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Distribution of N-mineralizing Enzymes in Soil Aggregate Fractions over 46 Years Application of Inorganic and Organic Fertilizers in a Tropical Rice-Rice System

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The stability of enzymes in soil has been attributed to enzyme association with organic matter and the protection provided within soil aggregates. Enzymes namely urease, amidase and protease significantly affects nitrogen (N) mineralization and their assessment is crucial to study the nutrient cycling. Therefore, the objective of this study was to evaluate the hypothesis that the long-term application of farmyard manure (FYM) and inorganic fertilizers (N-nitrogen; P-phosphorus; K-potassium) impact the distribution pattern of enzymes namely, urease, amidase and protease in different fractions of water stable soil aggregates, and whole soil at 0-15 cm and 15-30 cm soil depth. The treatments comprised of unfertilized control and different combinations of inorganic fertilizers and FYM *viz.* control, N, NP, NK, NPK, FYM, FYM+N, FYM+NP, FYM+NK and FYM+NPK. A significant difference in soil aggregate size distribution was observed at two sampling depths. Total water stable aggregates (WSA) ranged between 69.8-91.2% in which 0.1-0.053 mm aggregate fraction contributed (2.11-3.87%), whereas 0.25-0.5 mm aggregate fraction was having the highest (27.3-32.6%) contribution. The activities of three enzymes in whole soil as well in aggregate fractions were lowest in control and highest in FYM+NPK except for amidase, which was having highest activity in FYM alone treatment. Activities of all the three enzymes were highest in aggregate fraction of 5-2 mm. Activities of three enzymes in whole soil as well as in aggregate fractions were lower at 15-30 cm compared to 0-15 cm soil depth. It may be concluded from this study that long-term addition of FYM alone or in combination with inorganic fertilizer increases the macroaggregate (5-2 mm) and hence the overall activities of N mineralization enzymes.

Key words: Nitrogen mineralization, soil enzymes, soil aggregate, physical fractionation, farmyard manure

The nitrogen (N) cycle is one of the key element cycles occurring on the earth. At the macroscale level, N transformation processes are often observed to vary with soil systems, with seasons, and along the soil profile because of heterogeneity associated with soil environment (Vitousek *et al.* 1997). In turn, at the microscale level, microorganisms that mediate the processes and their activity are responsible for spatial variation in the soil ecosystem. Soil N mineralization is the most significant process that determines the quantity of N available to plants. Accordingly, assessment of enzymes involved in N dynamics in the soil is crucial to study the nutrient cycling specially in rice-rice cropping systems (Muruganandam *et al.* 2009). Soil enzymes are generally of microbial origin (Ladd 1978) and these are associated with viable proliferating cells and also can exist in extracellular manner. Urease enzyme catalyzes hydrolysis of urea fertilizers, amidase catalyzes the hydrolysis of aliphatic amides and similarly, proteases in the soil play a significant role in N mineralization (Ladd and Jackson 1982). Enzymes are stabilized in soil by adsorption to clay surfaces, humic colloids, entrapment, or copolymerization (Boyd and Mortland 1990). Since soil management that promotes stabilization of organic matter and promote aggregation would also promote stabilization of enzymes in the soil matrix. Enzymes also affect the soil quality because of their relationship to soil biology, ease of measurement, and response to soil

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management (Dick 1994). The stability of enzymes in soil is attributed to association with organic matter which is protected within soil aggregates (Boyd and Mortland 1990). Combined fertilization with organic manure and inorganic fertilizers increased invertase, protease, urease, acid phosphomonoesterase, dehydrogenase and catalase activities of water stable aggregates in different size fractions (Yi-Ren *et al.* 2013).

Various researchers reported that long-term application of inorganic fertilizer (N-nitrogen; P-phosphorus; K-potassium) along with organic manures increased the amount of larger aggregates (>250 μm) and the mean weight diameter (MWD) of aggregate fractions decreases at lower soil depth compared to surface soil (Brar *et al.* 2013; Tripathi *et al.* 2014; Padbhushan *et al.* 2016). Only few studies have tried to establish a relationship between soil aggregate size fractions and its associated enzymatic activities involved in N mineralization. Allison and Jastrow (2006) observed higher activities of certain enzymes such as cellobio-hydrolase, β-glucosidase, and N-acetyl glucosaminidase (NAG) in particulate organic matter fractions. They further suggested that plant-rich materials are actively degraded by enzymes synthesized by microbes ion these aggregate fractions. Fansler *et al.* (2005) observed greater β-glucosidase and NAG activities in microaggregate size fractions compared to macroaggregates.

There are very less studies in India which have analyzed the effect of rice cultivation on enzymatic activities particularly in soil aggregate fractions in tropical lowland conditions. Therefore, the objective of this study was to evaluate the hypothesis that the long-term application of farmyard manure (FYM) either alone or in combination with inorganic fertilizers impact the distribution pattern of enzymes namely, urease, amidase and protease in different fractions of water stable soil aggregates.

Materials and Methods

Study site

The study was conducted at the experimental farm of the National Rice Research Institute, Cuttack, India (20°25′ N, 85°55′ E). Mean annual maximum and minimum temperatures are 39.2 and 22.5 °C, respectively, and the mean annual temperature is 27.7 °C. Annual precipitation is about 1500 mm, of which 75-80% is received during June to September. The soil at the experimental site is an Aeric Endoaquept with a sandy clay loam texture (31% clay, 17% silt and 52% sand). Other soil properties of the experimental field at the beginning of the study were pH 6.6 (using 1:2.5, soil/water suspension), organic carbon 6.6 g kg^{-1} and total N 0.8 g kg^{-1}. Field experiment was started in 1969 and includes two crops per year with rice (*Oryza sativa* L.) as mono-crop in wet (July-November) and dry (January-May) seasons. The treatments were arranged in a randomized complete block design with three replications. The treatments include unfertilized control and different combinations of inorganic fertilizers and FYM *viz.* control, N, NP, NK, NPK, FYM, N+FYM, NP+FYM, NK+FYM and NPK+FYM. The FYM was prepared from Institute's dairy farm which contains 171-189 g soil organic C and 4-16 g total N kg^{-1}. The FYM (5 t ha^{-1}) was applied in the treatments receiving FYM during last week of May every year. The rate of inorganic fertilizer application were 60-40-40 and 80-40-40 N-P_2O_5-K_2O kg ha^{-1} for wet and dry seasons, respectively.

The soil sampling was done randomly from five spots in each replicated plots at two soil depths (0-15 and 15-30 cm) after harvesting of the rice crop in December 2014. Analysis of soil enzymes were done immediately after sampling.

Aggregate size fractions

For aggregate size fraction analysis, three undisturbed soil samples from each treatment were collected from two depths (0-15 and 15-30 cm). The moist soil was air-dried, and crumbled to pass through 8 and 5 mm sieves. Soil aggregates retained on the 5 mm sieve were separated by wet sieving (Camberdella and Elliott 1992). Sample of 100 g aggregates (in duplicate) were placed on first sieve of a nest of sieves with 5, 2, 1, 0.50, 0.25, 0.1 and 0.053 mm sieves to obtain five size fractions *i.e.* 5-2, 2-1, 1-0.5, 0.5-0.25, 0.25-0.1 and 0.1-0.053 mm. The water level was brought to the base of the top sieve to immerse soil samples for 3 min in Yoder's apparatus, and the sieves raised and lowered 3.7 cm 30 times per min for 10 min. After decanting floating organic materials, aggregate fractions retained on each sieve were dried at 65 °C for 48 h. Another set of aggregate analysis was done and enzymatic analysis was performed as soon as possible after separating different fraction of aggregate fractions. The data were analyzed to compute water stable aggregates (Kemper and Rosenau 1986) and MWD (Youker and McGuinnes 1957).

Analysis of enzymes in whole soil and aggregates

Urease activity was measured by the method of Tabatabai and Bremner (1972), and amidase activity was assayed using acetamide as the substrate (Frankenberger and Tabatabai 1980). Protease activity was assayed by determining the tyrosine released when 1 g of the oven-dry equivalent of field-moist soil sample was incubated with 5 mL of 50 *mM* Tris buffer (pH 8.1) and 5 mL of 2% Na-caseinate at 50 °C for 2 h. The aromatic amino acids thus released were extracted and the remaining substrate was precipitated with 0.92 *M* trichloroacetic acid and measured colorimetrically using folin-ciocalteu reagent at 700 nm. The activity of protease was expressed as mg tyrosine produced g^{-1} soil h^{-1}.

Statistical analysis

Aggregates were categorized into macro (5-2 mm), meso (2-1, 1-0.5 and 0.5-0.25 mm) and micro (0.25-0.1 and 0.1-0.053 mm) and their relative proportion was expressed as percentage of the total aggregates (Tripathi *et al.* 2014). The significant differences among different treatments were statistically analyzed by one way ANOVA at $P < 0.05$ using a SAS software package 9.2.

Results and Discussion

Aggregate size distribution and aggregate stability

A significant difference in soil aggregate size distribution was observed due to application of FYM and inorganic fertilizers compared to unfertilized control. At sampling depths of 0-15 and 15-30 cm under different treatments the total water stable aggregates (WSA) ranged between 69.8-91.2% (Table 1). The contribution of 0.1-0.053 mm aggregate fraction was least (2.11-3.87%), whereas 0.25-0.5 mm aggregate fraction was having the highest (27.3-32.6%) contribution in total WSA at two sampling depths (Table 1). The percentages of macro and meso aggregates were significantly higher when FYM was applied either alone or in combination with inorganic fertilizers compared to unfertilized control at both soil depths. Application of FYM alone increased macroaggregates (5-2 mm) by 162.6 per cent whereas mesoaggregates increased by 132 per cent in 2-1 mm fraction, by 283 per cent in 1-0.5 mm fraction over unfertilized control in 0-15 cm soil depth. The MWD was higher in FYM applied treatments than inorganic fertilizer alone and unfertilized control (Table 1).

Results of this study indicated that FYM application enhanced the water stable aggregates by

Table 1. Distribution of water stable aggregates in different treatments of long-term fertilizer experiment analyzed by wet aggregate analysis

	% Water stable aggregates						% Total WSA	MWD
	5-2 mm	2-1 mm	1-0.5 mm	0.5-0.25 mm	0.25-0.1 mm	0.1-0.053 mm		
				0-15 cm				
Control	2.14±0.60^{a}	3.50±0.55^{a}	2.12±0.75^{a}	29.3±0.15^{a}	31.2±0.62^{a}	3.29±0.41^{c}	71.5	0.43
N	3.58±0.20^{b}	4.59±0.29^{b}	4.12±0.54^{b}	30.2±0.44^{b}	30.0±0.37^{bc}	2.62±0.32^{abc}	75.2	0.52
NP	4.41±0.20^{c}	4.55±0.44^{c}	6.10±0.37^{c}	32.1±0.40^{ab}	30.4±0.31^{ab}	2.98±0.28^{bc}	80.6	0.55
NK	4.61±0.68^{cd}	7.22±0.25^{cd}	7.33±0.06^{d}	32.6±0.39^{cd}	27.7±0.75^{bc}	2.49±0.38^{ab}	82.1	0.61
NPK	5.31±0.68^{de}	8.09±0.09^{de}	7.65±0.20^{de}	32.6±0.38^{c}	28.0±0.49^{bc}	2.44±0.10^{ab}	84.2	0.64
FYM	5.62±0.30^{e}	8.12±0.47^{e}	8.12±0.13^{e}	28.2±0.46^{a}	30.4±0.78^{bc}	2.32±0.83^{a}	82.8	0.65
N+FYM	5.51±0.40^{e}	8.19±0.40^{e}	8.28±0.41^{e}	30.1±0.29^{b}	30.0±0.38^{bc}	2.31±0.41^{a}	84.4	0.65
NP+FYM	5.93±0.31^{e}	8.40±0.42^{e}	9.98±0.68^{f}	30.2±0.41^{b}	30.1±0.20^{c}	2.21±0.48^{a}	86.8	0.66
NK+FYM	5.63±0.25^{e}	9.61±0.16^{e}	10.97±0.59^{g}	31.3±0.33^{d}	28.1±0.50^{c}	2.21±0.13^{a}	87.9	0.67
NPK+FYM	7.85±0.37^{f}	11.17±0.43^{f}	13.94±0.22^{h}	30.4±0.26^{e}	25.3±0.28^{bc}	2.42±0.33^{a}	91.2	0.78
				15-30 cm				
Control	1.54±0.35^{a}	3.32±0.27^{a}	2.03±0.41^{a}	27.3±0.11^{a}	31.7±0.10^{a}	3.87±0.35^{a}	69.8	0.40
N	2.96±0.32^{b}	4.41±0.71^{b}	4.01±0.32^{b}	28.7±0.26^{b}	30.6±0.18^{b}	2.50±0.50^{b}	73.2	0.50
NP	3.64±0.27^{b}	4.47±0.26^{b}	5.89±0.48^{c}	30.2±0.44^{c}	31.7±0.58^{a}	2.57±0.63^{b}	78.5	0.52
NK	3.63±0.41^{b}	4.78±0.63^{b}	5.98±0.49^{c}	31.1±0.27^{d}	30.1±0.28^{bc}	2.65±0.43^{b}	78.3	0.53
NPK	4.63±0.51^{c}	6.58±0.38^{c}	7.02±0.22^{d}	32.2±0.65^{e}	28.3±0.37^{d}	2.26±0.81^{b}	81.0	0.60
FYM	4.22±0.52^{bc}	7.28±0.25^{d}	8.12±0.27^{e}	28.1±0.71^{b}	30.1±0.24^{bc}	2.13±0.42^{b}	79.9	0.60
N+FYM	4.23±0.33^{bc}	7.34±0.54^{d}	8.20±0.44^{e}	28.1±0.54^{b}	30.1±0.45^{bc}	2.46±0.39^{b}	80.4	0.60
NP+FYM	4.38±0.78^{bc}	7.58±0.41^{d}	8.24±0.61^{e}	29.5±0.22^{c}	29.6±0.26^{c}	2.12±0.35^{b}	81.4	0.61
NK+FYM	4.34±0.22^{bc}	8.70±0.47^{e}	9.90±0.30^{f}	30.1±0.42^{c}	27.8±0.20^{d}	2.11±0.34^{b}	83.0	0.63
NPK+FYM	6.48±0.46^{d}	9.89±0.49^{f}	11.16±0.60^{g}	29.6±0.16^{c}	26.6±0.21^{e}	2.29±0.23^{b}	86.1	0.72

Value±SD is provided; within the same aggregate fraction different small letters indicate significant difference at $p<0.05$

increasing the macroaggregates which is similar to Benbi *et al*. (2010), who observed that the amount of macroaggregates of different sizes increased by 18-105 per cent due to application of rice straw and FYM and decreased the microaggregates by 5-10 per cent in surface soil. Su *et al.* (2006) have also reported that long-term incorporation of FYM enhances the aggregate fractions (>2 mm and 250-2,000 µm) compared with the soils without FYM application. The increase in macroaggregates (>2 mm) proportion by FYM application may be due to incorporation of additional organic residues and available C to the soils compared with inorganic fertilizer application alone (Tripathi *et al.* 2014). Reduction in microaggregates proportion with FYM application may be attributed to binding of the microaggregates to macroaggregates due to secretion of mucilaginous substances (Sodhi *et al*. 2009). Various researchers have observed increased proportion of macroaggregates due to regular addition of organic matter through compost (Singh *et al.* 2007; Sodhi *et al.* 2009). Similar to our result, Singh *et al*. (2007) reported the positive effects of manure application on MWD.

Enzymatic activities in whole soil and aggregates

Urease activity

Enzyme activities varied widely among the treatments studied. Urease activity in surface soil (0-15 cm) was least in unfertilized control (217.4 µg urea hydrolyzed g^{-1} h^{-1}) and highest in FYM+NPK (301.5 µg urea hydrolyzed g^{-1} h^{-1}) (Fig. 1a). Urease activity reduced at 15-30 cm soil depth irrespective of treatments which varies from 185.3 µg urea hydrolyzed g^{-1} h^{-1} in control to 239.2 µg urea hydrolyzed g^{-1} h^{-1} in NPK+FYM (Fig. 1b). Trend of urease activity at 15-30 cm depth was similar to surface soil. Urease activity was significantly higher in FYM alone compared to inorganic fertilizer alone treatments irrespective of soil depth.

Urease activity was lowest in control and highest in FYM+NPK with 33.1-43.9 per cent increase over control across aggregate fractions (Fig. 2a). Macroaggregates (5-2 mm) had highest urease activity and microaggregate fraction (0.1-0.053 mm) had least urease activity among aggregate fractions. Urease activity was lower at 15-30 cm depth compared to

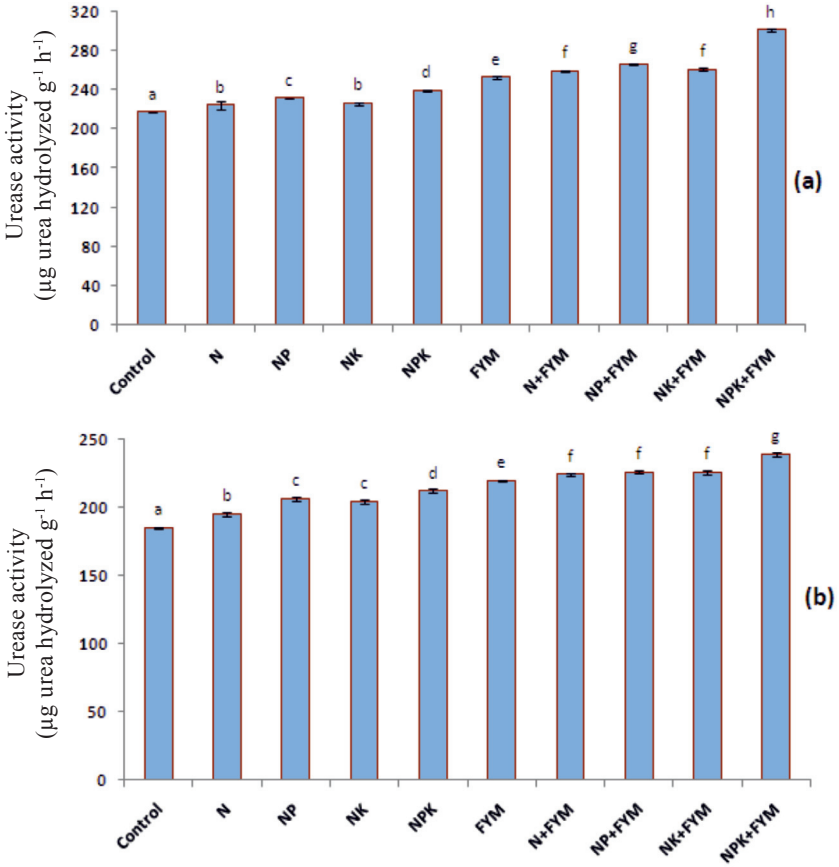

Fig. 1. Urease activity in whole soil (a) 0-15 cm and (b) 15-30 cm in different treatments (Bars indicate standard errors, different small letters indicate significant difference between treatments at $p<0.05$)

surface soil (0-15 cm) in all the aggregate fractions (Fig. 2b). The trend of urease activity at 15-30 cm depth in different aggregate fractions across treatments was similar to surface soil.

Analysis of enzymes related to N dynamics (urease, amidase and protease) was done in whole soil and soil aggregates to assess the effect of long-term application of FYM and inorganic fertilizers on these enzymatic activities and their distribution pattern across aggregate fractions. Our results showed an increase in urease activity by long-term application of inorganic fertilizer along with FYM which is against the findings of Dick *et al.* (1988), who reported decreased urease activity when inorganic N was applied for a long time but increased the urease activity when crop residues and manure were applied in a wheat-fallow system. They observed that increasing application of NH_3 based-N fertilizers decreased the urease activity and attributed the reason to the end product of the enzymatic reaction (NH_4^+) which suppressed urease activity. Higher urease activity by addition of inorganic fertilizer (urea as a source of N) in our experiment may be attributed to the fact that urea applied to flooded soil was hydrolyzed to NH_4^+ before being taken up by the growing rice crop. It may be possible that the treatments receiving inorganic fertilizer or FYM or both on a long-term basis might have resulted in the higher urease activity (Nayak *et al.* 2007).

Protease activity

Similar to urease activity, protease activity in surface soil (0-15 cm) was least in unfertilized control and highest in FYM+NPK (Fig. 3a). Protease activity reduced at 15-30 cm soil depth irrespective of treatments which varies from 4.29 µg tyrosine produced g^{-1} h^{-1} in control to 9.88 µg tyrosine produced g^{-1} h^{-1} in NPK+FYM (Fig. 3b). Trend of protease activity at 15-30 cm depth was almost similar to surface soil. Combined application of FYM and inorganic fertilizers resulted in significantly higher protease activity against inorganic fertilizer alone or

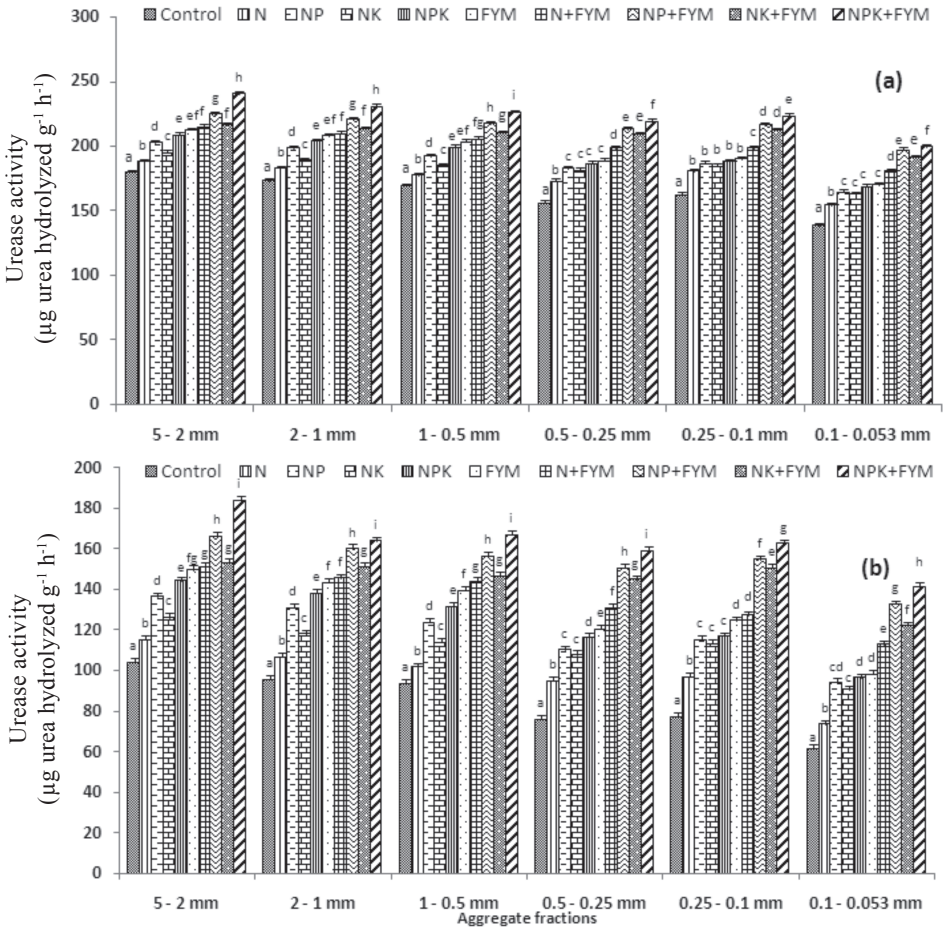

Fig. 2. Urease activity in different fractions of soil aggregates (a) 0-15 cm and (b) 15-30 cm in different treatments (Bars indicate standard errors and within the same aggregate fraction different small letters indicate significant difference at $p<0.05$)

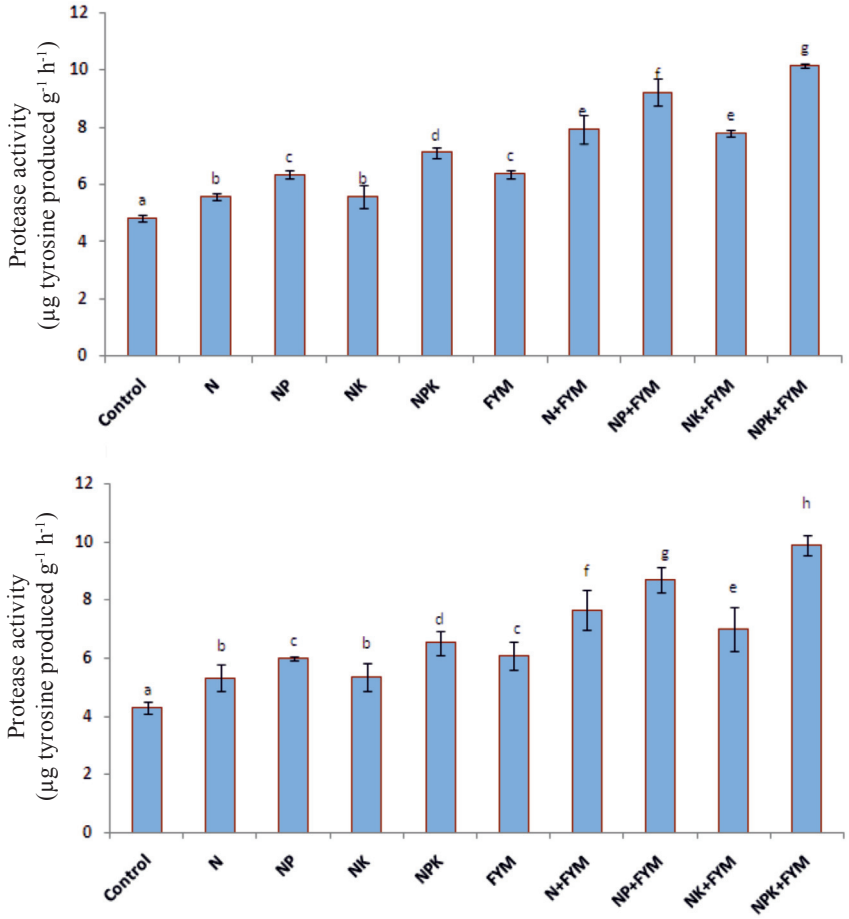

Fig. 3. Protease activity in whole soil (a) 0-15 cm and (b) 15-30 cm in different treatments (Bars indicate standard errors and within the same aggregate fraction different small letters indicate significant difference at $p<0.05$)

unfertilized control in all the aggregate fractions (Fig. 4a). In contrast to urease activity, protease activity was significantly higher in NPK treatments compared to FYM alone treatment with 21.4-131.0 per cent increase over FYM treatments across aggregate fractions in surface soil and 0.3-3.3 per cent increase in 15-30 cm soil depth (Fig. 4b). Protease activity decreased with soil depth in all the aggregate fractions.

The amount of proteases may be indicative of the biological capacity. Our results indicated that the application of FYM along with inorganic fertilizer increased the protease activity in comparison to inorganic fertilizer alone treatment across aggregate fractions. This higher activity might be attributed to more substrate availability in these soils. This indicated that there is greater biological activity in FYM applied plots and extracellular enzymes stabilize by making complex with humic substances (Colvan *et al.* 2001). Saha *et al.* (2008) also reported similar results where they found increased protease activity in treatments receiving organic amendments compared to other treatments.

Similar to our results which indicated higher enzymatic activities in macroaggregates, Ladd *et al.* (1996) investigated that active microbes may use newly introduced and more labile C in growth and enzyme production, thereby increasing enzyme activities in macroaggregates. The lower levels of enzyme activity in microaggregates may be due to lower amount of C and N available in microaggregate fractions (Tripathi *et al.* 2014) for microbial growth during degradation.

Amidase activity

Amidase activity in surface soil (0-15 cm) was least in unfertilized control (131.1 µg NH_4^+ g^{-1} h^{-1}) and highest in FYM (165.2 µg NH_4^+ g^{-1} h^{-1}) (Fig. 5a). Amidase activity was significantly higher in treatments receiving FYM in combination with inorganic fertilizers compared to treatments receiving inorganic fertilizers alone. Enzymatic activity reduced

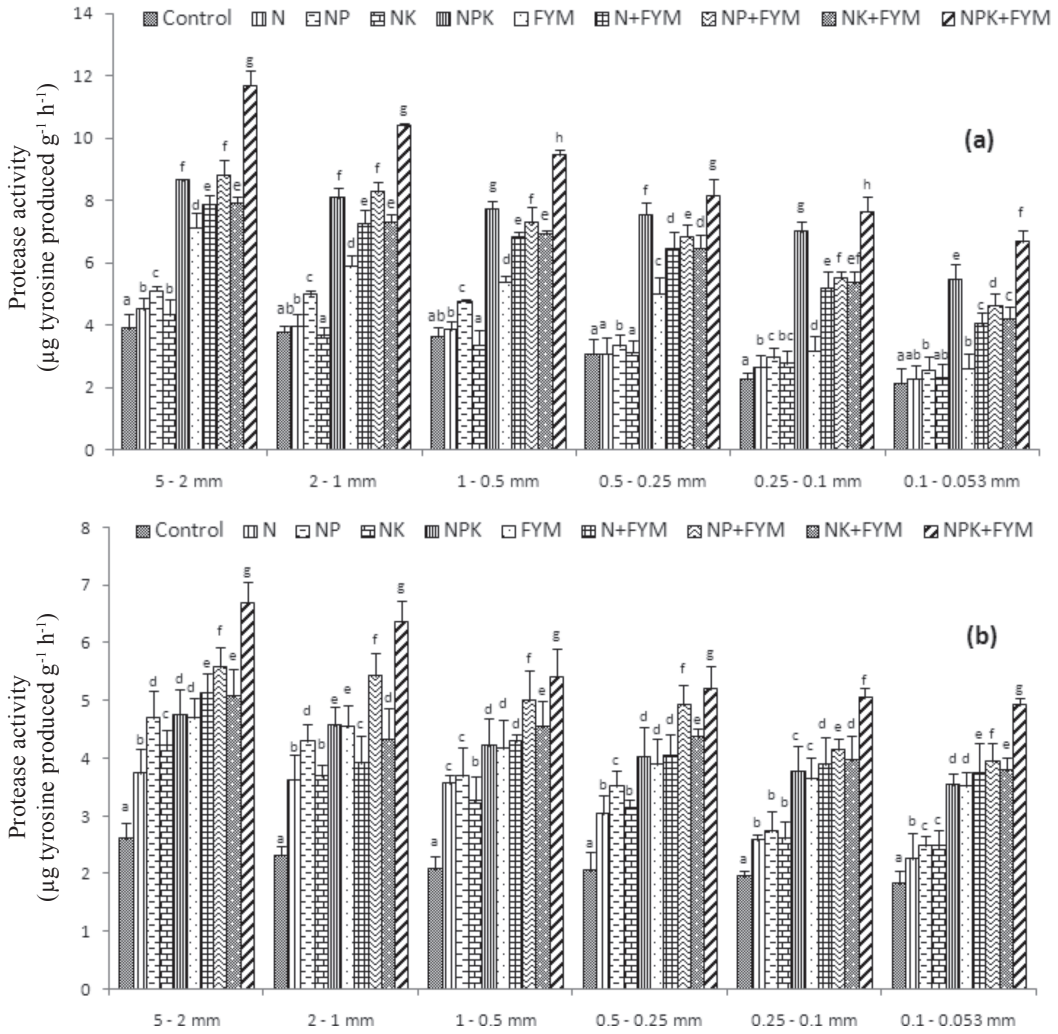

Fig. 4. Protease activity in different fractions of soil aggregates (a) 0-15 cm and (b) 15-30 cm indifferent treatments (Bars indicate standard errors and within the same aggregate fraction different small letters indicate significant difference at $p<0.05$)

at 15-30 cm soil depth irrespective of treatments which varied from 87.8 µg NH_4^+ g^{-1} h^{-1} in control to 119.9 µg NH_4^+ g^{-1} h^{-1} in treatment receiving FYM alone (Fig. 5b). Trend of amidase activity at 15-30 cm depth was similar to surface soil. Highest amidase activity was recorded in FYM alone treatment. Amidase activity was lowest in control and highest in FYM treated plots at both soil depths with 15.0-33.0 per cent increase over control across aggregate fractions in the surface soil and 23.9-48.3 per cent increase over control in 15-30 cm soil depth. Amidase activity was highest in 5-2 mm aggregate fraction and decreased with aggregate size except 1-0.5 mm where it was higher compared to other aggregate fractions (Fig. 6a). The least amidase activity was recorded in micro-aggregates (0.1-0.053 mm). Treatments receiving both FYM and inorganic fertilizers recorded significantly higher amidase activity compared to control and inorganic fertilizer alone treatments. The trend of amidase activity in different fractions of soil aggregates at 15-30 cm soil depth was similar to that at 0-15 cm (Fig. 6b).

Further, we found an increase in amidase activity by application of FYM whereas there was reduction in activity of amidase by the application of inorganic fertilizer alone. The similar results were reported by Dick *et al*. (1988), who also observed reduction in amidase activity with addition of inorganic N for long-term, whereas crop residues and manure additions increased the amidase activity in a wheat-fallow system.

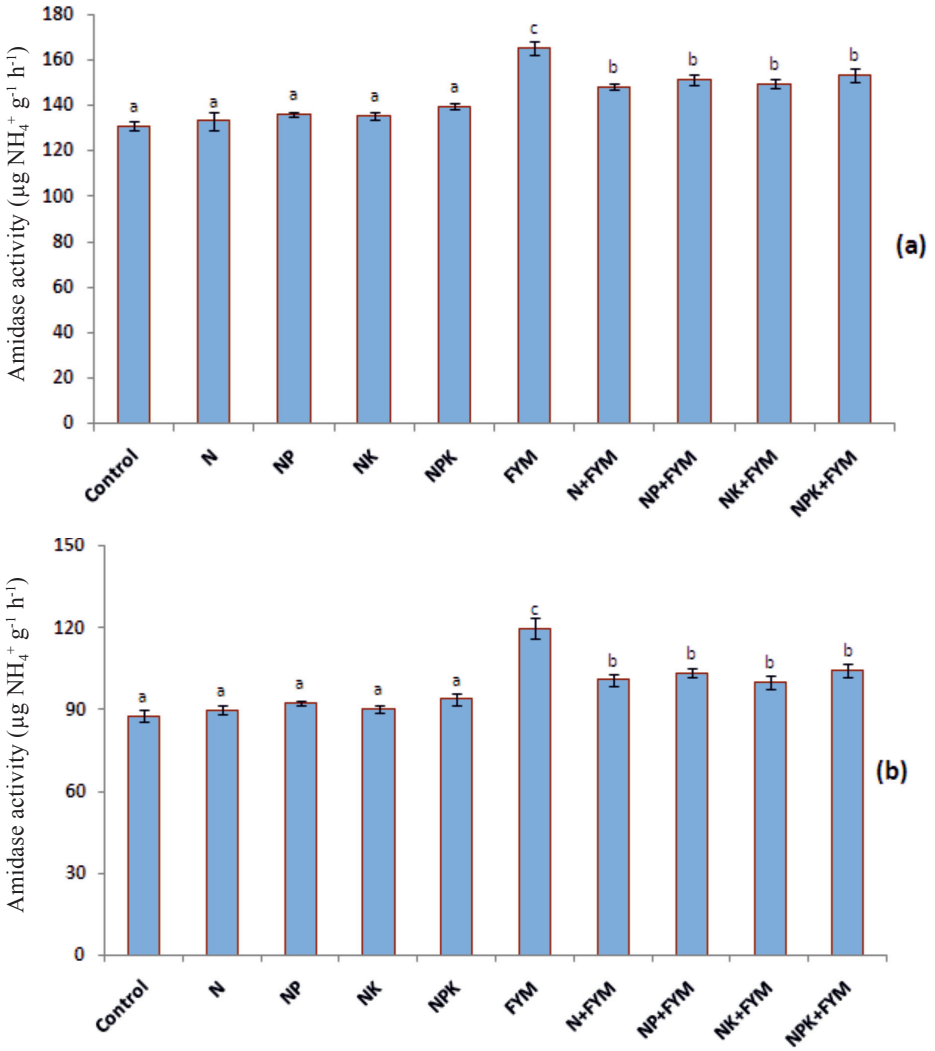

Fig. 5. Amidase activity in whole soil (a) 0-15 cm and (b) 15-30 cm in different treatments (Bars indicate standard errors and within the same aggregate fraction different small letters indicate significant difference at $p<0.05$)

It is clear from our results that continuous application of FYM alone or in combination with inorganic fertilizer resulted in an increase in macro- and meso-aggregate fractions, also the activities of N-mineralizing enzymes were reported high in macroaggregates.

Conclusions

Activities of enzymes (urease, protease and amidase) were assayed in whole soil as well as in soil aggregate fractions which was least in unfertilized control and highest in FYM+NPK across aggregate fractions except for amidase which recoreded highest activity in FYM alone treatment. Amidase activity was highest in FYM alone treatment with 21.4-131.0 per cent increase over control. Activities of all the enzymes reduced at 15-30 cm soil depth compared to surface soil in whole soil as well as in soil aggregate fractions. The reason for higher enzymatic activity in FYM applied plots may be attributed to higher organic matter content which provides a more positive environment for the accumulation of enzymes in the soil matrix. It was concluded from this study that continuous application of FYM alone or in combination with inorganic fertilizer results in increase in N-mineralizing enzymatic activities. The study of soil enzymes in soil aggregates will help us to understand the dynamics of nutrients especially carbon and nitrogen in soil.

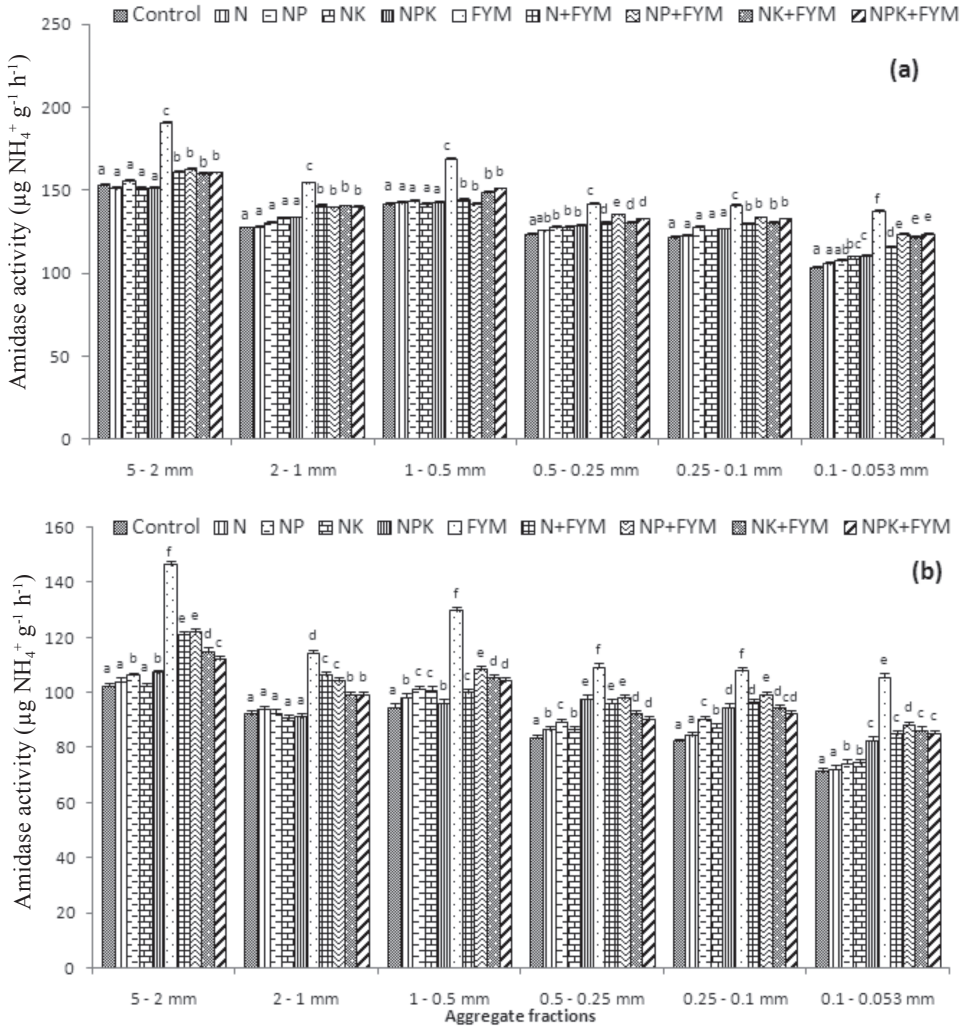

Fig. 6. Amidase activity in different fractions of soil aggregates (a) 0-15 cm and (b) 15-30 cm in different treatments (Bars indicate standard errors and within the same aggregate fraction different small letters indicate significant difference at $p<0.05$)

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