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RESEARCH METHODOLOGY

Introduction:

In generally, the research methodology is a process used to collect information and data for the purpose of making decisions in investigations. The methodology may include publication research, surveys and other research techniques include both present and past information. The research method involves qualitative and quantitative research, the qualitative research involves detail situation using research tools like surveys and observations. The quantitative research involves quantifiable data i.e. numerical and statistical explanations, used to solve problems in statistical and psychological contents.

The historical method, survey method and experimental method of research are used by most of the researchers in their research work. Most of the scientific issues are solved through experimental protocol. The experimental method has been used for the present study. This chapter is further divided into two major contents. The first content is to evaluate the toxicity of pesticides by using probit analysis explained in reference book ‘A statistical treatment of the sigmoid Response Curve’ by D. J. Finney published in the United States of America by Cambridge University Press, New York. The second content is to estimate the enzymatic activity of enzyme Amylase, Cellulase, Protease, Lipase and Urease respectively by using different methods explained in the reference book ‘Biochemical methods’ written by S. Sadasivam and A. Manickam published in the New Delhi by New age International Publication Limited.
3.1 Statement of the problem:

The present investigation is planned with the statement of problem “To study the effect of pesticides on enzymatic activities and behavior of earthworm from Nashik district”

3.2 Aim and Objectives of the study:

The major aim of the present study was to determinate the toxic effect of pesticide on the earthworm enzymatic activity and behavior.

Following are the objectives of the study-

1. To find acute toxicity of selected pesticides mostly used in agriculture.
2. To find out which pesticide is more toxic and broadly affect on enzyme secretion in earthworm.
3. To evaluate enzymatic activity of digestive enzyme secreted by earthworm.
4. To suggest policy guideline for farming practices.

3.3 Hypothesis of the study:

The hypothesis is a proposed explanation for a phenomenon. An assumption made for the sake of argument and interpretation of practical situation or condition taken as action. The pesticide is a synthetic material which could not degraded into soil, its residue remains in the soil, unfortunately it enters in the gut of earthworm by their feeding habit because humus soil is its natural food ultimately these residue critically affect on tissue and reduces its enzyme secretory activity.

1. The enzymatic activity is more in control earthworm than treated earthworm with insecticide, fungicide, herbicide, and Nematocide and Molluscicide pesticides.
2. Insecticide and fungicide pesticide are more toxic than herbicide, Nematocide and Molluscide pesticide
3.4 Variables of the study:

The variables or the elements used in the research for the purpose of all research are to describe and explain variance. The variables are the measurable characteristics may alter in groups and subgroups. In the present research work, dependant and independent variable are used. The independent variables are pesticides that affect on the enzymatic activity of the earthworm and dependant variables are enzyme which shows greater frequent variability in their appropriate secretary quantity through tissue of the body of an animal. The Insecticide, fungicide, herbicide, nematocide and molluscicide pesticides were used as independent variables and digestive enzymes like amylase, cellulase, protease, lipase and urease were used for the study. The enzyme amylase, cellulase, protease, lipase and urease were selected for the study because it is mostly activate on humus which contain cellulosic material degraded by detrivores like earthworms.

Following independent and dependant variables are used for the present study:

A) Independent variables – Pesticides

1. Insecticide
2. Fungicide
3. Herbicide
4. Nematocide
5. Molluscicide

B) Dependant variables – Enzymes

1. Amylase
2. Cellulase
3. Protease
4. Lipase
5. Urease
3.5 Research area:

The Nashik district was selected for the present study because it is well irrigated but excessively using synthetic pesticides in the field for protecting the crop from the pest. The Nashik is the most important highly developed city, to cultivate fruits and vegetables, supplies to the metropolitan cities like Mumbai (Maharashtra), Surat (Gujarat) and Indore (M.P.) in India.

The pesticides are non-biodegradable material which deposited remains in the soil for long period of time simultaneously accumulates in soil microbes and detrivores like earthworm which is the best friend of farmers. The pesticidal residue entered in the body of earthworm through soil which is the natural food and may affect on enzymatic activity causes heavy mortality hence the biodiversity of earthworm comes in danger due to excessive use of pesticides in Nashik district of Maharashtra.

The Nashik district is located in Maharashtra state on the coordinates between 18°33’ and 20°53’ North latitude and between 73°16’and 75°16’ East Longitude at Northwest part of the Maharashtra state, at 565 meters above sea level. Nashik district has an area of 15,530 square kilometers. It is bounded by Dhule district to the north, Jalgaon district to the east, Aurangabad district to the southeast, Ahmadnagar district, to the south, Thane district to the southwest, Valsad and Navasari districts of Gujarat to the west, and the Dang district to the northwest. It is Rhomboidal in shape with longer diagonal of about 170 km. from South West to North East with an area of 15582 sq. km. (6015 sq. miles). The district was formed in the year 1869 with the city of Nashik.
3.6 Toxicity Evaluation:

The toxicity is the concentration of chemical at which the animal comes in contact or inhale it in the body, it may affect on their various physiological functions and their rate at which it is activated. The toxicity is the degree to which a substance can damage an organism. The toxicidal effect affects on the whole body of an organism as well as the effect on a substructure of the organism such as cell and liver probably by their morphological character and physiological functions. In general the toxicity shown three entities through which it may affect on the body of an organism. It is either chemical form or biological or physical form. Mostly the chemical residue persists in the tissue, which disturb its functions. The biological toxicants include microorganisms that can induce disease in living organism. The biological toxicity measures difficult as its life cycle. The physical toxicants are the substances that interfere with biological processes.

The toxicant can be measured by its effect on the target organism especially their organs, tissue, cells because level of chemical stimulus depends on the doses of toxic substances. The toxicity can be measured by LD$_{50}$ by treating test organism with toxic pesticides comparing to know the toxicity through bioassay. It is too difficult to estimate the toxicity of chemical mixtures enhance its effects on the body of target organisms. The toxicity of the toxic substances may cause lethality to specific organ that damage vital organs or whole biological system of the organism. The acute toxicity affects lethal effect on dermal or inhalation of toxicants in the body of target animals.

The process of understanding the effect of a given pesticides on the biological process in a given test organism are much complicated by the fact that in any aquatic or terrestrial ecosystem introduce a set of new pesticide will change the complete
picture of the response pattern, suggesting that interaction of pesticide will have either enhanced the effect on the organisms. Therefore it is much more important to study the effect of pollutants both in isolated cases and in several combinations. It is particularly important for an ecologist or ecotoxicologist to decide about the safe level of pollutants in terrestrial environment.

The exposure of pesticide to environmental strata induces characteristic series of endocrine and other response that re termed primary, secondary or tertiary depending upon the biological organization being monitored. Thus, primary response describes changes in the endocrine level secondary responses constitute a series of hematological changes in the tissue referred in the body of an organism. Several aspects of the tertiary response can be used as indicators of the unfavorable environmental conditions.

**3.6.1 Selection of test animal:**

The Healthy and approximately same sized earthworms were collected from vermicompost unit and brought to the laboratory through the method described by Owa, et, al, (2003). The soil was carefully turned using a spade while the earthworm were handpicked into container and transported to the laboratory where they were washed with distilled water. The worms were kept in refrigerator for three to four hours in order to kill them without causing any harm or alteration to the digestive enzyme activity of the gut. The *Eudrilus eugeniae* is a species of earthworm which is widespread in warm regions. It is good species for vermicomposting in agricultural point of view. It is selected for the research because of its commonly distributed and available in Nashik district.
Plate No.2: *Eudrilus eugeniae*

**Systematic Position of Test Animal:**

Kingdom- Animalia

Phylum- Annelida

Class- Clitellata

Order- Haplotaxida

Family- Eudrilidae

Genus- *Eudrilus*

Species- *eugeniae*
3.6.2 Selection of pesticide:

To estimate the effect of pesticide on enzymatic activity on earthworm five major pesticides were selected for the experiment. These pesticides were used on large scale by the farmers in their agricultural field to control pest and weeds in Nashik district. The agriculture from Nashik city is well irrigated and cultivated in horticulture. For the present study purposes Insecticide: - Nuvan, Fungicide: - Bavistin, Herbicide:-Roundup, Nematocide- Furadan and Molluscicide- Snailkill pesticides were selected for the experiment because it has been used farmers on large scale in their agriculture. The pesticides are synthetic chemical manufactured in the industries for killing agricultural pest. Due to its toxicity, it is harmful to the pest. In soil ecotoxicological, model species are usually chosen from species that are easy to maintain and breed in laboratory conditions and for which molecular tools are available.

3.6.3 Physicochemical properties of pesticide:

The physicochemical properties of chemicals are essential for their studies because most of reactions and their rate depend on it. Even for understanding the molecular formula, information of its containing atoms is to be verifying for the studies for further research. Mostly the molecular weight is considered for calculating the number of moles of solute contains in the solution. Analytical analysis purpose there is molecular weight is too important for making different solution of treatments in bioassay.
1. Insecticide: Nuvan

(i) **Contain:** Dichlorvos

(ii) **Formulations:** 76% EC

(iii) **Producer:** Insecticide India Ltd., Rajasthan. (India)

(iv) **Agricultural Use:** It is used as an insecticide.

(v) **Control:** Insect and moths

(vi) **IUPAC Name:** 2, 2-dichlorovinyl, dimethyl phosphate

(vii) **Molecular Formula:** C₄H₇Cl₂O₄P

(viii) **Structural Formula:**

![Structural Formula](image)

(ix) **Molecular Weight:** 220.98g/mol.

(x) **Melting Point:** - 60 °C

(xi) **Boiling Point:** 140 °C

(xii) **Density:** 1.20 g/cm³
2. Fungicide: Bavistin

(i) Contain: Carbendazim

(ii) Formulations: 50% WP

(iii) Producer: BASF India, Mumbai. (India)

(iv) Agricultural Use: It is used as fungicide.

(v) Control: Fungus

(vi) IUPAC Name: Methyl, 1H- benzimidazol, 2- ylcarbamate

(vii) Molecular Formula: C₉H₉N₃O₂

(viii) Structural Formula:

(ix) Molecular Weight: 191.187 g/mol.

(x) Melting Point: 303 °C to 307 °C

(xi) Boiling Point: 50 °C

(xii) Density: 1.45 g/cm³
3. Herbicide: Roundup

(i) Contain: Glyphosate

(ii) Formulations: 41%SL

(iii) Producer: Monsanto India Ltd. Mumbai.(India)

(iv) Agricultural Use: It is used as a herbicide.

(v) Control: weeds

(vi) IUPAC Name: N- Phosphonomethyl glycine

(vii) Molecular Formula: C₃H₈NO₅P

(viii) Structural Formula:

![Structural Formula of Glyphosate]

(ix) Molecular Weight: 169.07 g/mol

(x) Melting Point: 184.5 °C

(xi) Boiling Point: 187 °C

(xii) Density: 1.704 g/cm³
4. Nematocide: Furadan

(i) **Contain:** Carbofuran

(ii) **Formulations:** 3%

(iii) **Producer:** Syngenta India Ltd. Pune. (India)

(iv) **Agricultural Use:** It is used as a nematocide.

(v) **Control:** Nematodes

(vii) **IUPAC Name:** 2,2, Dimethyl- 2,3 dihydro, 1- benzofuran -yl- methycarbamate

(viii) **Molecular Formula:** C_{12}H_{15}NO_{3}

(ix) **Structural Formula:**

![](image)

(x) **Melting Point:** 151°C

(xi) **Boiling Point:** 313.3°C

(xii) **Density:** 1.18 g/cm³
5. Molluscicide: Snailkill

(i) **Contain:** Metaldehyde

(ii) **Formulations:** 2.5 % (W/W)

(iii) **Producer:** P. I. Industries Ltd. Udaypur, (India)

(iv) **Agricultural Use:** It is used as a molluscicide

(v) **Control:** Snail and slugs

(vi) **IUPAC Name:** R-2, C-4, C-6, C-8, tetramethyl 1, 3, 5, 7-tetroxocane

(vii) **Molecular Formula:** C₈H₁₆O₄

(viii) **Structural Formula:**

![Structural Formula](image_url)

(ix) **Molecular Weight:** 176.212g/mol.

(x) **Melting Point:** 246 °C

(xi) **Boiling Point:** 110 to 120 °C

(xii) **Density:** 1.27 g/cm³
3.6.4 Preparation of toxicant: (Stock solution)

Solutions were prepared using above commercial grade Insecticide:-Nuvan, Fungicide:-Bavistin, Herbicide:-Roundup, Nematocide:-Furadan, Molluscicide:-Snailkill separately. All standards were prepared in tap water. 1 ml of pesticide per liter gives 1 ppm and different dilutions were prepared by adding required amount of tap water. Stock was then added in tray containing 5kg soil. By means of micropipette

PPM = parts per million:

PPM is a term used in chemistry to denote a very, very low concentration of a solution. One gram in 1000 ml is 1000 ppm and one thousandth of a gram (0.001g) in 1000 ml is one ppm.

One thousandth of a gram is one milligram and 1000 ml is one liter, so that 1 ppm = 1 mg per liter = mg/Liter.

PPM is derived from the fact that the density of water is taken as 1kg/L = 1,000,000 mg/L, and 1mg/L is 1mg/1,000,000mg or one part in one million.

Serial dilutions:

Making up $10^{-1}$ M to $10^{-5}$ M solutions from a 1M stock solution.

Pipette 10 ml of the 1M stock into a 100 ml volumetric flask and make up to the mark to give a $10^{-1}$ M soln.

Now, pipette 10 ml of this $10^{-1}$ M soln. into another 100 ml flask and make up to the mark to give a $10^{-2}$ M soln.

Pipette again, 10 ml of this $10^{-2}$ M soln. into yet another 100 ml flask and make up to mark to give a $10^{-3}$ M soln.

Pipette a 10 ml of this $10^{-3}$ M soln. into another 100 ml flask and make up to mark to
give a $10^{-4}$ M soln.

And from this $10^{-4}$ M soln. pipette 10 ml into a 100 ml flask and make up to mark to give a final $10^{-5}$ M solution.

(A) **PPM (PARTS PER MILLION) CONVERT INTO PERCENT SOLUTION**

Divide the ppm amount by 1,000,000 and multiply by 100 to get %. e.g.:

- $1$ ppm = $1/1,000,000 = 0.000001 = 0.0001\%$
- $10$ ppm = $10/1,000,000 = 0.00001 = 0.001\%$
- $100$ ppm = $100/1,000,000 = 0.0001 = 0.01\%$
- $200$ ppm = $200/1,000,000 = 0.0002 = 0.02\%$
- $5000$ ppm = $5000/1,000,000 = 0.005 = 0.5\%$
- $10,000$ ppm = $10000/1,000,000 = 0.01 = 1.0\%$
- $20,000$ ppm = $20000/1,000,000 = 0.02 = 2.0\%$

(B) **Parts per hundred convert into % to ppm**

Divide the % value by 100 and multiply by 1,000,000 to get ppm. e.g.:

- $1\% = 0.01 \times 1,000,000 = 10,000$ ppm
- $0.5\% = 0.005 \times 1,000,000 = 5,000$ ppm
- $0.1\% = 0.001 \times 1,000,000 = 1,000$ ppm
- $0.01\% = 0.0001 \times 1,000,000 = 100$ ppm

3.7 **Bioassay:**

Acute toxicity was calculated by bioassay studies carried out to determine the potency of pesticide through exposure system. Soil used for acclimatization and conducting experiment was clear tap water. The maximum sized active acclimatized earthworms were selected for the evaluation of toxicity. Acute toxicity tests data was
collected and analyzed statistically by mean of the probit methods on transforming the

toxicity curve into regression line (Finney 1951) which allow the average median
lethal concentration or LD$_{50}$ value to be calculated for 24, 48, 72 and 96 hours.

3.7.1 Screening test:

In order to fix concentration a preliminary range finding or screening test was

conducted. The test was conducted by static method. A group of 10 earthworms were

exposed to ten widely spread trays for different concentrations in ppm of commercial

grade Insecticide-Nuvan, Fungicide-Bavistin, Herbicide-Roundup, Nematocide-

Furadan, and Molluscicide-Snailkill for 24, 48, 72 and 96 hrs. Repeated above

experiment range of five concentration was fixed which gives mortality in the range

of 10% to 90%. The main aim of this test is to save time and labors.

3.7.2 Percent mortality:

The percent mortality calculated by Abbots formula (1925). Abbots formula gives the

percentage of the original number of animals died by the treatment.

\[
P = \left( \frac{(Om - Cm)}{(100 - Cm)} \right) \times 100
\]

Where,  
P = Corrected Mortality 

Om= Observed Mortality 

Cm= Control Mortality

It was observed that there was no mortality in control group of earthworm. The

mortality data obtained in the experimental animals for each dose was calculated by

Finney’s formula. (1971)

\[
P = \left( \frac{r}{n} \right) \times 100
\]

Where, P = Percent Mortality 

R = Mortality Observed 

n = Number of animals exposed
The obtained mortality data put into probit /log concentration transformation so as to plot probit regression line. These regression lines are plotted for the purpose of calculating the required concentration of pesticide to produce 50% mortality and 10% mortality. The standard error and Variance calculated by considering log LD$_{50}$. The chi square value and the fiducial limit of pesticide pollutant were calculated from regression equation. The lethal dose and safe concentration of pollutant was calculated by above formula.

3.7.3 Regression Line:

The regression line plotted between log concentration and probit kill describe by Finney (1971). The regression equation and regression line was calculated for earthworm when exposed for 24, 48, 72 and 96 hours to commercial insecticide-Nuvan, fungicide-Bavistin, herbicide-Roundup, nematocide-Furadan and molluscicide-Snailkill respectively. To trace regression equation and to plot regression line following steps were carried out are given below:

1. Table column number I, serial number of tough was entered.
2. Table column number II, the concentration of the pollutant in ppm.
3. Table column number III, Log base 10 concentration and headed “X”
4. Table column number IV, Number of animals used for the treatment.
5. Table column number V, observed mortality for 24, 48, 72 and 96 hours.
6. Table column number VI, Percent mortality (P) calculated by formula
   \[ P = \left( \frac{r}{n} \right) \times 100 \]
7. Table column number VII, Empirical probit value read from table no. I (Transformation of percentage of probit) from Finney’s “Probit Analysis” book. Page no.222
8. Table column number VIII, Expected probit value denoted by ‘Y’, it is calculated by plotted graph against ‘x’ (Log Conc. Of pollutant) and empirical probit. Considering maximum points provisional line was drawn. The expected probit values were read from the provisional line of the graph for the value of ‘x’ tabulated with two places of decimals.

9. Table column number IX, Weighing Coefficient (W) read from table II from Finney’s “Probit Analysis” book. Page no.226

10. Table column number X, weight ‘w’ (w = Wn) w is calculated by the product of weighing coefficient and number of animals exposed per treatment.

11. Table column number XI, working probit (y) ‘y’ value was read corresponding to each ‘y’ and ‘p’ and listed in column XI from Finney’s “Probit Analysis” book. Page no.240

12. Table column number XII, product of ‘wx’ (The ‘wx’ value obtained by multiplication of value from column no.III and IX)

13. Table column number XIII, product of ‘wy’ (The ‘wy’ value obtained by multiplication of value from column no. IX and XI)

14. Table column number XIV, XV and XVI the products of ‘w’ multiplied by (x)² and ‘w’ multiplied by ‘x’ and ‘y’ respectively. The summation of the products of column 14, 15 and 16 are \( \sum wx^2, \sum wy^2 \) and \( \sum wxy^2 \) respectively.

15. The value of \( \bar{x} \) and \( \bar{y} \) were calculated by using following equation,

\[
x = \frac{\sum wx}{\sum w} \\
y = \frac{\sum wy}{\sum w}
\]

16. The value of Regression coefficient ‘b’ was estimated by following formula:

\[
b = \frac{\sum wxy - x \times \sum wy}{\sum wx^2 - x \times \sum wx}
\]
17. The regression equation written as;

\[ y = \bar{y} + b (x - \bar{x}) \]

18. From the Regression equation value of ‘Y’ corresponding to the original value of ‘x’ calculated and entered in column XVII as improved expected probit ‘Y’. The value of improved and expected probit difference compared it remains less than 0.2 considered for correct calculations.

19. Finally the Regression line was plotted between log concentrations and improved expected probit.

3.7.4 Assessment of LD\textsubscript{50}: (50% lethal dose)

For the calculations of LD\textsubscript{10} and LD\textsubscript{50} of the pollutant from regression equation, \( y = 3.7184 \) and \( y = 5 \) (value from Finney’s table no. I) kept calculating ‘x’ value and Antilog of LD\textsubscript{10} and LD\textsubscript{50} were considered as pollutant content in ppm. The LD\textsubscript{10} and LD\textsubscript{50} values from 24, 48, 72 and 96 hours were calculated for the Insecticide-Nuvan, Fungicide-Bavistin, Herbicide-Roundup, and Nematocide-Furadan and Molluscicide-Snailkill respectively.

3.7.5 Calculations for accurate LD\textsubscript{50}:

The accurate LD\textsubscript{50} Value calculated estimating standard error, variance, chi-square and fiducial limit.

For the test of standard error, the variance value calculated by following formula;

\[ \psi = \frac{b^2}{\varepsilon w} + \frac{1}{\varepsilon w} \left( m - \bar{x} \varepsilon w - \bar{x} \varepsilon x^2 / \varepsilon w \right) \]

For the test of homogeneity of the data, chi-square value calculated by following formula;
\[ X^2 = \left( \sum w^2 y - y \times \sum w y \right) - b \left( \sum wx y - x \sum w y \right) \]

The value of chi-square was compound with the table of statistics for n-2 degree of the freedom (‘n’ is the number of experiments) should be the value is higher than the figure of chi-square for the 5% level.

The Fiducial limits \( m_1 \) and \( m_2 \) with 95% confidence were calculated from the variance by following formula;

\[ m_1 = m - 1.96 \sqrt{v} \quad \text{and} \quad m_1 = m + 1.96 \sqrt{v} \]

Where, \( m = \) calculated log LD\(_{50}\) and

\[ v = \text{variance} \) (Standard error of LD\(_{50}\))

The Lethal dose was calculated due to its importance from agricultural point of view.

The lethal dose was calculated by following formula;

Lethal dose = LD\(_{50}\) value X time of exposure

Hart, et.al. (1945) have proposed a formula for calculation of safe concentration of toxicant for the animals. The Safe concentration of toxicant calculated by following formula;

\[ \text{Safe Conc.} = \frac{48 \text{ hrs TLM X } 0.2}{S^2} \]

\[ S = 24 \text{ hrs TLM} / 48 \text{ hrs TLM} \]
Where TLM = tolerance limit or known LD$_{50}$ value, which is the concentration at which 50% of the test animals were killed OR mortalities (probit kill) in a particular time of period.

**3.8 Estimation of Enzymatic Activities:**

The control and Pesticide Treatment of Insecticide, Fungicide, Herbicide, Nematocide and Molluscicide pesticides the enzymes are isolated from the digestive tract by dissecting the animal and using following protocol mentioned in the reference book ‘Biochemical methods’ written by S. Sadasivam and A. Manickam published in the New Delhi by New age International Limited. Publications..

**3.8.1 Dissection of test animal:**

The earthworm was washed in distilled water and pinned down horizontally in the dissection tray with dorsal part downward. The ventral part was cut open longitudinally along the earthworm using sterilized dissecting kit. (Owa, et.al, 2013) The gut were removed in glassware. The total work carried out under sterilized condition by using 90% ethanol.

**3.8.2 Gut enzyme analysis:**

The gut enzyme analysis purposes Amylase, Cellulase, Protease, Lipase and Urease enzyme were estimated by using following protocol given the reference book of Biochemical methods by S. Sadasivam and A. Manickam, 3rd Edition, New Age International publication, New Delhi. According to that estimate the quantity of enzyme in the control and treated animal body tissue. The control and Pesticide Treatment of Insecticide, Fungicide, Herbicide, Nematocide and Molluscicide pesticides the enzymes are isolated from the digestive tract by dissecting the animal

3.8.3 Estimation of Amylase activity:


Principle:
The reducing sugar produced by the action of α-and or β-amylases react with dinitrosalicylic acid and reduces it to a brown coloured product, nitroaminosalicylic acid.

Material:
- Sodium acetate buffer, 0.1M pH 4.7.
- Starch, 1% solution
- Dinitrosalicylic Acid Reagent (DNS Reagent)
- 40 % Rochelle salt Solution (Potassium Sodium Tartrate)
- Maltose Solution

Procedure:

1. Pipette out 1ml of starch solution and 1ml of properly diluted enzyme in a test tube.
2. Incubate it at 27 °C for 15 min.
3. Stop the reaction by the addition of 2ml of dinitrosalicylic acid reagent.
4. Heat the solution in a boiling water-bath for 5 min.
5. While the tubes are warm, add 1ml potassium sodium tartarate solution.
6. Cool it in running tap water.
7. Make up the volume to 10 ml by addition of 6ml water.
8. Read the absorbance at 560nm.

9. Prepare a standard graph with 0-100µm maltose.

Calculations:
A unit of α-and or β-amylases is expressed as mg maltose produced during 5 min. incubation with 1% starch.

3.8.4 Estimation of Cellulase activity:
Principle: - The production of reducing sugar (glucose) due to cellulolytic activity is measured by dinitrosalicyclic acid method.

Materials:
-Sodium Citrate buffer, 0.1M (pH 5.0)
-Carboxymethyl Cellulose 1% (CMC) Dissolve 1g Carboxymethyl Cellulose in 100ml Sodium citrate Buffer 0.1m (pH 5.0)
-Dinitrosalicyclic Acid Reagent (DNS Reagent)
- 40 % Rochelle salt Solution (Potassium Sodium Tartrate)

Procedure:

1. Pipette out 0.45ml of 1% CMC solution at a temperature of 55 °C and 0.05 ml of enzyme extract.
2. Incubate the mixture at 55 °C for a 15 min.
3. Immediately after removing the enzyme substrate mixture from the bath add 0.5ml DNS reagent.
4. Heat the mixture in a boiling water-bath for 5 min.
5. While the tubes are warm, add 1 ml Potassium Sodium Tartrate solution.
6. Cool at room temperature.

7. Add water to make 5ml volume.

8. Measure the absorbance at 540 nm.

9. Prepared the standard graph with glucose in the concentration range 50µg to 1000 µg/ml.

Calculations:

Express the enzymatic activity as the mg glucose released per min per mg protein.
3.8.5 Estimation of Protease activity:


Principle:
The protease enzyme, performs proteolysis i.e. protein catabolism by hydrolysis of peptide bond between amino acids together in a polypeptide chain. The protease enzyme catalyzes the enzymatic degradation of proteins and peptides by hydrolytic cleavage of the peptide bond in an exergonic reaction.

Materials:
Enzyme extract- Homogenize the sample (g/5ml) material in ice-cold 25mM phosphate buffer (pH 7.2). Centrifuge the homogenate at 15,000 rpm for 15 min. and used the supernatant as enzyme source for the electrophoresis.

Procedure:

1. Incorporate casein (1% w/w) dissolve in 25mM phosphate buffer (pH 7.2) in the separating gel.
2. After electrophoresis, incubate the gel at room temperature for 1 hour in 2.5% Triton X-100 in water to removes SDS.
3. Transfer the gel to a bath containing 0.1M glycine NaOH, (pH8.3)
4. Incubate the gel at 37°C for 3-5 hours.
5. Fix the gel and stain by immersion in a 0.1% (w/v) solution of Amido black in methanol: acetic acid: water (30:10:60) for 1 hour.

7. Record the zymogram as soon as achromatic bands become evident in dark blue background.

Calculations: Express the enzymatic activity of protease enzyme as in mg.

3.8.6 Estimation of Lipase activity:


Principle:
The quantity of fatty acids released in unit time is measured by the quantity of NaOH required to maintain pH constant. The miliequivalent of alkali consumed is then taken as measures of the activity of the enzyme.

Materials:
- Substrate: Take 2ml of any clear vegetable oil, neutralize to pH 7.0, if necessary, and stir well with 25 ml of water in the presence of 100mg bile salts (Sodium tauracholate) till an emulsion is formed. Addition of 2gm gum arabic hastens emulsifications.
- 0.1 N NaOH
- Enzyme Source: Grind a known quantity of sample with a mortar and pestle. Homogenize the tissue with twice the volume of ice-cold acetone. Filter and wash the powder successively with acetone and ether, acetone: ether (1:1) Air dry the powder. This acetone powder can be stored in a refrigerator. Extract 1gm of the powder in 20 ml ice-cold water or a suitable buffer. Centrifuge at 15000rpm for 10min.and use the supernatant as enzyme source.

Procedure:

1. Take 20ml of substrate in a 500 ml beaker.
2. Add 5ml of phosphate buffer (pH 7.0).

3. Set the beaker on a top of a magnetic stirrer cum hot plate and stir the content slowly.

4. Maintain the temperature at 35°C. Dip the electrodes of a pH meter in the reaction mixture.

5. Add enzyme extract (0.5ml), immediately record the pH and set the timer on. Let it be pH at zero time.

6. At frequent interval (10 min.) or as the pH drops by about 0.2 units add 0.1 n NaOH to bring the pH to the initial value. Continue the titration for 30-60 min.

7. Note the volume of alkali consume.

Calculations: enzymatic activity express in mill equivalent per minute per gram.
3.8.7 Estimation of Urease activity:


**Principle:**

The urease enzyme is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

**Materials:**

- 0.1M phosphate buffer
- 0.5 ml urea

**Procedure:**

1. Take 4ml 0.1M phosphate buffer.
2. Add 0.5 ml urea into 1.0 gm of sample.
3. Incubate at 30°C for 60 minute.
4. After incubation, 10 ml of 2 ml KCl was added.
5. Then mixture was kept at 4°C for 10 minute to stop enzymatic reaction.
6. The suspension was centrifuged for 5 minutes.
7. The ammonium ions formed by urease in the supernatant was determined.

**Calculations:** enzymatic activity express in mill equivalent per minute per gram.
3.9 Statistical Interpretation:

The statistics is the study of collection, analysis, interpretation, presentation and organization of the data. The two main statistical methodologies are used in research for data analysis. One of them is descriptive statistical analysis which summarizes data from the sample by using measures of central tendency and standard deviation. Another method is standard statistical method that involves test of the relationship between two statistical sets which has been employed in the present research. The statistical interpretation is a mathematical term important in the research. Analysis and explanation of obtained data on the basis of what is typical and what derivates from the average, how the data varies on different parameters and lastly how is it distributed in the relation to variables that being measured.

The collected data was subjected to statistical analysis which includes descriptive statistics by using statistical package for the social science (SPSS) version 16.0 The Fisher test was used to analyzed the significance of the response using LSD (Least Significant Difference) The Null hypothesis assumed on equal distribution of organism in the two sides. The P-value was set at 0.01 and 0.05, the two tailed test was chosen for the test analysis.