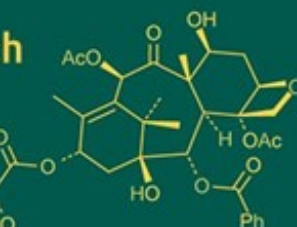
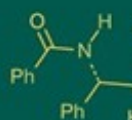
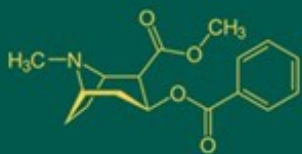


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
Indexing: Embase
IJABR 2026; SP-10(9): 2718-2722
www.biochemjournal.com
Received: 04-07-2026
Accepted: 12-08-2026
Published: 30-09-2026

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Phosphorus fractions dynamics in an Alfisols amended with biochemical activators under varying phosphorus levels: An incubation study

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9Sah.10245>

ABSTRACT

Phosphorus (P) fixation in an alfisols severely limits the efficiency of applied phosphatic fertilizers, with a substantial proportion of added P rapidly transforming into plant-unavailable aluminium (Al) and iron (Fe) bound forms. A laboratory incubation study was conducted to evaluate the influence of biochemical activators (phytase enzyme and humic acid), applied at two P levels through farmyard manure (FYM) as carrier, on the transformation of soil P fractions over a 90-day incubation period. Eleven treatments comprising an absolute control, the recommended dose of fertilizer (RDF), RDF plus FYM, and FYM-based treatments combined with phytase (@ 2 and 4 kg ha⁻¹) or humic acid (@ 2.5 and 5 kg ha⁻¹) at 100% and 75% of the recommended dose of phosphorus (RDP) were incubated in a completely randomized design with four replications, and soil samples were drawn at 5, 10, 15, 30, 45, 60 and 90 days after incubation (DAI) for sequential P fractionation. Saloid-P, aluminium-bound P (Al-P), iron-bound P (Fe-P), reductant soluble-P (reductant-P), occluded-P, calcium-bound P (Ca-P), organic-P and total-P were determined. Humic acid at 5 kg ha⁻¹ with 100% RDP (*T*₉) maintained the highest saloid-P, Al-P being reduced relative to the FYM + RDF treatment, and recorded the highest reductant soluble-P, occluded-P and total-P throughout incubation. Phytase application at 4 kg ha⁻¹ (*T*₅) sustained the highest saloid-P among phytase treatments and the most rapid decline in organic-P, confirming active phytate mineralization. Organic-P declined progressively in all treatments, with the steepest decline under full-dose phytase treatments. Total-P remained conserved within each treatment across the incubation period, confirming closed-system fraction inter-transformation. The results demonstrate that integration of phytase and humic acid with FYM as carrier substantially modifies the distribution of soil P fractions in favour of plant-available forms in P-fixing alfisols.

Keywords: Alfisols, Humic acid, Phosphorus fractionation, Phytase

Introduction

Phosphorus is an essential macronutrient required for energy transfer, membrane biosynthesis, nucleic acid synthesis and photosynthate translocation in plants, yet it is also the most chemically reactive of the major nutrients in soil. In acid alfisols, which dominate the red and lateritic soil regions of peninsular India, abundant amorphous and crystalline oxides and hydroxides of Al and Fe provide an enormous surface area for phosphate adsorption and specific chemisorption, resulting in P-fixing capacities frequently exceeding 70-80% (Sanyal and De Datta, 1991; Ahmed *et al.*, 2022) [2, 1]. Consequently, only a small fraction of applied phosphatic fertilizer remains available to the succeeding crop, with the remainder progressively transforming through a sequence of increasingly stable inorganic P fractions from loosely bound saloid-P to Al-P, Fe-P, reductant-P and occluded-P within sesquioxide matrices. Understanding the rate and direction of these transformations under different management practices is therefore fundamental to designing efficient P fertilization strategies for such soils.

Two biochemical approaches have received considerable research attention for improving P availability in fixation-prone soils. Phytase enzymes hydrolyse phytic acid (myo-inositol hexakisphosphate), the dominant organic P form contributing 30-65% of total soil organic P in many tropical soils, thereby releasing inorganic orthophosphate into the labile pool. Exogenous phytase application has been shown to significantly increase plant-available P and reduce the organic P fraction in incubated soils (Richardson *et al.*, 2001; George *et al.*, 2005) [4, 3]. Humic acid, in contrast, acts primarily on the inorganic P fractions where the carboxylate and phenolate functional groups of humic macromolecules form stable complexes with Al³⁺ and Fe³⁺ at sesquioxide surfaces, competitively blocking phosphate adsorption sites through ligand exchange and thereby reducing the conversion of labile P into Al-P and Fe-P (Gerke, 1992; Nardi *et al.*, 2002) [5, 6]. Farmyard manure additionally contributes low-molecular-weight organic acids that operate through a similar

but more transient ligand-exchange mechanism (Duffera *et al.*, 1999) [7] and serves as an effective protective carrier matrix for exogenously applied enzymes through adsorption onto humic colloid surfaces, extending their catalytic persistence in soil (Burns *et al.*, 2013) [8].

Despite extensive field-level evidence for the agronomic benefits of phytase and humic acid in *P*-fixing soils, relatively few studies have systematically tracked the temporal dynamics of the complete sequential *P* fractionation pattern under combined phytase and humic acid application at varying *P* doses using FYM as a common carrier. Such incubation-based fractionation data are essential for elucidating the mechanistic basis of field-level *P* availability improvements and for identifying the optimum combination of activator type and *P* dose for sustainable *P* management. The present study was therefore undertaken with the objective of evaluating the effect of phytase and humic acid, applied through FYM at two *P* levels, on the temporal transformation of saloid-*P*, Al-*P*, Fe-*P*, reductant-*P*, occluded-*P*, Ca-*P*, organic-*P* and total-*P* in an alfisol over a 90-day incubation period.

Materials and Methods

Soil characterization

The surface soil (0-15 cm) used for the incubation study was collected from the experimental field site, classified as an Alfisols. The soil was air-dried, ground and passed through a 2 mm sieve prior to incubation. The initial soil was acidic in reaction (pH 5.11 in 1:2.5 soil:water suspension) with an electrical conductivity of 0.11 dS m⁻¹, organic carbon content of 4.9 g kg⁻¹, available nitrogen of 299 kg ha⁻¹, available P₂O₅ (Bray and Kurtz No. 1) of 53.6 kg ha⁻¹, available K₂O of 301 kg ha⁻¹, saloid *P* of 8.15 mg kg⁻¹, Al-*P* of 55.16 mg kg⁻¹, Fe-*P* of 72.31 mg kg⁻¹, reductant-*P* of 61.13 mg kg⁻¹, occluded *P* of 15.96 mg kg⁻¹, Ca-*P* of 10.41 mg kg⁻¹, organic *P* of 98.54 mg kg⁻¹, total *P* of 321.66 mg kg⁻¹ and a *P*-fixing capacity of 79.5%, confirming the high *P*-sorption characteristics typical of alfisols.

Treatments and incubation procedure

The incubation study comprised eleven treatments laid out in a complete randomized design with four replications which include T₁: absolute control (no amendment); T₂: recommended dose of fertilizer for maize (RDF, 150:75:40 kg N:P₂O₅:K₂O ha⁻¹ equivalent applied through urea, single super phosphate and muriate of potash); T₃: RDF + farmyard manure (FYM) @ 7.5 t ha⁻¹; T₄: FYM + 100% RDP + phytase @ 2 kg ha⁻¹; T₅: FYM + 100% RDP + phytase @ 4 kg ha⁻¹; T₆: FYM + 75% RDP + phytase @ 2 kg ha⁻¹; T₇: FYM + 75% RDP + phytase @ 4 kg ha⁻¹; T₈: FYM + 100% RDP + humic acid @ 2.5 kg ha⁻¹; T₉: FYM + 100% RDP + humic acid @ 5 kg ha⁻¹; T₁₀: FYM + 75% RDP + humic acid @ 2.5 kg ha⁻¹ and T₁₁: FYM + 75% RDP + humic acid @ 5 kg ha⁻¹. In all FYM-based treatments (T₄-T₁₁), phytase or humic acid was thoroughly mixed with FYM prior to soil incorporation, allowing the organic matrix to act as a protective carrier for the biochemical activators.

Soil equivalent to 2 kg on dry basis was weighed into polythene-lined incubation containers for each treatment and replication, amended according to the respective treatment, thoroughly mixed and brought to 60% of water holding capacity. Moisture content was maintained by periodic addition of distilled water throughout the incubation period. Containers were incubated in the dark at ambient laboratory temperature for 90 days. Soil samples were drawn destructively from each replication at 5, 10, 15, 30, 45, 60 and 90 DAI, air-dried, ground to pass a 2 mm sieve and stored for *P* fractionation.

Sequential phosphorus fractionation

Soil *P* was sequentially fractionated following the scheme of Chang and Jackson (1957) [9] as modified by Petersen and Corey (1966) [10] and Williams *et al.* (1967) [11], widely employed for characterizing inorganic and organic *P* pools in acid soils. Saloid-bound *P* was extracted with 1N NH₄Cl, Al-*P* with 0.5N NH₄F at pH 8.2, Fe-*P* with 0.1N NaOH, reductant soluble *P* with sodium citrate-bicarbonate-dithionite; occluded *P* with 0.1N NaOH extraction and calcium-bound *P* with 0.25N H₂SO₄. Total *P* was determined following perchloric

acid digestion (Jackson, 1973) [12]. Phosphorus in all extracts was estimated colorimetrically by the ascorbic acid blue colour method using a UV-visible spectrophotometer at 660 nm.

Statistical analysis

Data generated for each *P* fraction at each sampling stage were subjected to analysis of variance (ANOVA) appropriate for a complete randomized design. Treatment means were compared using the critical difference (CD) at the 5% level of probability.

Results and Discussion

The temporal changes in saloid-*P*, Al-*P*, Fe-*P*, reductant-*P*, occluded-*P*, Ca-*P*, organic-*P* and total-*P* over the 90-day incubation period, as influenced by biochemical activators and phosphorus levels, are illustrated in Figure 1.

Saloid-*P*

Saloid-*P*, representing the most labile and readily plant-available inorganic *P* fraction, ranged from 7.95 to 18.69 mg kg⁻¹ across treatments and incubation periods. In all the treatments, saloid-*P* declined gradually with increasing incubation period as the freshly added soluble *P* progressively equilibrated with the more stable Al-*P*, Fe-*P* and occluded-*P* pools. The absolute control (T₁) maintained the lowest saloid-*P* throughout (8.34 to 7.95 mg kg⁻¹), reflecting the native, largely depleted labile *P* pool of this alfisols (Figure 1a). Among the biochemical activator treatments, treatment T₉ (FYM + 100% RDP + humic acid @ 5 kg ha⁻¹) consistently recorded the highest saloid-*P* at every sampling stage (18.69 mg kg⁻¹ at 5 DAI, declining to 17.47 mg kg⁻¹ at 90 DAI), followed closely by T₈ (FYM + 100% RDP + humic acid @ 2.5 kg ha⁻¹) and T₅ (FYM + 100% RDP + phytase @ 4 kg ha⁻¹). The superior saloid-*P* retention under humic acid treatments is attributable to the chelation of Al³⁺ and Fe³⁺ by carboxylate and phenolate functional groups of humic acid, which competitively occupies sesquioxide surface sites and thereby retards the diffusion-controlled conversion of saloid-*P* into Al-*P* and Fe-*P* (Gerke, 1992; Nardi *et al.*, 2002) [5, 6]. Among phytase treatments, the higher dose (T₅, T₇) maintained greater saloid-*P* than the lower dose (T₄, T₆) throughout incubation, consistent with continuous phytase-*P* hydrolysis replenishing the labile pool at a rate proportional to enzyme dose (Richardson *et al.*, 2001; George *et al.*, 2005) [4, 3]. Treatments at 75% RDP (T₆, T₇, T₁₀, T₁₁) recorded marginally lower saloid-*P* than their 100% RDP counterparts, reflecting the proportionally lower *P* input. The consistently higher saloid-*P* under T₉ throughout the incubation period indicates that humic acid at the higher dose provides the most sustained labile *P* supply among all treatments evaluated.

Aluminium-bound *P*

Aluminium bound *P*, the dominant inorganic *P* fraction in this alfisol, ranged from 43.54 to 66.29 mg kg⁻¹ and increased progressively in all treatments over the incubation period, confirming the continuous diffusion-controlled conversion of labile *P* into chemisorbed Al-*P* forms characteristic of acid soils dominated by Al-hydroxide surfaces (Chang and Jackson, 1957; Lindsay, 1979) [9, 13]. The absolute control (T₁) recorded the lowest Al-*P* throughout (43.54 to 47.24 mg kg⁻¹), consistent with its limited native labile *P* pool available for fixation (Figure 1b). The treatment T₃ (RDF + FYM, without any biochemical activator) recorded the highest Al-*P* at every stage (55.49 to 66.29 mg kg⁻¹), exceedingly even the inorganic RDF treatment (T₂: 50.22 to 57.42 mg kg⁻¹). This indicates that the additional organic matter from FYM, while beneficial for overall soil fertility, did not by itself prevent SSP-applied *P* from converting to Al-*P* in the absence of a specific *P*-fixation-blocking activator; rather, the enhanced microbial activity associated with FYM addition may have accelerated equilibration of applied *P* with reactive Al surfaces. In marked contrast, all biochemical activator treatments (T₄-T₁₁) maintained significantly lower Al-*P* than the treatment T₃ throughout incubation, with the magnitude of reduction proportional to both activator dose and *P* level. Among phytase treatments, the higher dose (T₅, T₇) consis

tently recorded lower Al-P than the corresponding lower dose (T_4 , T_6) at the same P level, indicating that more vigorous phytate hydrolysis maintained a larger proportion of P in the saloid pool rather than allowing its diffusion to Al-bound forms (Richardson *et al.*, 2001) [4]. Humic acid treatments produced the greatest reduction in Al-P relative to T_3 , with T_{11} (75% RDP + humic acid @ 5 kg ha⁻¹) recording the lowest Al-P among all fertilized treatments (46.63 to 51.77 mg kg⁻¹) demonstrating the strong Al-chelating capacity of humic acid even at the reduced P dose (Nardi *et al.*, 2002) [6].

Iron-bound P

Iron bound P followed a pattern broadly similar to Al-P, ranging from 74.24 to 91.59 mg kg⁻¹ and increasing progressively over the incubation period in all treatments, consistent with the high free Fe oxide content typical of red alfisols (Reddy *et al.*, 2005) [14]. The absolute control (T_1) recorded the lowest Fe-P throughout (74.24 to 79.84 mg kg⁻¹), and T_3 (RDF + FYM) recorded the highest (84.16 to 91.59 mg kg⁻¹), mirroring the Al-P trend and indicating that FYM addition without a specific activator favoured progressive P occlusion onto iron oxide surfaces (Figure 1c). All phytase and humic acid treatments (T_4 - T_{11}) maintained significantly lower Fe-P than T_3 at every stage. The dose-dependent reduction observed for Al-P was similarly evident for Fe-P. Within each P level and activator type, the higher activator dose consistently produced lower Fe-P than the lower dose ($T_5 < T_4$ and $T_7 < T_6$ for phytase; $T_9 < T_8$ and $T_{11} < T_{10}$ for humic acid), and treatments at 75% RDP recorded lower Fe-P than their 100% RDP treatments. The treatment T_{11} again recorded the lowest Fe-P among fertilized treatments (77.06 to 81.62 mg kg⁻¹). The chelation of Fe³⁺ by humic acid functional groups, forming soluble or weakly-bound humic-Fe-phosphate complexes that retain greater lability than crystalline Fe-P, has been documented as the principal mechanism un-

derlying reduced Fe-P formation under humic acid application (Gerke, 2010) [15]. The proportionally greater reduction in Fe-P under phytase treatments relative to Al-P may be attributed to the additional release of Fe²⁺/Fe³⁺ from phytate-metal complexes during phytase-mediated phytate hydrolysis (Leytem *et al.*, 2005) [16], liberating both P and Fe simultaneously and thereby limiting the pool of reactive Fe available for fresh Fe-P formation.

Reductant soluble P

Reductant soluble-P, representing P associated with reducible amorphous Fe and Mn oxide coatings, ranged from 61.34 to 72.41 mg kg⁻¹ and increased gradually in all treatments over the incubation period. The absolute control (T_1) recorded the lowest values throughout (61.34 to 64.54 mg kg⁻¹). Treatment T_9 (FYM + 100% RDP + humic acid @ 5 kg ha⁻¹) recorded the highest reductant soluble-P at every stage (67.64 to 72.41 mg kg⁻¹), followed by T_3 and T_8 (Figure 1d). The elevated reductant soluble-P under humic acid treatments, despite their reduced Al-P and Fe-P, is consistent with the mechanism proposed by Gerke (2010) [15], wherein humic-Fe complexes maintain Fe in amorphous, poorly crystalline forms that are more readily solubilized under the reducing conditions of the CBD extraction than the well-crystallized Fe oxides associated with the Fe-P fraction. Phytase treatments (T_4 - T_7) recorded intermediate reductant soluble-P values, with the higher dose treatments (T_5 , T_7) again exceeding their respective lower-dose counterparts. The consistent increase in reductant soluble-P across all treatments over the incubation period reflects the progressive transformation of freshly precipitated, poorly crystalline Fe-phosphate phases into the amorphous reductant soluble pool prior to their eventual crystallization into more stable occluded forms (Willett, 1989) [17].

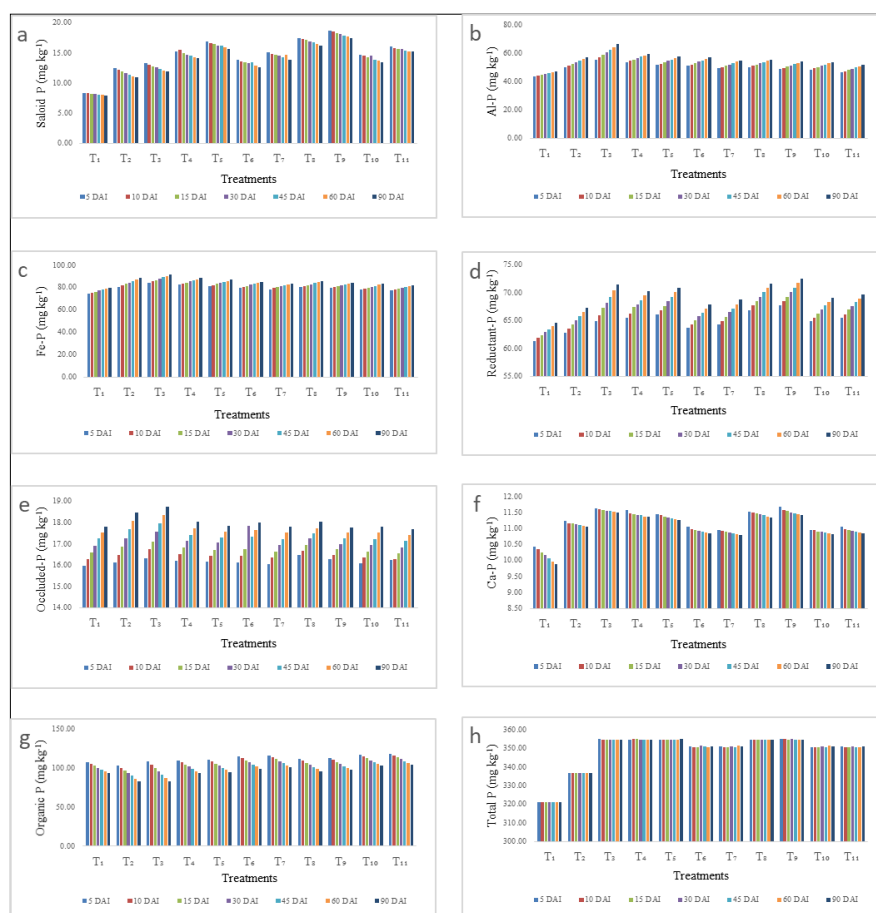


Figure 1. Influence of biochemical activators and P levels on a) saloid P, b) Al-P, c) Fe-P, d) reductant-P, e) occluded P, f) Ca-P, g) organic P and h) total P in soil at different days under incubation

Occluded P

Occluded-P, the most physically and chemically stable inorganic P fraction encapsulated within crystalline Fe/Al oxide aggregates, showed the narrowest range among all fractions (15.96 to 18.74 mg kg⁻¹) and increased only slightly over the incubation period in all treatments, consistent with the very slow turnover rate of this fraction (Kaiser *et al.*, 2003) [18]. The treatment T₃ (RDF + FYM) recorded marginally higher occluded-P than other treatments from 30 DAI onward (17.55 to 18.74 mg kg⁻¹), consistent with its correspondingly higher Al-P and Fe-P providing a larger pool for slow occlusion (Figure 1e). Absolute control treatment (T₁) and treatments receiving humic acid generally recorded the lowest occluded-P among fertilized treatments, suggesting that the reduced Al-P and Fe-P pools under humic acid application correspondingly limited the substrate available for occlusion. Given the inherently slow transformation rate of this fraction, the magnitude of treatment differences in occluded-P was considerably smaller than for the more labile inorganic fractions, indicating that a 90-day incubation period, while sufficient to capture dynamic changes in saloid-P, Al-P, Fe-P and reductant soluble-P, represents only the initial phase of occluded-P pool formation.

Calcium-bound P

Ca-P, derived principally from the Ca associated with the SSP source of P, ranged from 9.88 to 11.68 mg kg⁻¹. In contrast to Al-P, Fe-P and reductant-P, Ca-P declined gradually over the incubation period in all treatments, reflecting the inherent instability of Ca phosphate compounds at the acidic pH (5.11) of this alfisol, where thermodynamic favourability strongly favours dissolution of Ca-P and subsequent reprecipitation as the more stable Al-P and Fe-P (Lindsay, 1979) [13]. The absolute control (T₁), receiving no SSP, recorded the lowest Ca-P throughout (10.44 to 9.88 mg kg⁻¹) and also the steepest proportional decline, consistent with depletion of the limited native Ca-P pool without replenishment (Figure 1f). Treatment T₉ (FYM + 100% RDP + humic acid @ 5 kg ha⁻¹) recorded the highest Ca-P at the initial sampling (11.68 mg kg⁻¹ at 5 DAI) and maintained the highest or near-highest values throughout, closely followed by T₃ and T₈. The relatively higher retention of Ca-P under humic acid treatments may be attributed to humic acid-Ca²⁺ complexation, which maintains a fraction of the SSP-derived Ca in forms that continue to co-precipitate with orthophosphate as Ca-P rather than permitting its complete dissociation and reaction with Al³⁺/Fe³⁺ (Holford, 1997) [19]. The slower decline of Ca-P in T₃, T₈ and T₉ relative to T₂ indicates that FYM-based treatments, irrespective of activator, provide some buffering against the rapid dissolution of SSP-derived Ca-P in this acid alfisol.

Organic P

Organic-P declined consistently and substantially in all treatments over the 90-day incubation period, ranging from 83.30 to 118.66 mg kg⁻¹, reflecting progressive microbial mineralization of soil organic P compounds, predominantly phytate (Richardson, 1994) [20]. At 5 DAI, the highest organic-P was recorded in low dose of humic acid treatments, T₁₁ (118.66 mg kg⁻¹) and T₁₀ (117.56 mg kg⁻¹) followed closely by the reduced-P phytase treatments T₇ (116.61 mg kg⁻¹) and T₆ (115.36 mg kg⁻¹), all exceeding T₃ (109.12 mg kg⁻¹) (Figure 1g). This initial pattern reflects the larger native and FYM-derived organic P substrate pool available in treatments receiving a lower inorganic P dose, where less inorganic P competition for adsorption sites allowed a correspondingly larger organic P pool to persist at the start of incubation. By 90 DAI, the magnitude of decline differed markedly among treatments. Treatment T₂ (RDF alone) and T₃ (RDF + FYM) showed the steepest absolute decline (from 103.31 to 83.30 mg kg⁻¹, and 109.12 to 83.40 mg kg⁻¹, respectively), despite the treatment T₃ starting from a higher initial value than T₂. The full-dose phytase treatment T₅ declined from 111.11 to 94.89 mg kg⁻¹ and T₄ from 110.18 to 93.22 mg kg⁻¹, representing the steepest proportional decline among the biochemical activator treatments and confirming that exogenous phytase, protected by the FYM carrier matrix, actively ac-

celerated phytate-P hydrolysis throughout the incubation period (George *et al.*, 2005; Burns *et al.*, 2013) [3, 8]. Reduced-P treatments (T₆, T₇, T₁₀, T₁₁) retained the highest organic-P at 90 DAI (99.55 to 104.17 mg kg⁻¹), consistent with their larger initial organic P pools and the comparatively lower microbial P-mining pressure under reduced inorganic P input. The consistently higher organic-P retained in humic acid treatments relative to phytase treatments at the same P level (e.g., T₉: 97.50 mg kg⁻¹ vs T₅: 94.89 mg kg⁻¹ at 90 DAI) is consistent with the contrasting modes of action of the two activators. Phytase directly accelerates organic P mineralization, whereas humic acid acts primarily on inorganic P retention without a specific organic P-mineralizing function (Liu *et al.*, 2023) [21].

Total P

Total-P remained essentially constant within each treatment across the entire 90-day incubation period, with variation of less than 0.6 mg kg⁻¹ across all seven sampling stages for every treatment. This stability confirms that the incubation system functioned as a closed system with respect to phosphorus, with only inter-fraction transformation occurring and no net gain or loss of total soil P, thereby validating the internal consistency of the sequential fractionation procedure. Among the different treatments, total-P ranged from 321.00-321.20 mg kg⁻¹ in T₁ (absolute control) to 354.56-355.15 mg kg⁻¹ in T₄, T₅, T₈ and T₉ (the 100% RDP treatments with FYM and either phytase or humic acid), with T₂ (RDF alone, 336.60-336.79 mg kg⁻¹) and the 75% RDP treatments T₆, T₇, T₁₀ and T₁₁ (350.62 to 351.58 mg kg⁻¹) occupying intermediate positions corresponding to their respective P input levels (Figure 1h). The close similarity in total-P among T₃, T₄, T₅, T₈ and T₉ confirms that these treatments received equivalent total P inputs (100% RDP + FYM), with the biochemical activators influencing only the distribution of this P among fractions rather than the total quantity present, consistent with the designed treatment structure.

Conclusion

The 90-day incubation study demonstrated that humic acid and phytase, when applied through a FYM carrier, substantially altered the distribution of soil P fractions in this alfisols relative to FYM application alone. Humic acid at 5 kg ha⁻¹ with 100% RDP (T₉) consistently maintained the highest saloid-P and reductant soluble-P while reducing Al-P and Fe-P relative to RDF + FYM, indicating effective blockage of P fixation onto sesquioxide surfaces. Phytase at 4 kg ha⁻¹ with 100% RDP (T₅) produced the most rapid decline in organic-P, confirming active phytate mineralization, and sustained elevated saloid-P through continuous orthophosphate release. The dose-dependent responses observed for both activators, together with the conserved total-P within each treatment, confirm that the improvements in labile P fractions arose specifically from activator-mediated redistribution among inorganic and organic P pools rather than from any change in total soil P. These findings provide a mechanistic basis for the field-level improvements in P availability reported under integrated phytase and humic acid management and support their inclusion, mixed with FYM as carrier, as a component of sustainable P management strategies for P-fixing alfisols.

Acknowledgement

The author would like to thank the members of the advisory committee for their valuable guidance and insightful feedback throughout this research.

Conflict of interest

The authors declares that there are no conflicts of interest.

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How to cite this article: Priyadharshini T, Dhananjaya B, Ganapathi, Soumya T, Nandish M, Mallikarjuna H. Phosphorus fractions dynamics in an Alfisols amended with biochemical activators under varying phosphorus levels: An incubation study. *International Journal of Advanced Biochemistry Research*. 2026;SP-10(9):2718-2722. doi: <https://www.doi.org/10.33545/26174693.2026.v10.i9Sah.10245>