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RESEARCH PAPER

Influence of (Chicken Manure and Alfalfa Green Manure and Sheep Manure) on Alkaline Phosphatase and Asparaginase enzyme rate at different incubation temperatures.

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ABSTRACT

to reduce the negative impact of chemical fertilizers and increase soil fertility, organic fertilizers must be used which have a great potential to improve the biological, chemical, and physical properties of the soil. This study had been conducted to study the effect of different local manures (alfalfa, chicken, and sheep) on soil enzymes activity (Asparaginase enzymes and Alkaline Phosphatase), which they essential enzymes responsible in releasing nutrients needed by plants, at different conditions of incubation temperatures(28°C and 35°C) and duration (two and four weeks), results indicated that the highest Asparaginase enzymes activity (143.85ppm) was recorded in soils added by chicken manure after incubating it for 4 weeks at 28°C, while the lowest activity showed at 35°C incubated at the same period. the highest rate of Alkaline Phosphatase enzyme (150.92 ppm) was found after two weeks of incubation at 35°C in soils treated with alfalfa manure while at 28°C incubation temperature resulted in lowering of enzyme activity. Molecular analysis of soil showed difference among treated soils with different manures in quality of soil microorganisms. From results noticed that source of organic matter, incubation period and temperature have great effects on enzyme activity and type of soil microorganisms.

Keywords: temperature; Organic manure; microorganisms; Enzymes

1.INTRODUCTION

Intense and imprudent exploitation of land, frequently driven by the broadening need for food, has led to siltation in the cultural lands. For example, the adverse effects of mono-cropping carried out for long periods and the excess use of chemical pesticides and fertilizers have severely damaged soil health (Ernst et al., 2018). It further fiddles with product quality and human health (Ren et al., 2019) and causes chemical, physical, and biological degradation while reducing soil fertility (Mahajan and Sharma, 2008). Organic fertilizers, which are from local sources (Aksoy, 2001), form the very basis of soil fertility (Solomon et al., 2012). They are considered environmentally friendly and play a crucial role in plant nutrition (Aboudrare, 2009) by supplying essential nutrients, enhancing water retention, and influencing the bioavailability of heavy metals (Alvarenga et al., 2008). Different scientific research on green manure use in agriculture shows it as one of the methods to achieve high agricultural productivity across different regions (Yang et al., 2018). Hay alfalfa (Hoeppner et al., 2005) can provide an extra nitrogen source in organic crop rotations, and it might be considered a good quality forage for cattle. On the other hand, other farmers go for poultry manure and apply it to elevate soil nitrogen levels suitable for cereal production at a low cost (Lorent et al., 2016). Despite this, though, the utilization of animal manure in organic farming has been criticized because it contributes to nutrient overload (Hao et al., 2004), mainly when obtained externally; thus, it fails to yield nutrient cycles.

Microorganisms of the soil thrive in the presence of organic matter with high carbon content and the necessary elements for their flourishing. Soil enzymes that include microorganisms are essential in plant nutrition, being the leading players in all biochemical reactions, the stabilizers of the soil structure, and participators in the cycle of nutrients. The microbial community's tracking of soil enzyme activities becomes a barometer of the potential for soil organic matter degradation and nutrient availability (Waldrop et al., 2012). An example of such an enzyme is L-asparaginase. It hydrolyzates the L-asparagine amino acid compound into L-aspartic acid and ammonia (Meghavarnam and Janakiraman, 2015).

Different types of phosphatase enzymes are essential to convert complex organic P into inorganic P. Alkaline phosphatase, amidase, alpha-glucosidase, and dehydrogenase are the only kinds of phosphatase-enzymes that can be linked to the activity of microbes involved in the breakdown of soil, especially glucose (Frankenberger and Dick). Concentration on the degradation of diverse primary components comprising detrital organic matter such as glucosidases, phosphatases, and phenol oxidases has been the focus of recent research, which researches the ecological evolution of detrital organic matter to nutrient cycling. The soil temperature and organic matter content play a pinning role among the influencing factors of enzyme activities. Conversely, this temperature dependence can be explained by an adherence to basic thermodynamic laws. Promoting factors such as pH and time to the soil enzyme activity have also been proven to increase (R.C. and Jadhav, 2017). On the other hand, the soil organic carbon content emerged as an essential component in predicting the changes within soil enzyme activities and microbial biomass, with the soils containing more organic matter (SOC) displaying higher activities compared to soils with lower SOC.The purpose of the research was to determine the influence of three different organic residues (Chicken manure at 2%, Alfalfa Green manure at 2%, and Sheep manure at 2%) on the alkaline phosphatase and asparaginase rate changes under different temperature conditions.

2. MATERIALS AND METHODS

2.1. Soil Sample and organic residues Collections

The researcher took Rhizospheric samples from different sites in Ainkawa research center in Erbil governorate - Kurdistan region- Iraq from August to October 2022. Soil samples were collected at a depth

of 3-30 cm. Collected samples were performed in sterilized aseptic bags and subjected to microbiological, chemical and physical properties.
Three types of organic residues (chicken manure, Alfalfa green manure and sheep manure) were collected from Grdarasha farms. After sterilization process, they were dried and grind to powder, and sieved (2 mm).

2.2. Soil and organic manures analysis.

The samples were underwent some physical and chemical analysis included: soil texture, field capacity, EC, pH, and soil organic matter content. particle size distribution was performed by hydrometer method as described by Bowels (1976). Field capacity have been estimated according to the model prepared by Karim (2000). Electrical conductivity, Soil pH and Organic matter were measured for the soil saturated extract as described in (Ryan *et al*., 2003). For calculating C:N ratio in manures and soil, organic carbon and total nitrogen were determined. Organic carbon for manures were determined by titrimetric method according to Richards (1954). Amount of total nitrogen in manures were determined by the kjeldahl method as described in Rowell (1996).

2.3. The incubation experiments

Hundred grams of soil samples added by 2% of chicken manure, alfalfa green manure and sheep manure, individually, were placed in glass jars with three replications for each treatment and they closed tightly with plastic cover. The moisture content of all treatments was got to 80% of the field capacity by addition of distilled water, and they were incubated at two different temperature (28 °C and 35°C) for 28 days. Activity of alkaline phosphatase and asparaginase enzymes were measured twice, 14 and 28 days after incubating period, according to Tabatabai cited by (Page et al.,1982). The jars were opened each two days in order to maintain aeration.

2.4. Measurement of asparaginase enzyme activity.

The activity of asparaginase enzyme was measured after 14 and 28 days of incubation, according to Tabatabai cited by (Page et al.,1982). For asparaginase enzyme activity, five gram of sieved soil, 0.2 ml toluene, 9 ml of Tris (hydroxymethyl) aminomethane buffer (THAM), and 1 ml of 0.5M asparagine solution were added in a 50 ml volumetric flask, and Shaked for a few second, after mixing the contents, the flasks were incubated at 37°C for two hours. After incubation 35 ml of K CL (2.5 M)-Ag2SO4 (100 ppm) solution was added, Shaked for a few seconds, then allowed to cool to room temperature, and the contents was completed to 50 ml by the same solution and inverted for several times.
The released ammonium was measured according to (Bremner and keeney 1966). 1g. of soil, added by 9 ml of 2M KCl (10:1) and left for 30 minutes in a mechanical shaker then filterd by Whatman paper vol. 42. The released ammonium was determined in the distillation flask a filter the addition of 0.2g heavy MgO and receiving the ammonia gas in boric acid 10 which was added mixed indicator solution, then titrated with 0. 01M HCL.

2.5. Measurement the activity of alkaline phosphatase

The activity of alkaline phosphatase enzyme was tested using the process that Tabatabai (1982) illustrates. The sieved soil of 1 gram was added to 0.2 ml of toluene, 4 ml of modified universal buffer (MUB) having a pH of 11, and 1 ml of P-nitrophenyl phosphatase solution (0.025 M) to a 50 ml Erlenmeyer flask after 14 and 28 days of climate manipulation, respectively The flask was closed and shook briefly and incubated at 37°C. After 60 minutes, 4 ml of 0.5 M NaOH and 1 ml of 0.5 M CaCl2

and were added, followed by a brief swirl of the mixture, and then it was filtered via a Whatman no.2 fold filter paper. The absorbance of the yellow color of the nitrophenol that had leached from the filter was read using the spectrophotometer at 720 nm. The same procedure was used to prepare a standard curve but without soil.

2.6. Total counting of bacteria and fungi.

Both soil Bacteria and Fungi numbers were estimated by determination of viable plate count according to (Aneja, 2003). After preparation of serial dilution, soil suspensions were cultivated on nutrient agar plates then all plates were put in an incubator for one day at 28°C. to determine bacterial cell counts. While for Fungal population count soil suspensions were cultured on SDA (Sabouraud Dextrose Agar) then plates were put into an incubator for 7 days at 25°C.

2.7. Molecular identification of bacteria and fungi

This investigation involved the extraction of genomic DNA from bacterial and fungal cells using the GeneAll® ExgeneTM for Clinic Cell SV mini kit. The integrity of the isolated DNA was confirmed by electrophoresis on a 0.8% agarose gel (Figure 1). All bacterial and fungal isolates have been undergone PCR assay for more confirmation to the identity of all isolates, using universally conserved 16S rRNA gene for bacterial isolates (Figure 2), and ITS gene for fungal isolates (Figure 3) genes.

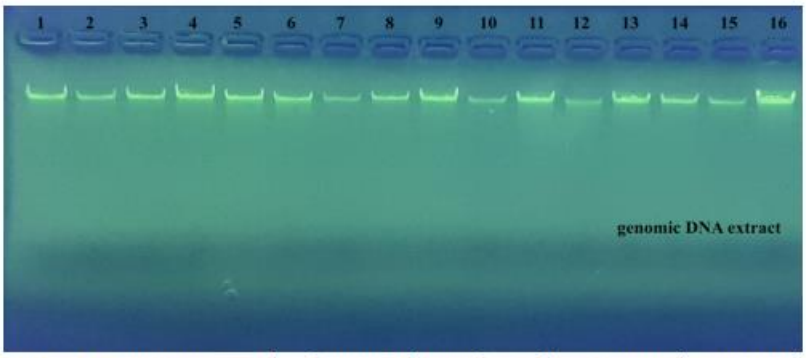

Fig. 1 Agarose gel electrophoresis of extracted genomic DNA of bacterial isolates (lanes: 1-9) and fungal isolates (Lanes: 10-16) represented the appearance of successfully extracted demonic DNA.

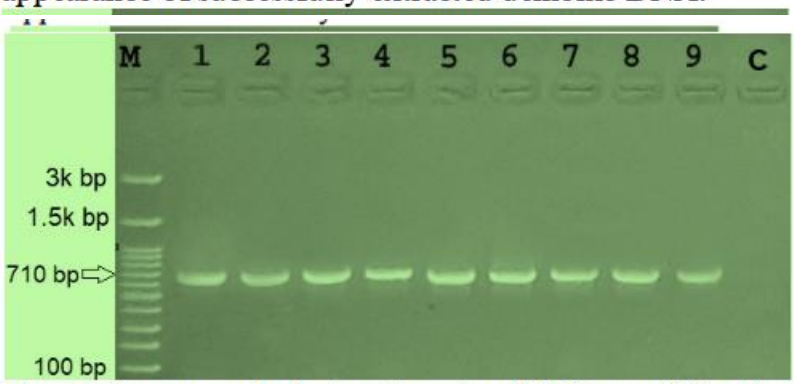

Fig. 2 Agarose gel electrophoresis of PCR amplification products for the 16S rRNA gene of nine bacterial isolates. M: The DNA marker (100 bp ladder), lanes **1**-**9** positive amplification of **710 bp** for 16S rRNA gene, C: Negative control.

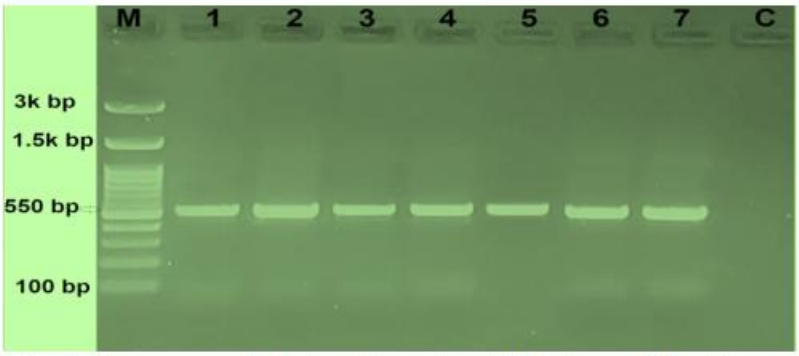

Fig. 3 Agarose gel electrophoresis of PCR amplification products for the ITS gene of seven fungal isolates. M: The DNA marker (100 bp ladder), lanes 1-7 positive amplification of 550 bp for ITS gene, C: Negative control.

2.8. Statistical analysis.

The data was illustrated as mean (M + S.E) and statistical analysis was carried out using available software (SPSS version 26.0). Data analysis was made using one way analysis of variable (ANOVA). The comparisons between groups were done using Duncan test was considered as statistically significant as mentioned by (IBM Corp, 2019).

3. RESULTS AND DISCUSSION:

3.1. Soil and organic manures analysis

Chemical and physical results of the laboratory analysis for the soil sample taken before starting experiment indicated that the textural class of the experimental soil was silty clay loam, by applying particle size distribution data (Sand 8.8%, Silt 61.3 %, clay 29.9 %) in soil texture triangle. The pH value of the soil was 7.94, Field capacity was 80%, Electrical conductivity was 0.298 Ds/m at 25˚C, and the result showed that organic matter content of the soil was (1.548%). The (C: N) ratio for chicken 2%, sheep 2%, alfalfa 2%, soil were (9:1), (16: 1), (11: 1), (9: 1) respectively.

3.2. Total counting of bacteria and fungi and their Molecular identification

Both soil Bacteria and Fungi were estimated by determination of viable plate counting according to (Aneja, 2003). The numbers of bacterial and fungal colonies were counted are 97.619 gm soil $*10^6$ and 4.571 gm soil $*10^6$. In molecular analysis, Sequence information was obtained from the NCBI database to characterize bacterial and fungal isolates. The results were analyzed and compared with similar sequences deposited in GenBank using the BLAST program. Based on GenBank reference sequences, Table (1) shows that 9 of the soil isolates (No. 1 – No. 9) 100% identity to different nine species of bacteria, and seven isolates (No. 10 – No. 16) showed 100% identity to different seven species of fungi due to nucleotide changes (microvariant). In all treated soil with manures, *(Staphylococcus pasteuri*) species of bacteria found while (*Salmonella enterica)* can be identified only in soils treated with sheep manure and *(Sulfitobacter donghicola)* in soils treated with chicken manure only. From the seven isolated species of fungi, four of them identified in all three types of manures2% which treated with soil.

Table (1): The GenBank NCBI accession numbers of bacterial and fungal isolates were indicated BLAST analysis to identify observed samples

Isolate No.	Identified Samples	ACCN of samples	Identic rate %	Host	Cou ntry
No. 1	*Priestia megaterium*	OQ155023	100	Soil-(sheep2%-chicken2%)	Erbil, Iran
No. 2	*Staphylococcus warneri*	OQ155024	100	Soil-(sheep2%-chicken2%)	Erbil, Iran
No. 3	*Sulfitobacter donghicola*	OQ155025	100	Soil- (chicken2%)	Erbil, Iran
No. 4	*Xanthomonas axonopodis*	OQ155026	100	Soil-(sheep2%-alfalfa2%)	Erbil, Iran
No. 5	*Bacillus aryabhattai*	OQ155027	100	Soil-(sheep2%-alfalfa2%)	Erbil, Iran
No. 6	*Bacillus halotolerans*	OQ155028	100	Soil-(alfalfa2%-sheep2%)	Erbil, Iran
No. 7	*Staphylococcus pasteuri*	OQ155029	100	Soil-(alfalfa2%-sheep2%-chicken2%)	Erbil, Iran
No. 8	*Flavobacterium agri*	OQ155030	100	Soil-(sheep2%-chicken2%)	Erbil, Iran
No. 9	*Salmonella enterica*	OQ155031	100	Soil-(sheep2%)	Erbil, Iran
No. 10	*Aspergillus niger*	OQ154256	100	Soil-(chicken2%-sheep2%)	Erbil, Iran
No. 11	*Penicillium chrysogenum*	OQ154257	100	Soil–(chicken2%-sheep2%-alfalfa2%)	Erbil, Iran
No. 12	*Fusarium oxysporum*	OQ154258	100	Soil-(alfalfa2%-sheep2%-chicken2%)	Erbil, Iran
No. 13	*Trichoderma viride*	OQ154259	100	Soil- (sheep2%-chicken2%-alfalfa2%)	Erbil, Iran
No. 14	*Botrytis cinerea*	OQ154260	100	Soil- (alfalfa2%-sheep2%-chicken2%)	Erbil, Iran
No. 15	*Cladosporium herbarum*	OQ154261	100	Soil- (chicken2%-sheep2%)	Erbil, Iran
No. 16	*Pseudocercospora eumusae*	OQ154262	100	Soil- (sheep2%-alfalfa2%)	Erbil, Iran

3.3. Effects of chicken, sheep, and alfalfa green manure on Alkaline Phosphatase and Asparaginase enzyme rate at two temperatures and two time incubation period.

The results in table (2) showed that the 28°C incubation temperature had positive effects on Asparaginase enzymes activity in treated soil with chicken, sheep, and alfalfa green manures during different incubation periods. The highest rate of Asparaginase enzymes was recorded in soils treated with alfalfa green manure after two weeks of incubation (135.10 ppm) and significantly differ from other treatments, while after four weeks of incubation the activity of Asparaginase enzyme generally was increased in all studied treatments and the highest rate was found in chicken manure treatments (143.85 ppm) comparing with sheep, alfalfa green manure and control treatments. In contrast, the results showed that the Asparaginase enzymes activity was reduced in all manures treatments after four weeks of incubation at 35°C temperature. the highest level of Asparaginase enzymes in treatments incubated at 35°C was recorded after two weeks of incubation in sheep manure (100.45 ppm).

Table (2): mean activity of asparaginase and Alkaline Phosphatase enzyme in treated soil with three types of manures at two temperature and two time of incubation.

Treated soil with manures	Asparaginase enzyme activity (ppm)				Alkaline Phosphatase activity (ppm)			
	Temp 1(28°C)		Temp 2 (35 °C)		Temp 1(28°C)		Temp 2 (35 °C)	
	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
Control	9.80 nmlk	4.90 nm	16.80 kj	16.55 kj	8.60 g	7.67 g	52.52 d	40.15 fed
Chicken2%	42.70 gf	143.85 a	99.75 b	33.25 ihg	18.36 gfe	17.43 gf	97.85 cb	59.30 d
Sheep 2%	57.75 e	46.20 f	100.45 b	39.55 hgf	16.86 gf	11.87 g	115.96 b	56.78 d
Alfalfa 2%	135.10 a	135.45 a	94.85 b	46.55 f	27.56 gfe	19.49 gfe	150.92 a	59.54 d

Alfalfa green manure treatments get the highest results for Alkaline Phosphatase enzyme activity after 2 weeks of incubation at both 28°C and 35°C incubation temperature (27.56 ppm, 150.92 ppm) respectively. The use of green manure planting systems has benefited the population and structure of soil biota. As a result, the soil C/N ratio in the upper soil layers changed because of the different C/N ratios of compost at the moment of application, consequently affecting the optimal mineralization of organic matter (Tejada et al., 2008). In the case of the studied green manures, the researchers discovered that alfalfa proved to be stimulating labile phosphorus fractions (NaHC03-Pi, NaOH-Pi), phosphorus uptake, as well as different soil enzyme activities, to some extent, better as compared with the conventional nitrogen fertilizer (Gao et al., 2016).

Analysis of four weeks of incubation unveiled the most pronounced differences of alkaline phosphatase observed in soil samples amended with sheep and chicken manure (11.87 ppm and 59.30 ppm) at the following temperatures, respectively (28°C and 35°C). Several selected soil properties seem to have an impact on soil enzyme activities. Specifically, L-asparaginase activities strongly bind to soil organic

carbon content, of which other enzymes such as phosphatase, urease, pyrophosphatase, and rhodanese demonstrated positive correlations. Frequently, the organic carbon content of soil samples reflects the level of biological epilogissement of soil because it has a linear relationship with enzyme levels of the soil. Conversely, total nitrogen content was strongly indicated with L-asparaginase activity in the surface soil region tested. The relationship of the enzyme with organic matter transformation and changes in N compounds was evident to some extent (Frankenberger & Tabatabai, 1991). Additionally, sheep manure has been seen to increase soil conductivity through electrical contact and decrease the amount of the metal complex extracted by DTPA in soils; thus, it poses a remarkable solution to soil pollution. All samples result for Alkaline Phosphatase enzyme gets high activity at two weeks of incubation compare to four weeks at 28°C and 35°C temperature. In most cases, the highest activity of the enzymes urease and phosphatase was observed at the first two weeks of the incubation which means that the number of the genera of urease and phosphatase producing bacteria were on its highest peak at the end of two weeks. The activity of the enzyme phosphatase decreased sharply in most cases, this is probably due to the adsorption of the enzyme phosphatase to clay particles in the presence of Ca^{+2} as bridge and this complexation will render the enzyme inactive and it may become unextractable by the extractants which being used in our method (Atroshy, 2004).

The recent studded indicated that the addition of chicken, sheep, and alfalfa green manure had positive effects soil enzymes Alkaline Phosphatase and Asparaginase rate, this may be due to the effect of different manure on total soil microorganisms' activity. Microbial biomass is proportional to the soil's organic matter, the more
the biomass, the more the enzymatic activity (killham 1996 and Alexander 1977, Dick,
et al. 1988). Soil physical and chemical properties included soil pH, moisture and organic matter content effect on enzyme activity. also, these manures may be used as a source of different nutrients in soil rather than great influence of incubation periods and temperatures on organic manure decomposition. These results of both enzymes affected by the moisture level in the samples, since a decreasing in both of enzyme's rates are noted, when both samples incubated for four weeks (Atroshy, 2004). Especially at 35°C temperature the activity of both enzymes declined after four weeks incubation. Microbes and, potentially, flora, are believed to react to elemental disparities in their supplies by generating enzymes aimed at the lacking element (Mooshammer, M. et al., 2014). According to the results presented in a study Conant et al. (2011), temperature is a key factor controlling the rate of decomposition of plant debris.

4. CONCLUSION:

Soil treated with organic manures have positive effect on enzymes activity. Activity of enzymes in treated soils with manures have obviously more than control samples. Alkaline Phosphatase and Asparaginase activity rate for samples treated with Chicken manure, sheep manure and alfalfa green manure increased in 35 °C compared to 28°C, this is true for control samples of them. Remaining all samples in incubator in the same temperatures after 4 weeks declined compare to 2week remaining samples in incubator except at 28°C, 2 weeks. The C:N ratio of soil and manures with activity and population of microorganisms have clearly observed on the enzymes activity rate because of highest results of treated soils of manures compare to control samples. Samples treated with chicken manure have gained good result for activity of Asparaginase enzyme according to temperature 28°C, 4 weeks incubation while samples contain sheep manure gets high result at 35°C, 2week period incubation. For observing the lowest result in Asparaginase enzyme, Samples treated with chicken manure gained. Alkaline Phosphatase recorded high value in treated soil with alfalfa

green manure in 2 weeks incubation time, at 28°C and 35°C, and the lowest was in sheep manure compare to other manure in 4 weeks incubation time, at 28°C and 35°C.

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