thereby enhanced. Realizing this, an attempt was made with an aim to determine the impact of microbial community on enzyme activities, its variation in kinetic properties, and to illustrate if the kinetics of enzyme activities can be used as an index of reclamation.

MATERIALS AND METHODS

(i) Study site

The present study was carried out in Basundhara (west) open cast colliery in the Ib valley of Mahanadi Coalfields Limited (MCL), Odisha, India (Geographical location: 22º 03' 58" - 20º 04' 11" north latitude and 83º 42' 46" - 83º 44' 45" east longitude). The coal mine overburden spoil have been grouped into six different age series (fresh: OB_0, 2 yr: OB_2, 4 yr: OB_4, 6 yr: OB_6, 8 yr: OB_8 and 10 yr: OB_{10}) since inception. Tropical dry deciduous forest was considered to be the natural vegetation of the study site, which experiences a semi-arid climate (1300 mm rainfall y^{-1}, annual average temperature 26°C, and relative humidity 15%).

(ii) Soil sampling

Sampling was done in accordance with the general soil microbiological method. Each mine overburden was divided into 5 blocks, and from each block five spoil samples were collected randomly from (0 - 15) cm soil depth by digging pits (15 × 15 × 15) cm^3 size. Samples collected from each block were referred as 'sub-samples', and were thoroughly mixed to form one 'composite sample'. Similar strategy has been followed for sampling from different age series mine overburden along with nearby forest soil (NF). The composite samples were homogenized, sieved (0.2 mm) and analyzed.

(iii) Enumeration of Soil Microbes

The soil microbial populations were enumerated by the standard spread plate dilution technique. Azotobacter populations (AZB) were estimated using azotobacter mannitol agar[41]. Arthrobacter populations (ARB) were enumerated using Arthrobacter selective media[42]. Rhizobia (RZB) were counted on yeast extract mannitol agar (YEMA) containing Congo red dye[43]. Total heterotrophic aerobic bacteria (HAB) were enumerated using nutrient agar[44]. Sulfur reducing bacterial population (SRB) was enumerated using sulphate reducing medium (Himedia)[45]. Actinomycetes count (ACM) was determined using starch casein agar[46] with 40 µl/ml streptomycin and 50 µl/ml griseofulvin. Yeast counts (YES) were estimated using

potato sucrose agar[47]. Fungal count (FUN) was performed using Rose Bengal agar with 50 µl/ml streptomycin[40].

iv) Soil enzyme activities

Amylase activity of different coal mine overburden spoil as well as nearby NF samples were determined by spectrophotometric method (540nm) by taking starch as substrate and incubated at 30°C for 24hr[48]. Invertase activity was estimated by using sucrose as substrate, incubated at 37°C for 24hr, and determined by taking absorbance at 540nm using spectrophotometer[49]. Protease activity was also determined by spectrophotometric method (700nm) with sodium caseinate as a substrate[50]. Urease activity of different mine overburden soil samples as well as NF was determined by titration method using 0.005 N H_2SO_4 with boric acid indicator[51]. The phosphatase activities of different mine overburden spoil as well as NF samples were determined by spectrophotometric method (400nm) using *p*-nitrophenyl phosphate as substrate[52]. Dehydrogenase activity in different soil profiles were measured by spectrophotometric method (485nm) following the reduction of 2,3,5- triphenylotetrazolium chloride (TTC) as an artificial electron acceptor to red-coloured triphenyl formazon (TPF) [23, 40].

(v) Kinetic parameters determination

The soil enzymes follow Michaelis Menten kinetics, despite soil being considered as a discontinuous, structured and heterogenous system[53]. Michaelis–Menten equation linearized by Lineweaver-Burk was used to determine Vmax and Km, and estimated by the intercept and slope respectively[21] in triplicates by taking six substrate concentrations such as amylase (5-50 mM), invertase (10-100 mM), protease (1-10 mM), urease (5-45 mM), phosphatase (10-50 mM) and dehydrogenase (10-90 mM) respectively.

(vi) Statistical analysis

Simple correlation analysis was performed to test the level of significance with respect to the variation in microbial communities on enzyme activities using SPSS Statistics 17.0. Besides, simple correlation analysis was also performed between soil physico-chemical properties, enzyme activities and kinetics (Vmax, Km and Vmax/Km). Further, principal components analysis (PCA) was performed in order to discriminate six mine overburden spoil and NF soil using Statistrix PC DOS Version - 2.0 (NH Analytical software).

RESULTS AND DISCUSSION

Each soil develops its own physico-chemical, biological, and microbiological characteristics due to the transformation of energy and matter during the development of soil. Such variation in microbial environment is the driving force that can able to segregate microbial communities among six different coal mine overburden spoil in chronosequence as well as the nearby NF soil, which is the function of variable availability of nutrient and energy, microclimatic conditions and the heterogeneity of vegetation cover that can bring to a shift in the microbial community[7].

(i) Microbial enumeration

The microbial community compositions in a chronosequence coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) as well as nearby NF soil in terms of CFU per gram soil have been presented (Table 1). The data suggested that the NF soil has higher microbial activity, and microbial population as compared to mine overburden spoil. Azotobacter belongs to family *Azotobacteraceae*, which is obligately aerobic, chemolithotrophic, dinitrogen-fixing bacteria. The nutrient deficient profile of OB_0 (2×10^{-1}) may be the possible reason for minimal azotobacter count as compared to OB_{10} (28×10^{-3}). Both the numbers and proportions of arthrobacter count decreases in OB_0 (17×10^{-2}) as compared to OB_{10} (50×10^{-3}) due to increasing soil acidity[42]. The numerical predominance of arthrobacters varied among mine spoil because of their nutritional versatility of extreme resistance to drying and starvation[54]. Being highly specific for their symbiotic relationship with leguminous plants to assimilate gaseous nitrogen (nitrogen-

fixing), higher rhizobial count was found in NF (25×10^{-4}) as compared to different age series mine spoil. Besides, the heterogeneity of vegetation cover may be the possible reason for such variation in rhizobial counts[55] with minimal in OB_0 (6×10^{-1}) as compared to OB_{10} (19×10^{-3}). The HAB population was found to be less in mine spoil as compared to NF (27×10^{-8}). Environmental stresses brought by mining activities and contamination by heavy metals in OB_0 (29×10^{-2}) as compared to OB_{10} (62×10^{-6}) could be adducted for the reduction in microbial population and diversity[7]. The SRB count was found to be highest in OB_0 (37×10^{-6}) and showed a decline trend over time (Table 1). Being nutrient deficient condition but enriched with pyrite contamination, OB_0 favors the enrichment of SRB[3,56]. Utilization of sulfur compounds, oxidation of inorganic P compounds, sulphate reduction accounted for OC mineralization[57] was exhibited by SRB, which indicates the importance in both sulphur and carbon cycles.

Table 1

Distribution and abundance of different microbial population in a chronosequence coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) as well as native forest soil.

Microbial community	CFUg^{-1} dry wt. Spoil						
	OB_0	OB_2	OB_4	OB_6	OB_8	OB_{10}	NF
Azotobacter (AZB)	2×10^{-1}	7×10^{-1}	13×10^{-2}	21×10^{-2}	18×10^{-3}	28×10^{-3}	65×10^{-4}
Arthrobacter (ARB)	17×10^{-2}	19×10^{-2}	26×10^{-2}	31×10^{-3}	33×10^{-3}	50×10^{-3}	38×10^{-4}
Rhizobia (RZB)	6×10^{-1}	8×10^{-1}	12×10^{-1}	21×10^{-2}	14×10^{-3}	19×10^{-3}	25×10^{-4}
Heterotrophic Aerobic Bacteria (HAB)	29×10^{-2}	31×10^{-2}	35×10^{-2}	22×10^{-4}	49×10^{-4}	62×10^{-6}	27×10^{-8}
Sulfur reducing bacteria (SRB)	37×10^{-6}	31×10^{-6}	19×10^{-4}	11×10^{-4}	18×10^{-3}	13×10^{-3}	15×10^{-1}
Actinomycetes (ACT)	3×10^{-2}	14×10^{-2}	18×10^{-2}	9×10^{-3}	12×10^{-3}	18×10^{-3}	51×10^{-3}
Yeast (YES)	3×10^{-1}	11×10^{-1}	14×10^{-1}	21×10^{-1}	12×10^{-2}	16×10^{-2}	7.8×10^{-3}
Fungi (FUN)	3×10^{-1}	5×10^{-1}	12×10^{-1}	11×10^{-2}	14×10^{-2}	23×10^{-2}	19×10^{-4}

The dominance of actinomycetes (aerobic, gram-positive bacteria), one of the major groups of soil population would be greatly influenced by geographical distribution including temperature, pH, OC content, aeration and moisture[58], which may be the possible reason for higher ACT count in NF ($51x10^{-3}$) as compared to OB_0 (3×10^{-2}). Being relatively acid-tolerant[59], least ACT count was observed in OB_0 as compared to OB_{10} (18×10^{-3}). The distribution of yeast population is also determined by a number of environmental factors such as pH, moisture, OC content, adequate aeration and relatively high concentration of utilizable substrates. This may be the possible reason for higher YES count in NF (7.8×10^{-3}), and minimal count in OB_0 (3×10^{-1}) as compared to OB_{10} (16×10^{-2}). Soil fungi are mostly opportunistic (zymogenous), which may occur either as free-living or in mycorrhizal association with plant roots primarily in topsoil important for stabilizing soil structure by forming macroaggregates. Higher fungal population in NF (19×10^{-4}) may be due to prevailing favourable moisture, sufficient organic matter[60], and humus accumulation that enhanced colonization in soil. Least fungal count in acidic soil[61] such as OB_0 ($3x10^{-1}$) has also been reported (Table 1).

(ii) Soil enzyme activities

The biochemical functions in soil subsystems are catalyzed by soil enzymes and hence considered to be a bio-indicator of soil fertility[12] because of their involvement in biogeochemical cycling[29,62]. The 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 coal mine overburden spoil[63]. Soil enzymatic studies can be potentially used to monitor and assess soil restoration process of perturbed ecosystem[23,64,65]. Besides, the enzyme activity indices appeared to be more informative and highly reliable as it responded more clearly to parent material properties, and hence considered to be an important indicator of mine spoil genesis over time[2,66]. A comparative analysis on the activities of soil

enzymes (amylase, invertase. protease, urease, phosphatase and dehydrogenase) indicated minimal activity in OB_0, which may be due to the reduced microbial population caused by the toxic effects and oxidative stress of mine spoil metal impurities, there interference in osmotic balance and nutrient deficiency[67].

Amylase is complex enzyme [*i.e.* α-amylase (E.C. No. 3.2.1.1)], which hydrolyzes starch to reducing sugar. Amylase hydrolyzes α-1,4-glucan links in polysaccharides containing three or more α-1,4-linked D-glucose units. The amylase activity showed a range of 1.253 to 4.571 μg glucose/g spoil/hr with minimum in OB_2 and maximum in OB_{10} (Table 2). It is evident from the data that the amylase activity is quite higher in NF soil (13.124 μg glucose/g soil/hr) 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[68], and diversity of microbiota[69,70]. Invertase (β-fructofuranosidase; E.C. 3.2.1.5) showed progressive increase from 6.642 μg sucrose/g spoil/hr (OB_2) to 348.331 μg glucose/g spoil/hr (OB_{10}) (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 associated toxic effects on soil microbes[71,72] inhibiting microbial growth, thus reducing the synthesis and secretion of enzymes. The interaction of heavy metals that causes changes in the active center and structure of soil enzymes make active amylase and invertase concentration decrease inhibiting the decomposition of starch and sucrose respectively.

Table 2

Enzyme activities of mine spoil samples collected from a chronosequence coal mine overburden ($OB_0 \rightarrow OB_{10}$) as well as native forest soil.

Parameters	Mine spoil collected from different age series coal mine overburdens						Native Forest soil (NF)
	OB_0	OB_2	OB_4	OB_6	OB_8	OB_{10}	
Amylase activity (μg glucose/g/hr)	nd	1.253 ± 0. 124	2.034 ± 0.112	2.263 ± 0.171	3.655 ± 0.279	4.571 ± 0.205	13.124 ± 1.153
Invertase activity (μg sucrose/g/hr)	nd	6.642 ± 0.498	25.228 ± 5.211	83.331 ± 4.781	121.013 ± 7.372	348.331 ± 4.636	849.335 ± 6.389
Protease activity (μg tyrosine/g/hr)	nd	3.042 ± 0.058	8.801 ± 0.534	23.692 ± 1.428	28.437 ± 2.127	39.266 ± 2.574	215.813 ± 12.911
Urease activity (μg NH_4^+/g /hr)	nd	3.354 ± 0.027	5.299 ± 0.121	9.463 ± 0.261	14.317 ± 1.032	20.121 ± 1.576	58.451 ± 2.834
Phosphatase activity (μg PNP/g /hr)	nd	nd	10.108 ± 1.005	26.495 ± 1.554	35.407 ± 2.901	49.617 ± 2.250	92.118 ± 3.107
Dehydrogenase activity (μg TPF/g /hr)	0.056 ± 0.011	0.144 ± 0.039	0.291 ± 0.034	0.458 ± 0.052	0.948 ± 0.041	1.275 ± 0.043	4.006 ± 0.115

[nd]: Not detectable. Values are mean ± SD, calculated from seasonal (summer, rainy, winter) mean values.

The protease activity was comparatively higher in NF soil (215.813 μg glucose/g soil/hr) with respect to different mine overburden spoil (Table 2). The progressive increase in protease activity from OB_2 (3.042 μg tyrosine/g spoil/hr) to OB_{10} (39.226 μg glucose/g spoil/hr) was due to the progressive improvement in available soil nutrients and proteinaceous substrate in soil organic matter facilitated by vegetation cover, associated difference in litter inputs and root exudation in course of time[73] and distribution of proteolyitc bacteria[31,34,70]. Urease (Urea amidohydrolase; E.C. 3.5.1.5) belongs to soil hydrolases, which is mostly an extracellular enzyme involved in urea hydrolysis (hydrolysis of soil amide N) into CO_2 and NH_3. Urease acts as intermediary enzyme in the transformation of organic nitrogen into inorganic forms[74]. Hence, emphasis on urease activity has been given in order to evaluate N supply to plants, because large N losses to atmosphere by volatilization mediated by these enzymes. Higher urease activity was exhibited by nearby NF soil (58.541 μg NH_4^+/g soil/hr). The urease activity showed an increasing trend from OB_2 (3.354 μg NH_4^+/g soil/hr) to OB_{10} (20.121 μg NH_4^+/g soil/hr), which may be due to the variation in physico-chemical properties of soil[27], moisture content[31], organic matter, gradual accumulation of N over time[28,38], and synthesis of urease enzyme by the increasing

microbial population[29]. However, urease activity in OB_0 was beyond detectable limit due to the nutrient deficient situation with altered geomorphic system.Acid phosphatase (Orthophosphoric monoester phosphohydrolase; E.C. 3.1.3.2) hydrolyzes orthophosphoric monoester to alcohol and orthophosphate. Phosphatase acts as intermediary enzyme in the transformation of organic phosphate into inorganic forms[74], and has a role in P cycling[75,76]. Wide variation in phosphatase activity was exhibited, which ranged from 10.108 μg PNP/g spoil/hr (OB_4) to 49.617 μg PNP/g spoil/hr (OB_{10}). The phosphates activity in OB_0 and OB_2 were beyond detectable limit (Table 2). Phosphatase activity appeared to be more dependent on the metabolic state of soil, biological activity of microbial population, and hence can be used as an index for microbial activity[76].

Dehydrogenase is an intracellular oxido-reductase group of enzymes involved in electron transport system of oxygen metabolism, regulating the metabolic reactions in soil, and is considered to be an index of overall microbial activity[11,40] and metabolic status of soil microbial community[69,77,78]. The dehydrogenase activity showed a consistent increase from OB_0 (0.056 μg TPF/g spoil/hr) to OB_{10} (1.275 μg TPF/g spoil/hr). The highest dehydrogenase activity was observed in NF soil (4.006 μg TPF/g soil/hr) as compared to different mine spoil (Table 2), which may be due to higher organic matter that support increased microbial activity and microbial biomass[79], and concentration of soil dehydrogenase[80]. Further, the variation in dehydrogenase activity with respect to different mine overburden spoil in chronosequence may be attributed to the change in microbial community composition with a change in the community of soil dehydrogenase[19].The relationship between successional level of microbial communities and enzyme activities determine the degree of variability in a chronosequence coal mine overburden spoil (Table 3), which has been substantiated by many workers[23,81,82].

Table 3
Simple correlation between microbial communities and enzyme activity.

Microbial community	Enzyme activity					
	Amylase	Invertase	Protease	Urease	Phosphatase	Dehydrogenase
AZB	0.952**	0.936**	0.989**	0.953**	0.848*	0.961**
ARB	0.972**	0.961**	0.998**	0.976**	0.895**	0.980**
RZB	0.961**	0.947**	0.993**	0.963**	0.866*	0.971**
HAB	0.944**	0.929**	0.985**	0.946**	0.835*	0.953**
SRB	-0.512	-0.447	-0.395	-0.490	-0.634	-0.458
ACT	0.991**	0.991**	0.982**	0.998**	0.972**	0.996**
YES	0.985**	0.976**	0.995**	0.987**	0.919**	0.993**
FUN	0.943**	0.925**	0.985**	0.944**	0.833*	0.952**

***** Correlation is significant p < 0.01, and * correlation is significant p < 0.05 (2-tailed test). (AZB: Azotobacter; ARB: Arthrobacter; RZB: Rhizobia; HAB: Heterotrophic aerobic bacteria; SRB: Sulfur reducing bacteria; ACT: Actinomycetes; YES: Yeast and FUN: Fungi)***

(iii) Kinetic parameters:

Kinetics study of amylase activity indicated an increasing trend in Vmax from OB_0 (2.013 μg/g soil/hr) to OB_{10} (12.629 μg/g soil/hr). Higher Vmax value was estimated in NF soil (56.788 μg/g soil/hr) as compared to different mine spoil (Table 4).

Table 4
Km and Vmax (µg/g spoil/hr) of different enzymes.

Enzymes	Kinetic parameters	OB0	OB2	OB4	OB6	OB8	OB10	NF
Amylase	Vmax	2.013	4.511	6.537	7.044	9.113	12.629	56.788
	Km (mM)	50.709	46.531	41.279	39.946	34.027	23.156	13.219
	Vmax/Km	0.0396	0.0969	0.1583	0.1763	0.2678	0.5453	4.2959
	R^2	0.983**	0.994**	0.814*	0.883**	0.859*	0.958**	0.984**
Invertase	Vmax	8.193	13.482	31.199	90.251	135.115	411.528	927.368
	Km (mM)	46.531	39.069	37.062	33.151	30.573	21.708	12.192
	Vmax/Km	0.1760	0.3450	0.8418	2.7224	4.4194	18.9574	76.0636
	R^2	0.905**	0.815*	0.807*	0.897**	0.926**	0.913**	0.951 **
Protease	Vmax	2.949	6.766	14.044	32.754	41.394	75.942	270.593
	Km (mM)	18.372	16.057	13.043	12.803	12.549	11.019	10.169
	Vmax/Km	0.1605	0.4213	1.0767	2.5583	3.2985	6.8919	26.6095
	R^2	0.991**	0.952**	0.839*	0.965**	0.882**	0.884**	0.935**
Urease	Vmax	2.544	5.629	9.406	15.567	22.588	29.307	65.518
	Km (M)	0.089	0.075	0.062	0.054	0.048	0.041	0.031
	Vmax/Km	28.5842	75.0533	151.7096	288.2777	470.5833	714.8048	2113.4838
	R^2	0.912**	0.947**	0.959**	0.922**	0.864**	0.951**	0.897**
Phosphatase	Vmax	3.106	5.126	14.308	29.671	39.384	53.608	95.608
	Km (M)	0.091	0.078	0.064	0.053	0.049	0.037	0.028
	Vmax/Km	34.1318	65.7179	223.5625	559.8301	803.7551	1448.8648	3414.5714
	R^2	0.899**	0.905**	0.894**	0.945**	0.911**	0.944**	0.932**
Dehydrogenase	Vmax	0.225	0.351	0.419	0.706	1.356	2.129	5.831
	Km (M)	0.178	0.152	0.126	0.085	0.071	0.054	0.022
	Vmax/Km	1.2640	2.3092	3.3253	8.3058	19.0985	39.4259	265.0545
	R^2	0.923**	0.991**	0.988**	0.951**	0.958**	0.929**	0.981**

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

Soil invertase can catalyze the rupture of β-glucose bonds resulting in sucrose hydrolysis, which reflects the transformation mechanism of OC, and serves as an evaluation indicator of soil maturity and fertility level. Comparison of Vmax value of invertase activity showed similar trend *i.e.* progressive increase from OB_0 (8.193 µg/g soil/hr) to OB_{10} (411.528 µg/g soil/hr). The Vmax in NF soil (927.368 µg/g soil/hr) was found to be comparatively higher than different coal mine spoil. The Km value of soil amylase was found to be highest (*i.e.* 50.709 mM) in OB_0, but decreased with increasing soil moisture[83]. The Km value estimated in NF soil was found to be minimal *i.e.* 13.219 mM. Similar trend was also exhibited in case of invertase activity, where Km value ranges from 46.531 mM (OB_0) to 12.129 mM (NF). Further, Vmax/Km value of amylase was estimated to be lowest in OB_0 (0.0396) as compared to NF soil (4.2959). Similar trend was also exhibited in soil invertase *i.e.* minimum in OB_0 (0.1760) and maximum in NF soil (76.0636).

The amylase, invertase activity and its kinetic parameters showed positive correlation with different physico-chemical parameters (Table 5 & 6).

Table 5
Simple correlation between soil properties and amylase activity.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	Amylase activity	Vmax	Km	Vmax/Km
Amylase activity	0.763*	-0.630	0.805*	0.995**	0.862*	0.963**	0.959**	0.954**	1			
Vmax	0.645	-0.504	0.693	0.996**	0.760*	0.906**	0.993**	0.991**	0.984**	1		
Km	-0.907**	0.793*	-0.942**	-0.881**	-0.972**	-0.982**	-0.783*	-0.772*	-0.922**	-0.847*	1	
Vmax/Km	0.583	-0.435	0.634	0.985**	0.708	0.872*	0.999**	0.998**	0.968**	0.997**	-0.807*	1

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

Table 6
Simple correlation between soil properties and invertase activity.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	Invertase activity	Vmax	Km	Vmax/Km
Invertase activity	0.729	-0.571	0.780*	0.972**	0.843*	0.953**	0.943**	0.936**	1			
Vmax	0.732	-0.571	0.784*	0.965**	0.846*	0.953**	0.933**	0.925**	0.999**	1		
Km	-0.922**	0.814*	-0.950**	-0.884**	-0.973**	-0.977**	-0.782*	-0.772*	-0.922**	-0.926**	1	
Vmax/Km	0.636	-0.478	0.690	0.985**	0.762*	0.909**	0.983**	0.979**	0.986**	0.982**	-0.859*	1

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

Higher amylase and invertase activity in mine spoil over time may be due to the available nutrients leading to an increase in microbial biomass[30,84] and microbial diversity[69] causes increased microbial enzyme production, and hence higher Vmax[73,85]. Besides, the type of organic matter was shown to influence amylase and invertase activity more than the quantity of organic matter. Further, the substrate diffusion rate affects Km due to the typical heterogenic system of soil. The stronger enzyme-substrate affinity (lower Km value) in higher moisture content may be caused by higher diffusion rate because of more water solubility[10]. The potential reason of lower Km in NF as compared to different mine spoil may be due to the higher water holding capacity because of higher organic matter content[10]. The lowest value of Vmax/Km in OB_0 may be attributed to the extreme dryness in mine overburden spoil that limits the solubility and restrict the movement of available OC as energy source and thus limits the microbial respiration.

The Vmax of soil protease varies from 2.949 to 270.593 µg/g soil/hr with minimum in OB_0 and maximum in NF soil. The Km and Vmax/Km value of soil protease ranged from 18.372mM (OB_0) to 10.169mM (NF), and 0.1605 (OB_0) to 26.6095 (NF) respectively (Table 4). Such variation in protease activity among different coal mine overburden spoil as well as NF soil was contributed by the progressive improvement in OC, TN and EP over time[32,33] and the distribution of proteolyitc bacteria[31,34,70]. The gradual accumulation of proteinaceous substrate facilitated by the vegetation cover over time and the associated difference in litter inputs and root exudation in NF soil as compared to OB_0 may have contributed to increased Vmax value[73]. The protease activity and its Vmax exhibited a positive correlation with MC, pH, OC, TN and EP (except BD), whereas its Km value showed negative correlation with all the tested soil properties except BD (Table 7).

Table 7
Simple correlation between soil properties and protease activity.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	Protease activity	Vmax	Km	Vmax/Km
Protease activity	0.654	-0.507	0.698	0.995**	0.766*	0.911**	0.992**	0.990**	1			
Vmax	0.693	-0.541	0.740	0.994**	0.805*	0.936**	0.979**	0.975**	0.995**	1		
Km	-0.980**	0.978**	-0.977**	-0.700	-0.947**	-0.869*	-0.550	-0.540	-0.636	-0.674	1	
Vmax/Km	0.674	-0.521	0.722	0.993**	0.789*	0.927**	0.983**	0.979**	0.996**	1.000**	-0.657	1

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

Comparisons of Vmax of urease activity showed similar trend *i.e.* progressive increase from 2.544 µg/g soil/hr (OB_0) to 65.518 µg/g soil/hr (NF) (Table 4). Factors contributing higher Vmax in OB_{10} as compared to OB_0 may include the absence of disturbing, gradual establishment of vegetation cover in course time, which mitigate the problem of runoff of the residual soil nutrients[29]. The greater difference in Km value of urease activity, which varies from 0.089 M (OB_0) to 0.031 M (NF), suggested that the binding status and

origin of soil urease is dissimilar. Further, the different mine spoil undergo different pedogenic processes resulted variation in their physico-chemical properties[68] unlikely to have similar urease origin. The catalytic efficiency (Vmax/Km) of urease in OB_0 (28.5842) was estimated to be lowest than NF soil (2113.4838), which may be due to the variation in soil organic matter content and successional changes in soil texture over time[28,86]. Further, the variances in kinetic parameters of urease may be due to the variation in the soil physico-chemical properties[27,87], moisture content[32,84], soil depth, heavy metals, temperature and pH, microbial community[88], organic matter and gradual accumulation of N[28,38], and synthesis of urease by increased microbial population[29]. The urease activity as well as Vmax exhibited a positive correlation with clay %, WHC, MC, pH, OC, TN and EP (Table 8). Km of urease had no significant correlation with urease activity suggesting that it is independent of enzyme concentration[19].

Table 8
Simple correlation between soil properties and urease activity.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	Urease activity	Vmax	Km	Vmax/Km
Urease activity	0.755*	-0.610	0.797*	0.993**	0.857*	0.963**	0.960**	0.955**	1			
Vmax	0.814*	-0.673	0.852*	0.977**	0.904**	0.985**	0.926**	0.919**	0.994**	1		
Km	-0.995**	0.949**	-0.998**	-0.766*	-0.986**	-0.925**	-0.625	-0.614	-0.605	-0.856*	1	
Vmax/Km	0.736	-0.586	0.782*	0.991**	0.845*	0.958**	0.963**	0.958**	0.999**	0.992**	-0.788*	1

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

The Vmax value of soil phosphatase showed a range from 3.106 to 95.608 µg/g soil/hr with minimum in OB_0 and maximum in NF soil. The Km value of phosphatase varied from 0.091 M (OB_0) to 0.028 M (NF), and Vmax/Km ranged from 34.1318 (OB_0) to 3414.5714 (NF) (Table 4). The phosphatase activity, Vmax and Vmax/Km showed positive correlation with all the tested variables except BD (Table 9).

Table 9
Simple correlation between soil properties and phosphatase activity.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	Phosphatase activity	Vmax	Km	Vmax/Km
Phosphatase activity	0.875**	-0.742	0.906**	0.935**	0.945**	0.995**	0.860*	0.852*	1			
Vmax	0.878**	-0.745	0.909**	0.936**	0.948**	0.996**	0.860*	0.852*	1.000**	1		
Km	-0.992**	0.937**	-0.997**	-0.766*	-0.986**	-0.930**	-0.627	-0.616	-0.915**	-0.918**	1	
Vmax/Km	0.776*	-0.625	0.820*	0.976**	0.877**	0.974**	0.935**	0.928**	0.980**	0.981**	-0.834*	1

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

Estimation of dehydrogenase activity is pre-requisite, as they are an integral part of soil microorganisms and involved in organic matter oxidation. Higher Vmax value was observed in NF (5.831 µg/g soil/hr) as compared to OB_0 (0.225 µg/g soil/hr) (Table 4), which may be due to higher organic matter in NF soil that support increased microbial activity and microbial biomass, consequently the concentration of soil dehydrogenase[80]. The variation in Km of dehydrogenase from 0.178 M (OB_0) to 0.022 M (NF) with respect to different coal mine spoil as well as NF soil can be explained on the basis of the capability of the enzyme catalyzing same reaction can have different sources in soil, and thus different Km values[23]. Km value reflects the apparent affinity of the enzyme for the substrate: the smaller the Km value, the greater the affinity[19]. The Vmax/Km of dehydrogenase was found to be maximum in NF (265.0545) and minimum in OB_0 (1.2640), which may be due to the changes in microbial community with a change in the community of

dehydrogenase[19]. Therefore, the Vmax and Km of dehydrogenase act as useful markers to assess the changes in microbial activity, since they represented the quality and affinity of dehydrogenase. The dehydrogenase activity, Vmax and Km is positively correlated with different variables (Table 10).

Table 10
Simple correlation between soil properties and dehydrogenase activity.

Parameters	Clay	BD	WHC	MC	pH	OC	TN	EP	DH-ase activity	Vmax	Km	Vmax/Km
DH-ase activity	0.728	-0.578	0.775*	0.992**	0.840*	0.954**	0.966**	0.962**	1			
Vmax	0.725	-0.567	0.773*	0.986**	0.840*	0.954**	0.960**	0.955**	0.999**	1		
Km	-0.988**	0.915**	-0.990**	-0.783*	-0.987**	-0.939**	-0.650	-0.640	-0.805*	-0.804*	1	
Vmax/Km	0.600	-0.446	0.652	0.987**	0.726	0.885**	0.997**	0.995**	0.982**	0.979**	-0.689	1

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

Further, in order to discriminate six coal mine overburden spoil in chronosequence as well as NF soil, principle component analysis[89] was performed on the basis of their soil enzyme activity and kinetic parameters, which explained the maximum variance (components Z1and Z2) and their cumulative percentage of variance was 99% (Figure 1). Thus, the study clearly revealed that the soil enzyme activity and kinetic parameters can serve as an integrative measure of soil quality.

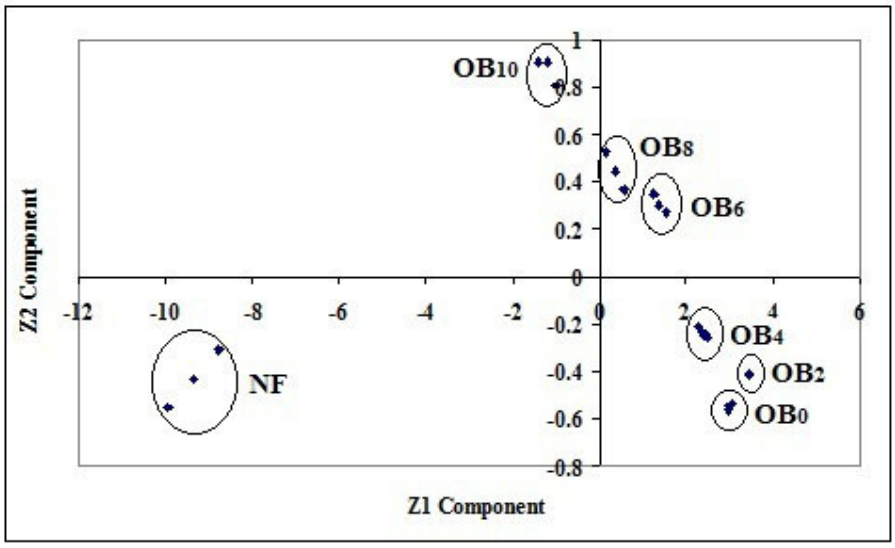

Figure 1
Principal component analysis based on different enzyme activities and kinetic parameters in a chronosequence coal mine overburden spoil as well as native forest soil.

CONCLUSION

Heterogeneity in soil physicochemical properties within the landscape is related to the abundance and distribution of microbial communities, which may be the possible reason for such variation in microbial activity. Due to the dispersed nature of soil profiles, a consistent and appealing approach is pre-requisite for soil quality assessment and sustainable development. During the early stage of development at the reclaimed sites, the soil microbial community responded quickly to functional changes, because they

take the advantage of nutrients that are readily available in disturbed sites. Nevertheless, the enzyme activities and kinetic parameters are also very sensitive to anthropogenic disturbances, and show a quick response to the induced changes. Chronosequence studies on coal mine overburden spoil indicated the gradual accumulation of labile sources of carbon inputs supplemented by the vegetation cover lead to changes in microbial community over time and hence an increase in overall microbial activity. The kinetic parameters are sensitive index of the changes in enzyme activity contributed by the variation in soil microbial community over time, and therefore proposed as a measure of success of reclamation efforts.

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