COURSE
GUIDE

ANP 309
ANALYTICAL TECHNIQUES FOR ANIMAL PRODUCTION II

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MODULE ONE: ARTIFICIAL INSEMINATION TECHNIQUES IN ANIMAL BREEDING

Unit 1 : Meaning of AI and Significance of AI

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1.0 Introduction

Insemination could be either a natural or artificial process of depositing semen in the genital tract of the female animal. Insemination can be by natural mating through a variety of methods depending on livestock management system (*i.e.* pen mating, flock mating). Artificial insemination involves the proper evaluation and extension of semen samples before the female animal can be inseminated. It is also essential to adopt proper techniques for handling the sperm cells once semen has been collected and utilise proper equipment for the insemination.

1.2 Objective

At the end of this unit, you will

- Able to define artificial Insemination
- Differentiate between natural mating and artificial insemination
- know the importance of AI in animal breeding program
- be able to identify the advantages and limiting factors associated with AI

1.3 Main content

1.3.1 Definition of Artificial insemination (AI)

It is the deposition of viable spermatozoa inside the female reproductive tract during oestrus period in order to achieve fertilization of the ovum (ova). Deposition of semen by a male into the reproductive tract of a receptive female under normal conditions and environment is referred to as natural mating. Artificial insemination on the other hand is the placement of semen by unnatural means into the reproductive tract of a receptive female.

1.3.2 Importance of AI

It is one of the most important tools employed to fast track the genetic improvement of livestock. This will enable the breeder to maximise the use of potentially selected males for desirable traits for extensive or multi-locational testing which would not have been possible using a single bull at a time. This would increase the genetic progress made with a limited period of time.

1.3.2.1 The benefits of AI;

The benefits associated with using AI are numerous and cannot be overemphasized. They include the following;

a. It minimizes the risk of transmission of venereal diseases which may occur in natural mating system.

b. It allows for proper planning of breeding programs. This is because the breeder can time production based on availability of funds, inputs (*e.g* feed, forage *etc*), climate. The ovulation of a population breeding female livestock can be synchronised and inseminated at the same time to minimise production cost.

c. It is cost effective as viable semen of excellent potential sires can be preserved for years in facilities without the associated cost of keeping live animals (feed cost, veterinary bills, heating bills, labour, housing).

d. Genetically excellent males which have non-genetic deformities can be utiised for breeding purposes. Wide spread use and availability of genetically superior sires is guaranteed. One bull can breed 500,000 cows in a lifetime. After death, semen can be used. Frozen semen of between 40 and 45 years have been used successfully for insemination

e. It aids the chances of enhanced genetic progress with a short period of time.

f. It promotes introduction of new genetic resources from other countries. This is because it is easier to import frozen semen from one country to another unlike the associated challenges of importation of live animals (quarantine, customs, recent threat such as the Ebola virus)

1.3.2.2 The limitations associated with AI

There are associated challenges with the use of AI especially in a developing economy. They are many and not limited to the following points;

a. The technique of AI requires skilled and experienced manpower. Where these skilled personnel are lacking, the practice of AI is very limited.

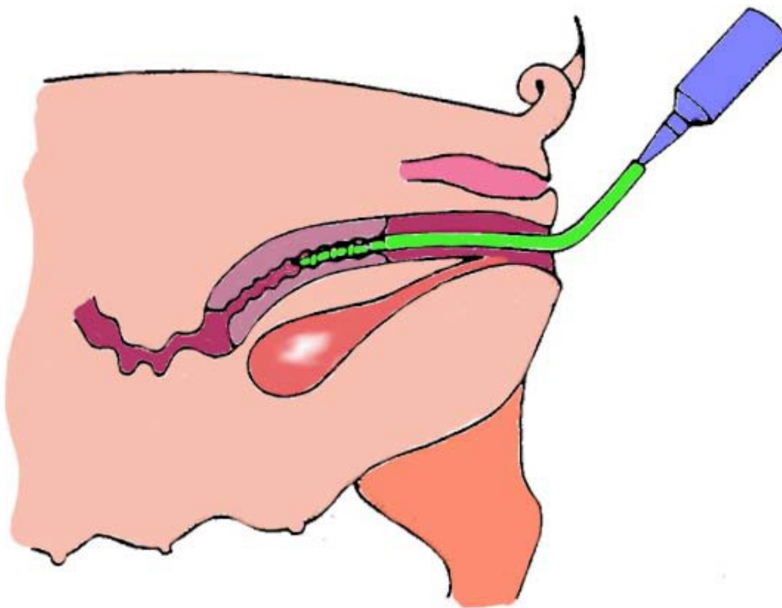
b. AI requires good infrastructural availability such as constant power supply, good transportation systems (good road network) and excellent communication system. These infrastructures are lacking in the developing economies of the world.

c. The challenge between culture and acceptability of AI technology by the major players in the large ruminant production system, the Fulani pastoralists, who are vehemently opposed to the practice.

d. The management practice in the large ruminant production system which is essentially nomadic is not amenable to the practice of AI. The practice works best for livestock reared under the intensive system.

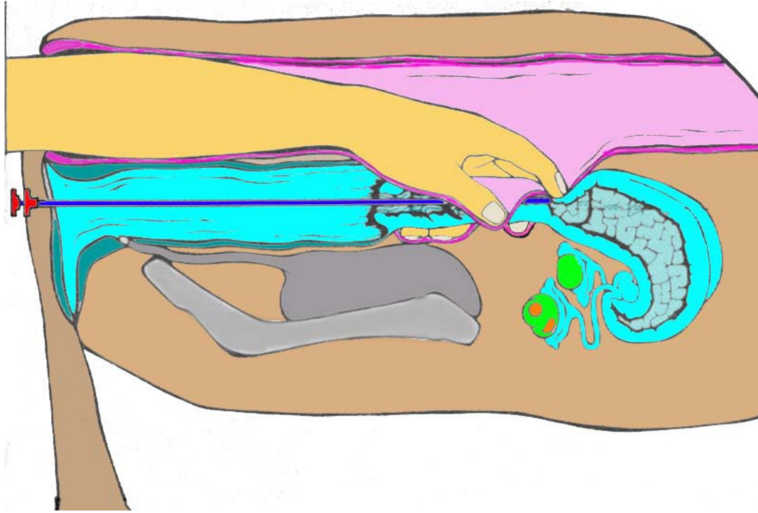
1.3.3 Tools required for artificial insemination

Spiral tip Foam Intrauterine (all for sow)



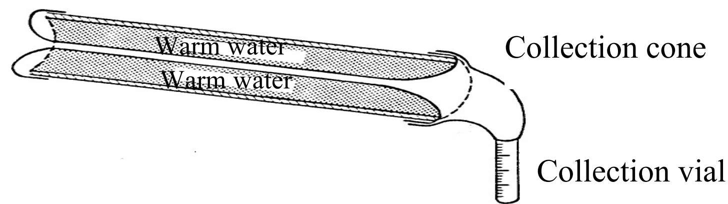
Artificial Insemination in the Sow

Insemination of the Cow



Rectal-Vaginal Approach

Inner lining



Artificial Vaginas

Other important equipment include liquid nitrogen tank for storing semen over many years

Semen Storage straws

1.4 Conclusion

There are many benefits associated with the artificial insemination of spermatozoa in to the reproductive tract of a female livestock on heat which exceed natural mating. These full potentials of these benefits are yet to be harnessed in the developing economies due to lack of

functional infrastructures and skilled manpower coupled with a need for attitudinal change of the owners of livestock. This will enhance progress in the livestock industry.

1.5 Summary

- Insemination of spermatozoa into female reproductive system could be by natural mating or artificial process.
- Artificial insemination involves the proper evaluation and extension of semen samples before insemination.
- Proper techniques and equipment are required for handling the sperm cells once semen has been collected.
- AI aid to fast track the genetic improvement of livestock.
- There are associated benefits to the use of AI such as cost effectiveness, cross-border introduction of genetic materials with minimal bureaucracy.
- The limitations associated with the use of AI are the usual challenges of the underdeveloped economies such as lack of skilled personnel, infrastructure *etc*.

1.6 Tutor marked Assignment

1. Define Artificial Insemination. How is this mating system different from Natural mating?

2. You read in NAPRI Annual Report that a superior Bull exist in NAPRI. Describe the process of utilizing the bull to improve your dairy herd without physically carrying the bull to your farm.

3. What are the disadvantages of artificial insemination?

4. List some advantages of artificial insemination.

1.6 References/further reading

Osinowo O.A. 2006. Introduction to animal reproduction.

http://www.powershow.com/view/1cb0cc-MjM3N/Artificial_Insemination_powerpoint_ppt_presentation

Unit 2: Semen collection techniques in farm animals

2.1 Introduction

2.2 Objectives

2.3 Main content

2.3.1 Factors that enhance semen collection

2.3.2 The methods of semen collection suitable for different farm animals

2.3.3 The Aspiration method

2.3.4 Massage method

2.3.5 Electroejaculator

2.3.6 Gloved-hand technique

2.3.7 Artificial vagina

2.4 Conclusion

2.5 Summary

2.6 Tutor marked assignment

2.7 References/ Further Reading

2.1 Introduction

Normal production and maturity of spermatozoa takes place only at a temperature which is several degrees lower than the temperature of the abdomen hence the need for the males to have

descended testes. During ejaculation of semen, there is a simultaneous mixing in the urethra of a dense mass of spermatozoa and seminal plasma fluid of associated accessory glands which gives rise to the semen which is released from the penis. It is the action of involuntary smooth muscles that propels spermatozoa from the tail of the epididymis and vas deferens through to the urethra. The strong contraction of the urethra delivers the semen in the course of ejaculation.

2.2 Objectives

- You will understand the factors which will enhance semen collection
- You will also learn the various methods of collecting semen farm animals
- You will learn how to analyse semen on the farm and in the lab

2.3 Main Content

2.3.1 Factors that enhance semen collection

In AI, viable spermatozoa is usually collected by artificial means and outside the genital tracts of the female livestock. Prevention of contamination of the semen is very critical to enable the technician carry out proper semen quality and evaluation. This means that there are certain factors that you must considered for effective collection of the semen.

They include the following;

a. Between breed differences

b. The specie of livestock

c. The frequency of semen collection

d. The procedure employed for semen collection

e. The age of the male from which the semen is to be collected

f. Factors attributable to individual differences within breed/specie

g. The amount of stimuli the male livestock is exposed to prior to semen collection

Between breed differences

The quantity of semen varies between breeds. The differences could be due to differences in body weight and scrotal size. This has been reported for Holstein-Friesian bulls which have a higher concentration of spermatozoa per ejaculation than the *Bunaji* breed.

The specie of livestock

Specie differences in semen quantity have been documented. The volume per ejaculate in the boar is higher than the bull. The spermatozoa concentration per ejaculate of the bull is higher than the boar.

The frequency of semen collection

The quantity and quality of the semen per ejaculate usually declines with successive ejaculation.

The procedure employed for semen collection

The electro-ejaculator method of semen collection yields a significantly higher volume than the artificial vagina (AV) method. Semen collected using artificial vagina method has a higher concentration of spermatozoa than the electro-ejaculator method.

The age of the male from which the semen is to be collected

Older males have higher concentrations of mature spermatozoa than younger males. This because younger males may not have attained sexual maturity, hence their spermatozoa may not be mature.

Factors attributable to individual differences within breed/specie

Within breed and specie, individual differences may be observed due to age of each animal, difference in size of scrotum, libido and health status.

The amount of stimuli the male livestock is exposed to prior to semen collection

When the animal has been sexually stimulated preparatory to semen collection, it eases semen collection and yield optimum semen volume. Stimuli such as false mounting or the use of teasers greatly aid semen release.

2.3.2 The methods of semen collection suitable for different farm animals

Semen can be collected using different methods which is entails direct stimulation of the musculature of the male reproductive tract or simulation of the movement of the vagina on the *glans penis*. Whatever method to be employed for collection, it is necessary to take care of the pre-collection welfare of the male and stimulation. This will ensure an increase in quality and quantity of semen yield. It is also essential to ensure cleanliness to prevent contamination of the semen collected.

It is noteworthy that some methods of semen collection are more suitable for some specie than others. The double-handed method is best suited for poultry, the gloved-hand method for boars and the artificial vagina method is commonly used for bulls.

The following are the methods of semen collection;

2.3.2.1 The Aspiration method: this is one of the earliest methods of semen collection. The male is allowed to mate naturally with the female and the semen is sucked out of the vagina with a spoon, sponge or syringe. The major limitations associated with this method are;

- The method does not give a good representation of the ejaculate in terms of quality and quantity in low.
- There a is high likelihood of bacterial infection
- There could be damages to the vagina due to the introduction of spoon into it
- There a is high likelihood of contamination of semen with vagina secretion

2.3.2.2 Massage method: There are two types of massage method depending on the specie from which semen are to be collected. They are;

a. Massage method employed for bulls and stallions: this is because they have big rectum. The rectum of the animal is cleaned before the hand of the technician is inserted into it. The inserted hand is used to massage the ampulla and vesicular glands of the male from the front to the back. This stimulates ejaculation. There is a high likelihood of contamination from urine. The ejaculate also contains a higher proportion of seminal secretion than semen from natural ejection.

b. The double-handed massage method: The cock is held and one hand is used to gently and rapidly make strokes over the back of the bird towards the tail to induce stimulation. The other hand is used to gently massage the abdomen until a good stimulation is achieved (the legs will stiffen and the tail feathers raised). This is accompanied by erection of the copulatory appendages in the cloaca, semen is expelled from the swollen

papilla by gently pressing inward on inward on either sides of the cloaca with the thumb and forefinger of the hand that was used for the back massage.

2.3.2.3 Electroejaculator: An electric probe which consists of a small variable voltage transformer of about 12-60 volts and a built-in rheostat to control the flow of the current can be used. This bi-polar probe; which has two copper rings or electrodes which fits into a metallic, glass or wooden rod; when inserted into the rectum of the male animal will transmit intermittent voltage. This will cause stimulation of the musculature of the reproductive tract and then ejaculation occurs. The method is useful for bulls that are unable to mount or use artificial vagina. It is also used for rams and billy-goats. It is not widely used in boars as they tend to have fatty tissues. The integrity of the spermatozoa ejaculated is not compromised. The semen volume is usually lower and contains more contaminant than ejaculate from natural mating.

2.3.2.4 Gloved-hand technique: This method is only suitable for the boar. This is because it enables the technician to exert the apt amount of pressure required on the *glans penis* to enable the penis-locking action.

The technician's hands and gloves should be washed well with water, disinfectant and rinsed with 70 percent alcohol. The screw-like end of the boar's penis should also be washed with soap and water, then disinfected.

The boar should be led into the room housing the dummy and allowed to make several false mounts. The rubber-gloved hand of the technician holds the screw-like end of the boar's penis to create the penis-locking action in the cervix of the sow. The ejaculate should be collected in a flask holding three large test-tubes. This is because the boar's semen is released in fractions; pre-sperm, sperm rich and post-sperm. The pre-sperm portion is a clear fluid containing very few

spermatozoa and lots of prenuptial secretions. The sperm rich fluid has a distinct creamy colour with a relatively high concentration of spermatozoa and includes secretions from the vesicular gland. The post-sperm portion has the highest volume compared to the other two portions, it contains sparse number of spermatozoa and lots of secretions from the bulbo-urethral glands.

2.3.2.5 Artificial vagina: this is a suitable method for bulls, boars and occasionally rams. It was not commonly used for boars until recently. The practice requires the provision of an artificial vagina, a teaser or dummy and the life male animal from which the semen is to be collected.

The procedure works such that when the male animal from which semen is to be a collected attempt to mounts a teaser or dummy at least two times, it is pushed down. By the third attempt to mount, the erect penis is directed into the AV and ejaculation occurs. The artificial vagina consist of a solid cylindrical tube with a thin-walled rubber lining which forms a jacket that is filled with warm water of a temperature of 40-44°C. The rubber is usually stretched so that the AV mimics the temperature and pressure of the natural female vagina. An insulated and calibrated glass tube is usually attached at one of the ends into which the ejaculate is collected. This collection tube maybe covered with a muff (for insulation) to prevent a sudden change in the temperature of the semen collected.

2.3.4 Conclusion.

The success of the artificial insemination procedure is dependent of the collection of excellent quality and quantity of semen. Thus it entails the technician having a knowledge of the factors that will enhance the collection process.

2.3.5 Summary

- The collection of semen for artificial insemination is a very sensitive process which requires knowledge of the factors that will enhance yield and quality of the spermatozoa.
- The methods of semen collection suitable for different farm animals vary due to specie difference. However, some of the techniques for particular specie may be applied for another.

2.3.6 Tutor marked Assignment

2.3.7 References/ Further Reading

Osinowo O.A. 2006. Introduction to animal reproduction.

Unit 3: Evaluation of semen quality

3.1 Introduction

3.2 Objectives

3.3 Main content

3.3.1 Physical variables to consider during semen evaluation

3.3.2 Colour and appearance

3.3.3 Semen volume

3.3.4 Motility of the spermatozoa

3.3.5 Semen concentration

3.3.6 Live to dead semen

3.3.7 Sperm morphology

3.3.8 pH

3.3.9 Biochemical tests during semen evaluation

3.3.10 Respiratory Rate

3.3.11 Methylene blue reduction time

3.5 Conclusion

3.6 Summary

3.7 Tutor marked Assignment

3.7 References/ Further Reading

3.1 Introduction

It is highly important to evaluate the semen that will be used for AI. Evaluation of the semen will help save time and cost ensuring successful fertilization after insemination. The procedure entails both physical (visual) and biochemical evaluation.

3.2 Objectives

- To know the parameters that should be evaluated in the collected semen.
- To know the significance (s) of all the physical and biochemical properties that will signify good semen quality suitable for AI.

3.3 Main content

3.3.1 Variables to be evaluated for in the semen

a) Physical variables to consider during semen evaluation

Colour and appearance:

The normal semen colour should be creamy-white in appearance although there may be specie variation and sometimes individual variation. *E.g.* most bull semen is milky-white which can vary to a creamy colour and occasionally, a yellow semen may be obtained. Any other type of colour is an indication of a pathological condition. If the semen looks translucent, it is an indication of low spermatozoa concentration. The appearance of a relatively opaque appearance

is an indication of high sperm concentration. If the semen has bloody stains, urine, dirt, hair, it is indicative of contaminated semen sample.

Semen volume

Sexually mature males of different species produce differing volumes of semen containing different amounts of spermatozoa related to the physiological function of the sexual glands. The volume and concentration of are related although there are no direct correlations between semen volume and fertility. It is desirable that the male releases a sufficient volume of ejaculate which would aid the success of the AI if the procedure of insemination needs to be repeated. Differences observed in the volume of ejaculate in individuals may be due to differences in thrust at the time of collection hence it is important not to distract the animal during the procedure. The type of nutrients fed to the male animal, the breed of animal (within breed differences could be due to age differences and size of animal), health status, and frequency of semen collection are some of the factors which also affect semen volume.

How to measure the semen volume

Specie	**Volume (ml)**
Boar	80-200
Bull	5-8
Billy	0.3-1.5
Ram	0.8-1.2
Horse	60-100

Semen Quality Analytical Procedure

Volume

Collect the semen in a calibrated pipette, then read the volume read and enter the value appropriately.

Colour

You will depend on your eyes (I hope you are not colour blind)

The colour of the semen must be consistently be either milky or greyish in colour.

Morphology

You must check for both unstained samples and those stained with Nigrosin/Eosin stain.

On a glass slide, place one drop of unstained semen making a smear Then view the smear.

For the staining, Nigrosin-eosin stain smear was made by

Place two drops of semen together with a drop of 10% Nigrosin and 2 drop 5% eosin in a vial,

Mix them together thoroughly but gently.

Make a smear from the mixture and view under high power (x100) microscope.

Mass motility:

You must evaluate the motility immediately after semen collection.

Place a drop of semen on a warmed glass slide then observed with a microscope using low power (X10, X40) without cover slip. The mass activity can be graded 0 meaning no motility triple + for highest motility.

Sperm count (Concentration)

You will require Neubauer hemacytometer for sperm count.

Dilute the semen with normal saline at 1:400;

Fill the hemacytometer with the diluted semen through the capillary action of the red cell pipette. Then mount the hemacytometer and count the sperm cells

Live and dead ratio

Determined Live- dead ratio using nigrosin/eosin stain (5% eosin, 10% nigrosin).

Live sperm cells will not absorb the stain.

The cells that you observed to stain pink were so coloured because they have absorbed the stain. Only dead cells can absorb the stains.

When a high number of the spermatozoa are found to be clear, it means they've not absorbed the stain and were therefore live sperm cells, while a small number of them will be stained pink which means they've absorbed eosin component and are therefore dead.

3.3.2 Motility of the spermatozoa:

This is a measure of the rate of movement of the spermatozoa. This is usually carried out microscopically using an ordinary light microscope and viewing a drop of the semen on a glass slide with a cover slip. The rate of motility varies with species, health status, and the medium in which it is observed. Semen sample with motility above 65 percent is considered good for use in AI. The evaluation of motility is a subjective estimate mainly influenced by the thickness of the

semen and the temperature of the slide on which the semen is placed (idea temperature should be 37-39^oC under x40 magnification).

The importance of test for motility is because if there are no movements, there is an odd chance that the sperm will reach the oviduct which is necessary for fertilization of the eggs or ova. When the tail of the sperm cell is impaired, motility may cease or the sperm cell may move in circles or backwards. The condition, circular or reverse, may also be indicative of cold shock or that the media is not isotomic with the semen. Oscillatory motility is indicative of aged semen.

Gross motility of spermatozoa can be determined by examining a drop of raw undiluted semen on a pre-warmed slide under a light microscope at x40 magnification. A scale of 0-5 or a percentage can then be used to assess the motility. The ratings are as follows;

1. A 5-point score means a motility of 80 percent or more. This is when virtually all the spermatozoa is viewed to have a progressive forward movement.
2. A 4-point score means good motility. This is when approximately 70-80 percent of the spermatozoa are in vigorous, rapid movement.
3. A 3-point score is good. This is when 60-70 percent of the sperm cells are motile.
4. A 2-point score is fair, indicating that 30-60 percent of sperm cells are motile.
5. A 1-point score is indicative of poor motility. Less than 30 percent of the sperm cells are motile resulting in sluggish movement.
6. 0-point (zero point) means all the sperm cells are dead.

Specie	Motility (%)
Boar	70
Bull	65
Billy	75
Ram	75
Horse	65

It is rare to see semen samples with motility higher than 90 percent. Semen samples with motility less than 50 percent should not be used for insemination.

3.3.3 Live to dead sperm

Live to dead sperm can be estimated using eosin-nigrosin stain technique. The proportion of live to dead cell is estimated by supra-vital staining in which the cells that are alive when the stain was applied do not absorb the stain while the dead sperm cells take up the red eosin and appear red against the dark background of the nigrosin.

3.3.4 Semen concentration

The determination of the semen concentration is very important because it is used to know the ideal dilution rate of the semen. It is also used to predict the fertility of the male. The sperm concentration is influenced by the following factors. They include, the stage of sexual development and maturity of the male animal, the status of the development of the testes, over- or under-feeding which could result in the deposition of excess fat on the testes, the deficiency of essential nutrients required for sperm cell formation, species difference, seasons of the year and frequency of semen ejaculation.

There are several methods for determining semen concentration and they include;

a. Haemocytometer count
b. Estimation of total nitrogen
c. Weight of dry semen
d. Visual comparism with standard capacity tubes by passing light through
e. Electrical resistance of sperm suspension
f. The spectrophotometer method

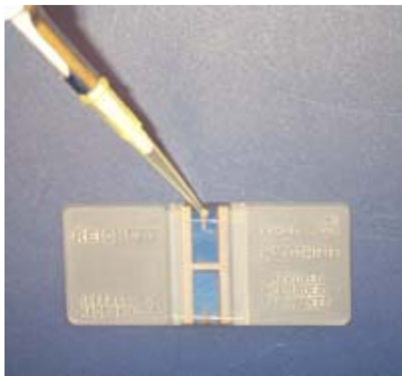
However, the two most commonly used methods are the haemocytometer count and spectrophotometer methods.

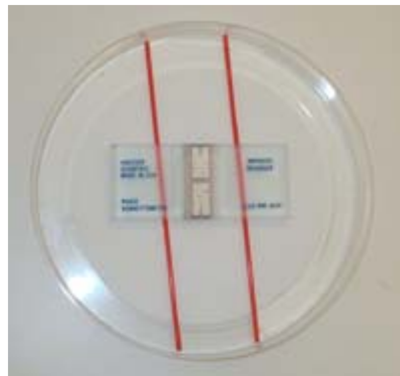
The haemocytometer count is used to determine semen concentration by using a white blood cell pipette to draw up to the half-way mark on the stem. A spermicide (such as 5% triphenyltetrazaline chloride or 5% chlorozine mixed with a physiological saline solution) solution is added to the parallel mark at the top of the bubble chamber. The preparation is mixed thoroughly using standard pipette shakers. A drop of this mixture is added to both sides of the haemocytometer slide. The haemocytometer slide is then kept in a humid or damp chamber for one hour to incubate. This will increase the accuracy of the sperm cell count as they would have precipitated into the haemocytometer instead of floating. The haemocytometer is partitioned into 5x5 squares. The number of sperm cells in the middle and outside squares are counted. The sperm cells that lie inside the squares are counted and those on the top and right hand sides too are counted diagonally from top left to bottom right.

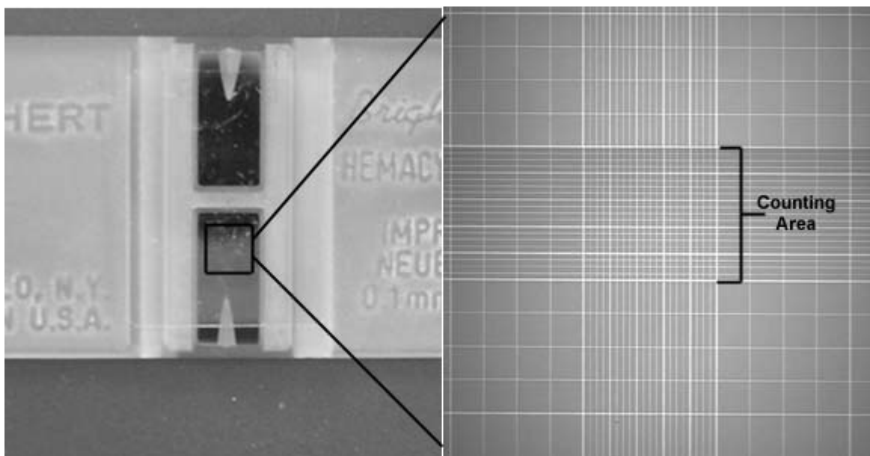
The sperm cell concentration is calculated by reference to the number of sperm cell per ml of semen as;

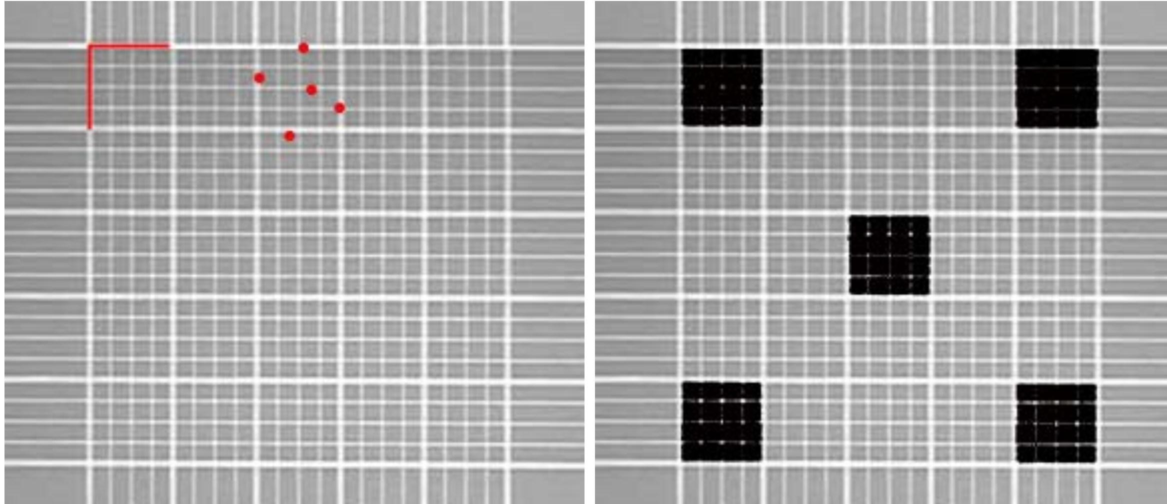
Sperm cell concentration = Number of sperm cell count X Dilution faction X multiplication factor

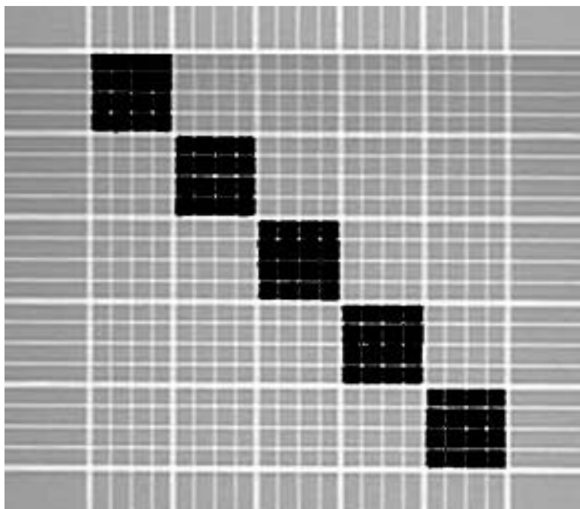
The spectrophotometer method works by taking absorbance readings of diluted semen at 600nm calibrated against haemocytometer counts. The rate of semen dilution is based on the volume of the ejaculate. When the information on semen volume, motility and concentration are known, the total number of sperm in an ejaculate can be estimated.











3.3.4 Sperm morphology:

It is essential to evaluate semen samples microscopically as they always contain any of the following; abnormal sperm cells, bacteria and protozoa; per ejaculate. The presence of these conditions may aid diagnosis for cause of sterility or poor conception rate. A diluted semen sample will be examined and the result will determine whether to accept or reject for particular semen sample for AI.

The normal sperm cell consists of the head, neck, mid-piece and the tail. These parts must be present and structurally complete so as to ensure fertility. When there is a deviation from the normal structure, it is regarded as an abnormality. In any ejaculate, there will always be abnormal sperm cells but in instances that the proportion of abnormality exceeds 5%, then the fertility of the semen sample would be impaired. Such semen samples should be rejected. These abnormalities are in the forms of damaged heads or tails or those with separate component parts.

Sperm cell abnormalities are classified as primary or secondary abnormalities. Primary abnormalities occur during the process of spermatogenesis. They include giant head, double heads, double midpiece, double tail, abnormal shape of head, dented head. Secondary abnormalities are those morphological aberrations that had taken place during the passing of the sperm cells through the male duct or *in vitro* handling of the sperm cell. The secondary abnormalities include cytoplasmic droplets on midpiece or tail, bent tail, detached head cap (acrosome).

A phase contrast microscopy at x1000 magnification under oil-immersion is used to assess sperm morphology.

3.3.6 pH

The pH of a fresh ejaculate is dependent on the secretions from the other glands. Variation in pH is influenced by breed or specie difference. For example, bull pH is 6.7 while boar is slightly higher; semen of bulls and dogs are slightly acidic while that of rabbit is alkaline. The pH of poultry is between 7.2 -7.6. The pH is also influenced by method of semen collection. The pH from massage method is about 8.0 while that from AV is 6.3. Normal sperm cells can survive at

about neutral pH hence should be kept in diluents that will aid in maintaining that pH until required.

3.3.7 Biochemical variables to consider during semen evaluation

In evaluating semen samples, there are a number of biochemical and metabolic processes which should be monitored. They include; a measure of respiratory rate, methylene blue reduction time (MBRT) and rezasurine reduction time (RRT). These tests are carried out by evaluating or measuring the metabolic rate of freshly collected or stored semen samples.

3.3.8 Respiratory Rate

This test employs the use of the Warbug apparatus and the respiratory rate of the sperm cell is manometrically measured. There is a high correlation between the respiratory rate and sperm motility, and between respiratory rate and semen concentration. Thus it is expected that the higher the motility and concentration of the semen sample, the faster the rate at which carbon dioxide is produced.

3.3.9 Methylene blue reduction time

The principle of this test is based on the phenomena that active cells use oxygen therefore semen samples containing a high concentration of active spermatozoa will use up oxygen more rapidly than those containing poor quality sperms. The use of oxygen will result in an excess of hydrogen ion that is free to combine with the chloride ion in methyl blue to form a leukemethylene complex and the time required for the blue colour to bleach out. There is a positive correlation between reduction time and sperm cell concentration. There is also an inverse relationship between reduction time and number of dead sperm cells. *For example*, good

quality boar semen will require 1-3 minutes to bleach out whereas a medium quality semen sample requires 3-5 mnutes and poor quality semen requires 5-10 minutes

3.4 Conclusion

The importance of evaluating semen quality of ejaculate meant for AI purposes will aid in the prior determination of semen samples that are potentially fertile.

3.5 Summary

- There are both physical and biochemical tests involved in semen quality evaluation
- The physical variables to evaluate in collected semen meant for AI are as follows; colour and appearance, semen volume, motility of sperm, live to dead sperm, semen concentration, semen pH and sperm morphology.
- The biochemical tests for semen quality evaluation are to measure metabolic ratr of semen samples. They include respiratory rate and methylene blue reduction time.
- Both physical and biochemical evaluation of semen samples collected for AI will save time and resources by pre-determination of the potential of fertility of spermatozoa.

3.6 Tutor marked Assignment

3.7 References/ Further Reading

Osinowo O.A. 2006. Introduction to animal reproduction.

http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/hemacytometer.html

Unit 4 Techniques involved in the storage of collected semen

4.1 Introduction

4.2 Objectives

- To know the methods which can be used for semen storage
- To know the common semen diluents used for semen extension
- To know the factors that can influence the survival of sperm cells during storage

4.3 Main content

4.3.1 An overview of the storage duration of semen of different livestock

4.3.2 The methods of semen storage

4.3.3 The methods of storing semen

4.3.3.1 Short term

4.3.3.2 Long term

4.3.4 Types of storage methods for frozen semen

4.3.5 The factors that affect viability of sperm cell during preservation

4.4 Conclusion

4.5 Summary

4.6 References/ Further Reading

4.1 Introduction

The pertinent reasons for using the semen of genetically superior males to inseminate a larger number of females than is naturally possible is the ability to preserve and store their sperm cells successfully until required. The sperm cell is held in a frozen, motionless state till it is thawed. The preservation process entails slowing down the metabolic rate of the spermatozoa. The preservation process can thus achieved by either cooling down the sperm cell or by adding a chemical to inhibit the sperm cell metabolism. The process also entails the addition of the following substances which have different functions during cooling or freezing;

Diluents	**Function**
Glucose	The supply of energy source to sperm cell
Egg yolk, milk	For maintenance of the integrity of the sperm cell
Milk	For the prevention of 'cold shock' during cooling
Glycerol or erythritol	To prevent the crystalisation of the cellular water as this could occur due to frozen storage.

4.2 Objectives

- To know the methods which can be used for semen storage
- To know the common semen diluents used for semen extension
- To know the factors that can influence the survival of sperm cells during storage

4.3 Main Content

4.3.1 An overview of the storage duration of semen of different livestock

Semen of different classes of livestock species can be extended and stored successfully at 5°C for 1-4 days. Fresh semen of sheep, swine and poultry has been used for AI but not in cattle. The fresh semen of stallions does not maintain integrity beyond 24 hours and boar semen beyond 48 hours.

The sperm cells can be kept in a frozen state in a motionless condition until it is exposed to heat. This frozen semen is in current use for AI in goats, fish and cattle.

4.3.2 The methods of semen storage

The methods which can be used for semen storage

The common method of storing semen is in liquid nitrogen. Liquid nitrogen is the fourth coldest substance in existence. It has a temperature of -196 ^{o}C or -32 ^{o}F. Various methods are being employed to preserve the integrity of sperm cells but the commonest are either rapid re-warming of frozen sperm cell instead of slow re-warming or the thawing of frozen semen in iced water for 8-10 minutes before use. The following are the common ways of storing semen for AI; short term and long term storage.

4.3.2.1 Short term

This is usually for a short period varying from few hours to few days. The integrity of the sperm cell is lost with increase in storage time leading to low viability. There are three methods for liquid storage of semen. They include;

i. Ambient temperature storage of semen

ii. Chilled storage of semen at 5 ^{o}C

iii. Flow dialysis. This could be either by continuous but a time-consuming replacement of the buffer surrounding the dialysis bag containing the semen or a complete replacement of the fluid used for dialysis every 24 hours.

4.3.2.2 Long term

Long term storage of semen is by deep freezing. This is achieved by cooling to 5°C and keeping the cooled semen in appropriate storage medium than frozen in liquid nitrogen. The advantage of the long term storage of semen is that the sperm cells retain their fertility for years.

4.3.3 The following are types of storage of frozen semen;

I. Ampoules of about 0.5-1 ml capacity made of glass or straw materials.

II. Pellets: The semen stored in pellets are thawed in physiological saline solution (0.9% concentration)

III. Straws: Long straws of 2½ -5" length can hold frozen semen of 0.25-0.5 ml volume.

IV. Shell freezing: This method results in the highest percentage of live sperm cells than the previous processes. The dead and abnormal sperm cells can also be removed from frozen semen by passing the semen through glass fibre. This prevents the incidence of premature abortion in the female who has been artificially inseminated with abnormal or dead sperm cells which has resulted in

fertilisation. Another advantage of this method is that it increases the viability of the sperm cells and ensures a higher conception rate.

V. Lyophilisaton of semen: the technique employs the use of a vacuum t change some of the moisture to a vapour state by passing the liquid form so that the semen so treated may lose about 90-95 percent of its moisture so that less space is required for storage. A major disadvantage of this method is that, only 5-15 percent of motile sperm cells are recovered when the semen is extended and results in poor conception rate.

4.3.4 The factors that affect viability of sperm cell during preservation

These are the determinants of the survival of sperm cells which are being preserved for AI. This factors when considered will ensure that viable spermatozoa are present after thawing in the future and fertilisation of the female ovum (ova) will occur. They include;

i. The quality of the diluents used

ii. The storage duration

iii. The storage method employed

iv. The quality of the ejaculated semen prior to storage

4.4 Conclusion

It is possible to preserve and store the semen of genetically superior male livestock artificially and for a longer duration outside their bodies. This can be achieved by slowing down the

metabolic process of the sperm cells, however suitable diluents are needed to ensure the survival of viable spermatozoa.

4.5 Summary

- Semen of livestock can be stored until needed
- Preservation of sperm cells can be achieved by slowing down the metabolic rate of the cells
- Suitable diluents should be added prior to storage to supply energy to the sperm cells, prevent crystallization of cellular water and cold shock during cooling
- The semen of different livestock can endure varying duration of short term storage
- There are short term and long term methods of storage
- Factors such as storage duration, integrity of the semen prior to collection, type of the diluents *etc* need to be considered before embarking on preservation because it can affect fertility when the spermatozoa is inseminated in future

5 References/ Further Reading

Osinowo O.A. 2006. Introduction to animal reproduction.

Unit 5: Semen extension, insemination and factors that may affect fertility after insemination

5.1 Introduction

5.2 Objectives

- To know the reasons for extending semen samples
- To know the common semen diluents used for semen extension
- To understand the art of insemination

5.3 Main content

5.3.1 Semen extension

5.3.2 Characteristics of an ideal medium for semen extension

5.3.3 The types of semen extender

5.3.4 Insemination of the female livestock with stored semen

5.3.5 Methods of insemination

5.3.6 Factors that can influence fertility during insemination

5.4 Conclusion

5.5 Summary

5.6 Tutor Marked Assignment

5.7 References/ Further Reading

5.1 Introduction

The objective of extending semen is to increase the volume of the ejaculate so that a higher number of females can be mated with a given male than is possible in natural mating. For example, by employing semen extension and AI , one ejaculate of a bull of 5ml which contains 0.8-1.2 x 10^9 sperm cells/ml can be extended such that 1ml by volume of extended semen can contain 12 x10^6 sperm cells when inseminated into the female, the cow, will result in a successfully ensuring fertilization of the ovum. Thus, an average ejaculate from a bull when extended can be used t inseminate about 300-500 females.

5.2 Objectives

- To know the reasons for extending semen samples
- To know the common semen diluents used for semen extension
- To understand the art of insemination

5.3 Main Content

5.3.1 Semen extension

5.3.1.1 Characteristics of an ideal medium for semen extension

- A good semen extender should have the ability to increase the volume of ejaculate in order to get more does for AI insemination.

- It should also ensure the survival and longevity of the sperm cells. It should guarantee that the viability and fertility of sperm cells over extended periods of time.
- The ideal semen extender should provide adequate nutrient for the sperm cells, protection of the sperm cells from the harmful effect of rapid cooling
- The ideal extender should contain a buffer to prevent change in pH because lactic acid is produced. pH should be 7 or slightly alkaline.
- The ideal extender should ensure the osmotic pressure, about 290-320mOsm, that will aid the survival of the sperm cells.
- Antibiotics need to be added to the extenders *e.g.* Penicillin, streptomycin to inhibit the growth of microorganisms, sulphanilimide is also added to act as metabolic inhibitor and anti bacterial agent.

5.3.1.2 The types of semen extender

i. Egg yolk/ egg yolk citrate: only the egg yolk portion of the egg is used because the albumin contains lysozyme which is believed to be toxic to sperm cells.
ii. Milk extenders: this is in form of boiled whole milk or boiled skimmed milk diluents.
iii. Fruits and vegetables juices have been used to extender bovine semen. Examples include tomato broth, carrot juice as well as coconut milk.
iv. Other extenders include the following; blood plasma and serum, seminal fluid, starch solution and various alcohol solutions, glycerols and sugars.

It is important to note that if the semen will be frozen, glycerols and sugars should be added. Antibiotics such as penicillin, neomycin and streptomycin must be added to whatever type of extender used to enhance the keeping quality of the semen. Another advantage of inclusion of antibiotics into the extender is that it can increase conception rate by 5-12 percent.

5.3.1.3 Insemination of the female livestock with stored semen

For a successfully insemination that will lead to fertilisation to occur, the female animal needs to be on oestrus. It is also important to use highly fertile and viable sperm cells. The right sperm cell hand techniques and the use of proper equipments for the insemination process is equally necessary.

In cows, the semen is deposited in the anterior vagina while in sows, the semen is placed in the cervix. Hence, in cows and ewes, semen is deposited intra vagina while in sows and gilts, semen is intra uterine. Thus, the procedure for insemination varies with the specie anatomical structure.

Methods of insemination

- The cow can be inseminated by placing the semen in the cervical canal with the aid of a syringe or speculum
- Semen can be put in gelatin capsules which can then be inserted into the uterus where the temperature of the uterus melts the capsules and release the sperm cells
- Intra uterine deposition of semen can be by the use of a disposable or sterilized catheter
- In poultry, a microsyringe can be used to deposit semen at the opening

- The recto-vagina technique using the universal inseminating gun can be used. It is the most hygienic method and has the highest fertility rate compared to the previous methods.

5.3.1.4 Factors that can influence fertility during insemination

To ensure good fertility rate, the following conditions must be strictly addressed;

a. Oestrus detection: in most mammals, heat is detected when the female animal is in the stage of oestrus that it is willing to allow the male to service it. At this time, the egg (ovum) is released or it is said that ovulation has taken place. There are accompanying signs. The signs in cows include the degree of turgidity of the swollen uterus as it is less turgid when on heat and at ovulation, it is flabby. The cow is checked twice a day (morning and evening) for heat. Detection of heat in the ewe and the sows are normally done using a teaser.

b. The timing of insemination: Knowledge of ovulation is required so as to ensure a successful conception rate. Various livestock have differing timing of insemination. In sows, insemination is done on the first day of heat detection or very early on the second day. Cows are inseminated at the middle or at the tail-end of oestrus. Ewes are normally inseminated at the middle or send half of the detection of heat. Poultry are inseminated only when the oviduct is free of egg. Rabbits can be inseminated any time because they are spontaneous ovulators.

c. Restraining the female: it is an essential factor that will ensure conception. The cow should be restrained in a squeeze chute or left standing alone undisturbed or distracted. Ewe should be secured in an elevated crate or placed with the hind legs over a rail. The sow can be inseminated unrestrained as it is usually docile when on heat.

5.4 Conclusion

It is possible to extend semen for AI from few ejaculates to inseminate more females than is possible during natural mating. However, high conception rate is guaranteed if proper semen extension techniques and the ideal mediums are utilised. The method of insemination employed depends on the anatomical variation of the different livestock specie.

5.5 Summary

- Ejaculates can be extended to inseminate more females than is possible in natural mating
- The ideal medium for use in semen extension should ensure viability of sperm cells, have adequate nutrients for sperm cell survival, be of the optimum pH and osmotic pressure
- The media for extension can be from plant or animal source.
- Insemination technique is dependent on the anatomical structure of the specie of livestock
- The methods of insemination of semen are varied and some methods guarantee higher conception rate than others
- A high fertility rate at insemination is a function of good oestrus detection, correct timing of insemination and proper restraint of the female to be inseminated

5.6 Tutor marked Assignment

5.7 References/ Further Reading

Osinowo O.A. 2006. Introduction to animal reproduction.

Module 2: Digestibility trials (*in vivo* and *in vitro*)

Unit 6: An overview of digestibility trials.

6.1 Introduction

6.2 Objectives

- To know the importance of digestibility trials
- To enable you identify procedures that will be able to know feed resources that can be used for livestock feeding in developing countries

6.3 Main content

6.3.1 Definition of digestibility trials

6.3.2 Significance of digestibility trials

6.3.3 Meaning of *in vivo* and *in vitro* digestibility

6.3.4 Merits and limitations of *in vivo* trials

6.3.5 Merits and limitations of *in vitro* trials

6.4 Conclusion

6.5 Summary

6.6 Tutor Marked Exam

6.7 References/ Further Reading

6.1 Introduction

Digestibility trials when carried out on life animals are often times expensive to run, time-consuming and even difficult. These limitations have led to the need to carry out digestibility trials using laboratory methods that can mimic the biological conditions of the digestive system of the live animals. True digestibility will normally make allowance for endogenous losses. Endogenous losses are as a result of the action of digestive enzymes, shed-off intestinal cells and microbial matter. To quantify endogenous losses, the faecal output of the animal will be means.

6.2 Objectives

- To know the importance of digestibility trials
- To enable you identify procedures that will be able to know feed resources that can be used for livestock feeding in developing countries

6.3 Main Content

6.3.1 Definition of digestibility and digestibility trials

Digestibility can be said to be the proportion of a feedstuff which has been absorbed from the digestive system into the bloodstream of an animal. Digestibility is also a measure of the differences between intake and faecal output with allowance made for that part of the faeces that is not a product of feed (they include, the cell shed from intestinal walls, bacteria, residues of digestive juice). Digestibility trials give a measure of the relative feeding value of forage.

6.3.2 Significance of digestibility trials

Chemical analysis of forage cannot give a good picture of the feeding value of every type of forage. Biological methods are still the best for evaluation of feeding value of forages, whether the trial is carried out on the live animal or under a simulated condition (similar conditions as found in the rumen of the life animal). Biological methods will give the optimum results of the type of forage suitable for different ruminant species under varying conditions.

6.3.4 Meaning of *in vivo* and *in vitro* digestibility

In vivo digestibility involves feeding the livestock the test forage orally to evaluate the feeding value of the feed.

In vitro digestibility involves simulation of the rumen condition (anaerobic environment, having a neutral pH) of the species of livestock and introducing the test forage to evaluate the feeding value.

6.3.5 Merits and limitations of *in vivo* trials

The merits of *in vivo* digestibility trials are;

- Some of the procedures employed for *in vivo* trials can be used to estimate two different types of feed within the same trial period
- The trial can also be used for estimating digestibility in grazing animals

The limitations of *in vivo* digestibility trials are;

- It can be expensive to conduct as it involves provision of all the aspects of management of the live animals with the attendant costs
- It is labour-intensive

- There is the welfare concern of feeding untested forages to animals
- The animals may be stressed due to the limitations imposed on their movement during feeding and feacal collection

6.3.6 Merits and limitations of *in vitro* trials

The merits of *in vitro* digestibility trials are;

- It is a more efficient predictor of digestibility than some procedures employed for *in vivo* trials
- It can be used to evaluate multiple samples of forage types in a single trial

The limitations of *in vitro* digestibility trials are;

- There are animal welfare concerns because some *in vitro* procedures will require animals to be fistulated
- It is labour-intensive
- Digestibility may be influenced by variability in composition and activity of the digestive enzymes or innoculum used
- The procedure does not explain the mechanism of digestibility

6.4 Conclusion

Digestibility trials are necessary for evaluation of feed resources and for the determination of the effects of a feed resource on the rumen environment. There is the need to utilise *in vivo* and *in*

vitro techniques to observe and measure the animal's response to dietary manipulations given the limited feed resources in the developing economy.

6.5 Summary

- There is need to know true digestibility and not only apparent digestibility
- Digestibility trials aid in identification of true measure of feeding value of forages
- Biological methods are the most suitable means of evaluating the value of the feed resources
- *In vivo* digestibility trial is oral feeding of test forage and collection of feacal samples
- *In vitro* digestibility trial is done by putting representative samples of test forage in a simulated rumen environment to evaluate feeding value
- There are associated merits and limitations to the use of both methods of digestibility

6.6 Tutor marked Assignment

6.7 References/ Further Reading

- Tilley J.M.A and Terry R.A. 1963. A two stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Soiety*18: 104- 111
- Adesogan A.T, Givens D.I and Owen E. Measuring chemical composition and nutritive value in forages. Field and laboratory methods for grassland and animal production research. CABI publishing.

Unit 7: Protocols for *in vivo* digestibility trials

7.1 Introduction

7.2 Objectives

7.3 Main content: *In vivo* digestibility

7.3.1 Methods of *in vivo* digestibility

7.4 Conclusion

7.5 Summary

7.6 Tutor marked assignment

7.7 References/ Further Reading

7.1 Introduction

There is a need to exploit methods of digestibility trials which do not require sophisticated facilities and equipments. The measurements involved should be the minimum needed to set up feeding trials. This will require observing and measuring the livestock response to dietary manipulations based on the available feed resources. This will aid in the identification of several feed resources that are cheap and readily available locally.

7.2 Objectives

- Understand the techniques involved in conducting *in vivo* digestibility trials

7.3 Main Content

7.3.1 *In vivo* digestibility

7.3.1.1 Methods of *in vivo* digestibility

Materials used for in vivo techniques are expected to meet a set of digestibility standard conditions. These conditions include the animal protocols as well as diet preparations.

Animal Protocol

You must prepare the animal according to the breed, treatment and surgical modifications to be carried out on such animals. You must remember to sought the approval of the Animal Ethics Committee of your institution. Remember that animals have to be cannulated before you can collect digesta.

Certain factors are very important for preparing diet(s) for in vivo digestibility studies. Such factors include feedstuff variety, diet type (compound or single feedstuff), source (commercial or experimental), sample quantity and sample particle size.

You can do In vivo digestibility studies on ruminal digestion in which digestibility processes in the rumen of a ruminant animal is observed using fistulated steers.

There are several techniques which can be applied to *in vivo* digestibility. They include;

i. Direct or total/complete collection

ii. Difference method

iii. Regression method

iv. Indirect method

Direct or total/complete collection: this method entails housing the animal in metabolic cages/crates or they can be housed in pens. The animals housed in pen are fitted with collection bags. The quantity of feed is served to the animal is measured and proximate analysis of feed is carried out and noted. The total faeces voided during the experiment are also collected. The dry weight content of the faeces will be determined and proximate analysis carried out on the dry faecal samples.

The calculations involved in total collection method are as follows;

1. **Digestibility (g/kg)** $= \frac{\textbf{Nutrient in feed – Nutrient in feaces}}{\textbf{Nutrient in feed}} \times 1000$

2. **DMD (g/Kg)** $= \frac{\textbf{DM in feed – DM in feaces}}{\textbf{Dry matter in feed}} \times 1000$

Where;
DMD = Dry matter digestibility
DM = Dry matter

3. **OM (g/Kg)** $= \frac{\textbf{OM in feed – OM in feaces}}{\textbf{OM in feed}} \times 1000$

Where;
OMD = Organic matter digestibility
OM = Organic matter matter

4. D**OMD (g/Kg)** $= \frac{\textbf{OM in feed – OM in feaces}}{\textbf{DM in feed}} \times 1000$

Where;
DOMD = Digestible organic matter content

5. TDN = DCP + DCF + DNFE +DEE (2.25)

Where;
TDN = Total digestible nutrient
DCP = Digestible crude proteins
DCF = Digestible crude fibre
DNFE = Digestible nitrogen-free extract
DEE = Digestible ether extract (2.25)

***Note that Digestible organic matter content (DOMD) and Total digestible nutrient (TDN) are used to estimate energy values of feed resources**

Difference method: this method is used to calculate digestibility of two types of feeds fed concurrently. This implies that there are no interactions between the digestibility of the two feeds. There should also be a priori knowledge of digestibility and the feacal dry matter output (DMO) of the basal diet.

4. **Test feed DMD =** $\frac{\textbf{Test feed DMI} - (\textbf{Faecal DMO} - \textbf{Base feed DMO})}{\textbf{Test feed DMI}} \times 1000$

Where;
DMI = Dry Matter Intake
DMO = Dry Matter Output

Regression method:

Use the regression method to estimate digestibility of two feeds fed simultaneously. To do this, the animal will be served the two feeds of differing ratios. Estimates of the digestibility of each of the ratios are determined. A regression model is made fitting the regression of test feed versus digestibility. This then extrapolated to estimate digestibility of test feed.

7.4 Conclusion

There are numerous techniques for conducting *in vivo* digestibility trials depending on the number of feeds (singly or two different types fed concurrently) fed and the mode of collection of the digesta.

7.5 Summary

- *In vivo* trials do not require elaborate facilities/equipment to be conducted
- There are four (4) techniques which can be employed for *in vivo* trials
    - Parameters such as digestibility, dry matter digestibility, /organic matter digestibility, total digestible nutrients can be estimated from *in vivo* trials

7.6 Tutor marked Assignment

1. Define in vivo. What are the necessary animal protocols for in vivo technique?
2. Dry matter intake of a sheep was 200g, the dry mater output from the feaces of the same sheep was 50g. Calculate the dry matter digestibility.
3. List the 4 techniques that can be applied to in vivo technique

7.7 References/ Further Reading

Adesogan A.T, Givens D.I and Owen E. Measuring chemical composition and nutritive value in forages. Field and laboratory methods for grassland and animal production research. CABI publishing.

Bindelle, J., Buldgen, A., Lambotte, D., Wavreille, J. and Leterme, 2007. Effect of faecal donor and of pig diet composition on in vitro fermentation of sugar pulp. Animal Feed Science and Technology, 132: 212-226

Unit 8: Protocols for *in vitro* digestibility trials

8.1 Introduction

8.2 Objectives

8.3 Main content: **Methods of *in vitro* digestibility**

8.3.1 Rumen fluid-pepsin *in vitro* digestibility (IVOMD)

8.3.2 Rumen fluid-Neutral detergent

8.3.3 Enzyme-based assay

8.4 Conclusion

8.5 Summary

8.6 References/ Further Reading

8.1 Introduction

In vitro technique is the use of an artificial system to mimic a natural dynamic microbial ecosystem. The features of *in vitro* digestibility trials should be such that it is simple in outlook, repeatable and can be routinely practicable. It should have the ability to accommodate the investigation of all forage types while being able to handle large numbers of samples at a time. It should be laboratory-based.

Laboratory *in-vitro* digestibility trials mimics the forage digestion similar to that which could occur in animals (*in vivo*), however, the results obtained does not take into account factors such as palatability of the feedstuff, specie of the animal, the condition of the animal, dietary balance

etc. The animals used for *in vitro* experiments have to be fistulated. *In vitro* experiments could be labourious. The results of the experiments tend to be influenced by differences in the composition and activity of the innoculum or digestive enzymes. *In vitro* digestibility trials cannot show the kinetics involved in digestion.

8.2 Objectives

- Understand the techniques involved in conducting *in vitro* digestibility trials

8.3 Main Content

8.3.1 The use of an in vitro technique ?

Count bacteria

Microbial metabolism and growth

Simulate rumen conditions

predict feed quality; protein, fiber

microbial ecology; simulate rumen digestion

8.3.2 Methods of *in vitro* digestibility

8.3.2.1 Rumen fluid-pepsin *in vitro* digestibility (IVOMD)

This is a laboratory technique for determining the digestibility of dried forages. This procedure was developed by Tilley and Terry (1963). It involves incubation first with rumen liquor and then with acid pepsin solution. Apparent digestibility of the forage (in rumen fluid) is measured

after 48 hours and same measurement is carried out on forage (in pepsin) after 48 hours. This method gives an accurate prediction of *in vivo* digestibility for most forages.

The limitations of this method of estimating digestibility are;

i. There are variations in innoculum composition and activity due to differences in the diets of host (fistulated) animals, the specie of livestock used for the experiment, the timing of collection of the samples, the processing techniques used for the test samples.

ii. Challenges associated with analytical issues. There is the need to maintain anaerobic conditions using media which are of optimum pH and temperature. It is challenging filtering the samples for analysis due to high viscosity. *In vitro* experiments are associated with characteristic offensive odours. There is the need to prevent pathogenic infection by maintaining high standards of hygiene.

8.3.2.2 **Rumen fluid-Neutral detergent**

The procedure is similar to true digestibility. This procedure was developed by Van Soest *et al.* (1966). It gives a higher digestibility estimate than Rumen fluid-pepsin *in vitro* digestibility. The procedure requires the use of rumen fluid.

Example;
Weigh 0.5g of test forage in replicates.

Add 10 ml of filtered rumen fluid, obtained from a bull on a diet of known digestibility.

Add 20 ml of McDougal's solution to each 0.5g sample.

In order to simulate anaerobic rumen conditions, flush the tubes with CO_2 and seal with a Bunsen one-way valve.

Incubate the samples for 48 hr at 39' C and agitated every 12 hr to ensure that the rumen fluid and sample are properly mixed.

After the 48-hr digestion, each sample should be refluxed *i.e.* boiled with 100 ml of neutral detergent for 1 hour (Van Soest *et al*. 1966).

The samples can be filtered and rinsed with acetone to remove all neutral detergent, dried at 100°C

The washed samples will then be ashed

8.3.3 **Enzyme-based assay**

There are numerous methods of enzyme-based assay for *in vitro* digestibility. They include;

i. Cellulose
ii. Neutral detergent-cellulase
iii. Neutral detergent-cellulase + gammanase
iv. Pepsin cellulose

Enzyme-based assay are not without limitations. These are due to variations in enzyme activity due differences in the source of enzymes and differences in the batch of production of the enzymes. The results of trials where enzymes have been used represent the effect of a few specific enzymes and not all digestive enzymes. Equations derived from enzyme-based trials are usually limited to the mode of action on specific specie of livestock. Some components of some feedstuff may not be effectively digested by synthetic enzymes which may lead to a biased conclusion such as underestimation of feed digestibility.

8.4 Conclusion

In vitro digestibility trials have the advantage of being able to test for a larger number of forage (more than two forage types) samples under the same condition concurrently.

8.5 Summary

- *In vitro* digestibility trials should be repeatable and practicable.
- A large number of forage samples can be tested at the same time under the same simulated rumen condition.
- Difference in composition and activity of innoculum or digestive enzymes influence the results from *in vitro* trials.
- There are three main methods for carrying out *in vitro* trials using either rumen liquid or enzymes as catalyst.

8.6 Tutor marked assignment

1. What is the difference between in vivo and in vitro techniques?

2. Name 4 enzyme based assay in vitro techniques of digesitbility.

References/ Further Reading

- Adesogan A.T, Givens D.I and Owen E. Measuring chemical composition and nutritive value in forages. Field and laboratory methods for grassland and animal production research. CABI publishing.
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- Tilley J.M.A and Terry R.A. 1963. A two stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Soiety*18: 104- 111

- Van Soest P.J., Wine R.H. and Moore L.A. 1966. Estimation of the true digestibility of forages by the *in vitro* digestion of cell walls. *Proceedings of the 10th International.Grassland. Congress, Helskinki, Finish Grasssland Association* pp.438-441

Module 3: Biological assays involving PER, NPU, AD, ID and BV

Unit 9: Practical applications of biological assay

9.1 Introduction

9.2 Objectives

9.3 Main content:

9.3.1 The significance of biological assays

9.3.2 The types of biological assays

9.3.3 Protein Efficiency Ratio (PER)

9.3.4 Net Protein Utilisation (NPU)

9.3.5 Apparent digestibility (AD)

9.3.6 True digestibility (TD)

9.3.7 Biological Value (BV)

9.4 Conclusion

9.5 Summary

9.6 Tutor marked assignment

9.7 References/ Further Reading

9.1 Introduction

There are varying methods for characterizing the nutritional merits of feeds and feedstuff; they include the chemical and biochemical analytical techniques. The assessment of a protein diet is usually progressive, starting from the very simple to the more complex tests. The appraisal starts with nitrogen and amino acid analysis, then through a series of specific chemical evaluations, and finally the biological tests. Previously, animal experiments have been widely used to assess the protein quality of feed. The results obtained from such experiments are regarded as sufficient in provision of all the information regarding the protein quality of the feed. There is however a limit to the quantity and quality of information that can be derived from animal assay procedures which should be highlighted.

Biological techniques are highly recommended in the developing economy especially in areas where standard laboratory with up-to-date equipments are lacking. Biological assay techniques require fewer and common simple equipments which can be obtained in the open market.

9.2 Objectives

To be able to measure and observe the response of livestock in feeding experiments

9.3 Main content

9.3.1 The significance of biological assays

Biological assay of feed is of utmost importance to the identification of feed resources which can be utilised for livestock feeding in developing economies. The capability of a feed to satisfy the protein requirements of livestock is a function of both the quantity of protein and the nutritional quality of the feed. The significance of biological assays for assessing protein quality is to

measure nutritional quality as a characteristic of a test protein. The method of choice of protein assessment should have values which range from zero to one (O-1) or from zero to one hundred percent (0 to 100 %).

9.3.2 The types of biological assays

The following are feed protein quality evaluation methods.

9.3.2.1 Protein efficiency ratio (PER)

It is the simplest method of determining the nutritive value of an experimental feed. The growth rate of the young livestock is measured. The weight gain is related to the amount of protein ingested by the animal, the index that is obtained is then termed as the protein efficiency ratio. This method has been adopted by AOAC for evaluating the quality of protein in the diet. Thus, the equation,

$$\textbf{PER} = \frac{\text{Weight gain of a test animal}}{\text{Intake of the specific feed protein during the trial period}}$$

Biological assays which are dependent on nitrogen-balance methods such that nitrogen intake and nitrogen excreted are determined for animals fed experimental diets containing the test protein or a protein-free diet, and then the nitrogen retention is estimated indirectly. The faecal and urinary nitrogen excretion of metabolic and endogenous origin can then be determined.. This can be used for the estimations of apparent digestibility (AD), true digestibility (TD), net protein utilization (NPU) and biological value (BV).

9.3.2.2 Net protein utilization (NPU)

It is the ratio of amino acid which has been converted to protein to the ratio of amino acids supplied. It could also be expressed as the product of biological value and digestibility.

NPU = BV x D

Experimentally, NPU can be determined by measuring the dietary protein intake and nitrogen excreted by the experimental animal. The equation will thus be;

$$\mathbf{NPU} = \frac{((\mathbf{0.16\ X\ (24-\ hour\ protein\ intake\ in\ grams)}) - ((\mathbf{24-hour\ urinary\ urea\ nitrogen}) + \mathbf{2}) - (\mathbf{0.1\ x\ (Ideal\ body\ weight\ in\ kilogram\ )}))/}{(\mathbf{24-hour\ protein\ intake\ in\ grams})}$$

The value of NPU can vary from one (1) to zero (0). A value of one indicates that there is a 100 percent utilization of the dietary nitrogen as protein and a value of zero is an indication that none of the nitrogen supplied was converted into protein. Examples of feedstuff such as milk or egg have NPU of one (1) on the NPU chart.

9.3.2.3 Apparent digestibility (AD)

This is estimated as the difference between nutrient contained in the feedstuff ingested and nutrient contained in the faeces voided by the animal.

AD = Nutrient ingested - Nutrient voided in faeces

Apparent digestibility does not account for nutrient lost as methane gas or metabolic waste in faeces.

9.3.2.4 True digestibility (TD)

This is the portion of the feedstuff ingested by the animal which has been absorbed into the bloodstream of the animal. True digestibility corrects for endogenous and microbial effect of nutrients lost in faeces. Thus, TD could be defined as the difference between nutrient intake and

nutrients in faecal output taking into account nutrients that is not derived from digested feed residues (such sources are the cells of the intestinal tracts that has been shed, microbial matter such as bacteria residue and digestive juices or enzymes).

9.3.2.5 Biological Value (BV)

This is the proportion of the absorbed nitrogen that is retained for maintenance and / or growth. When BV is defined in terms of carcass nitrogen, the apparent BV is expressed as a ratio to unity (recommended) or as a percentage. Determination of biological value of a protein diet is based on nitrogen-balance methods in which nitrogen ingested and excreted are determined in animals fed diets containing the test protein diet or protein-free diet and nitrogen retention is estimated indirectly. The procedure allows for the determination of faecal and urinary nitrogen excretion of metabolic and endogenous origin.

9.4 Conclusion

Biological assay of diets/feedstuff given to livestock is essential as this will provide relevant information on the protein quality of the feed served the animal. This in turn will aid productivity due to the knowledge of the protein status and the availability to the livestock.

9.5 Summary

- Biological assay of feedstuff given livestock is essential as it provides relevant information on the protein quality and utilisation by the animal
- Biological assay of feedstuff for protein value is one of the final test for chemical/biological evaluation of feedstuff

- Biological assay can be done using few, common and simple equipments (example, weighing scale) to evaluate.
- There are five (5) types of biological assay considered in this study and they include; PER, NPU, AD, TD and BV.

9.6 Tutor marked assignment

1. Define Biological value.
2. What is the relationship between PER and NPU?
3. What are the differences between True digestibility and apparent digestibility?

9.7 **References/ Further Reading**

- Gil A., Martinez de Victoria E and Olza J. 2015. Indicators for the evaluation of diet quality. *Nutr Hosp.* 31(Supl. 3):128-144. DOI:10.3305/nh.2015.31.sup3.8761
- Kant A.K. 1996. Indexes of overall diet quality: a review. *J Am Diet Assoc* 96: 785-91.

Module 4:

Separation and characterisation of components involving the use of chromatography, electrophoresis, radio-isotopy, manometry, colorimetry, spectrometry

Unit 10: Techniques involved in separation and characterisation of components involving the use of chromatography and electrophoresis

10.1 Introduction

10.2 Objectives

10.3 Main content:

10.3.1 Available techniques involving chromatography

10.3.2 Column chromatography

10.3.3 High performance (pressure) liquid chromatography

10.3.4 Adsorption chromatography

10.3.5 Ion exchange chromatography

10.3.6 Hydrophobic chromatography

10.3.7 Gel filtration chromatography

10.3.8 Affinity chromatography

10.3.9 Separation of proteins using electrophoresis

10.3.10 Agarose gel

10.3.11 Polyacrylamide gel

10.4 Conclusion

10.5 Summary

10.6 Tutor marked assignment

10.7 References/ Further Reading

10.1 Introduction

There are various techniques for the separation and characterisation of proteins. They include chromatography and electrophoresis. The choice of technique for a particular mixture requires knowledge of the protein molecules of the mixture. A mixture of fluids can be easily separated from one another due to differences in solubility in different solvents and if the constituents of the mixture have differing molecule sizes. However, for proteins which have similar physical and chemical properties, then chromatography has to be the technique employed to purify the mixture. Electrophoresis is a major technique for laboratory protein separation because it is relatively easy and cheap to carry out.

10.2 Objectives

- To understand the techniques involved in the separation of proteins,

10.3 Main content:

10.3.1 Available techniques involving chromatography

Chromatography works by putting the substances to be separated into a structure which consists of two phases, that is: a mobile phase and a stationary phase. These substances will then be

separated based on differences in their interaction between these two phases as the mobile phase moves across the stationary phase.

In liquid chromatography technique, the substance (solute) to be analyzed is dissolved in the solvent. The solvent then flows across a solid matrix (which is the stationary phase). The solutes then intermingle with the stationary phase by reversibly binding to the stationary phase. The potency of the binding between the solute and the stationary phase will determine how fast the solute is carried by the mobile phase.

10.3.2 Column chromatography

A column chromatography consists of a central component known as the column. A pump is included to control the rate of flow of buffers through the column. The solvent used in the mobile phase will need to gradually change and this is with the aid of a gradient maker. The elution of the different components of the solute can be noted during chromatography either with a spectrophotometer which measures the absorbance of material coming off the column or for protein chromatography, monitor the A280.

Chromatography which is used for protein purification scheme will include a fraction collector**.** A fraction collector is a apparatus that routinely collects the liquid flowing from the column into separate tubes. Each tubes are then evaluated for the proteins investigated for. The quantities of the proteins will be plotted against either the fraction number, volume or time. These three variables can be easily determined from the flow rate and volume of the individual fractions.

10.3.2 High performance (pressure) liquid chromatography

High Performance (Pressure) Liquid Chromatography is an improvement in technology over the column chromatography. The flow rates in column chromatography are restricted due to the compression of the support matrices present in the columns. This technology employs the use of resins which can endure the rigours of packing and high flow rates such that high resolution are enhanced. Another advantage is that separation of substances can be done under higher pressures such as high flow rates which will result in increased resolution.

10.3.4 Adsorption chromatography

The thin-layer chromatography (TLC) and paper chromatography are typical examples of adsorption chromatography. Adsorption chromatography is normally utilised for separation of small molecules *e.g.* lipids, nucleotides, simple sugars, amino acids. Although adsorption chromatography is not commonly used in protein purification, hydroxyapatite columns have been utilised successfully in exceptional situations to separate proteins that were otherwise not easily separated by other techniques.

Hydroxyapatite is a crystalline form of calcium phosphate. It is has been used as a stationary phase medium to analyse proteins or nucleic acids. It involves a non-specific interaction between the positive calcium and negative carboxyl groups on the proteins and the negative phosphate and positive amino groups on the protein.

10.3.5 Ion exchange chromatography

Ion exchange chromatography (IEC) is a variant of adsorption chromatography. It is based on charge-charge interactions. The stationary phase includes fixed charges on a solid support. The fixed charges on the stationary phase are either positive or negative. These charges are referred

to to as **anion exchange** or **cation exchange** chromatography respectively. The mode action is such that the substances which are to be separated will replace the counter ions associated with the chromatography medium and firmly bind to the exchanger by way of electrostactic interactions. In the situation that some solute molecules are electrostatically bound to the exchanger and other solute molecules are not bound is used to separate solutes (proteins).

A typical example is the mixtures of adenine nucleotides, consisting of adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) can be separated this method. Adenosine, is uncharged hence will not bind to the anion exchanger. With an increment in the concentration of formate to the column, AMP will be the first to be eluted, next is ADP and lastly the ATP. The sequence of elution is related to the overall negative charge of the nucleotides (*i.e* the number of phosphate groups).

10.3.6 Hydrophobic chromatography

Hydrophobicity is a chemical property that causes the aggregation of nonpolar compounds with each other in an aqueous environment. Proteins can be separated according to differences in their hydrophobicities. The media for hydrophobic chromatography is a support matrix made of agarose and long chain hydrocarbons covalently bound to it. Examples include octylagarose (8 contiguous methyl groups) and phenyl-agarose. They (octyl-agarose and phenyl-agarose) provide a hydrophobic surface for proteins to interact with instead of aggregating with each other. Highly hydrophobic resins such as octyl-agarose are most suitable for weakly hydrophobic proteins, while less hydrophobic resins such as phenyl-agarose are more suited for proteins of intermediate hydrophobicity. Proteins can bind to hydrophobic columns when the conditions that will promote hydrophobic interactions exist as these conditions will determine the extent of

binding. *E.g* an increase in ionic strength increases hydrophobic interactions. Ammonium sulphate $(NH_4)_2SO_4$ and sodium chloride NaCl are examples of salt that are used in hydrophobic chromatography.

10.3.7 Gel filtration chromatography

This technique is also known as molecular sieve chromatography or size exclusion chromatography. The technique separates proteins on the basis of individual molecule size. The protocol is such that the test substance (proteins)is passed over a column made of small beads (composed of cross-linked polymers). The pore size is determined by the extent of cross-linking of the polymers. Proteins (solutes) which .are smaller than the pore easily go into the gel matrix and are retained on the column for a longer duration. However, proteins (solutes) molecules which are bigger than the pore size will not enter the matrix or beads but pass through the column unhindered. The duration of retention of solute in the gel matrix is inversely proportional to the size of the molecule of the protein (solute).

Gel filtration has been used to determine the molecular weight of a protein. This is possible if the columns are calibrated by using markers (proteins) of known molecular weight. These markers whose molecular weights are known are passed over the column and the Kav determined for each protein as follows:

Kav = Ve - Vo/Vt - Vo.

Where;

Vo = void volume (It is the volume of the substance which is too large to enter the matrix of the support medium.

Vt = total volume (is calculated from the volume of the column bed *i.e* $\pi r2$ x length)

Ve = elution volume

10.3.8 Affinity chromatography

Affinity chromatography involves proteins interactions with specific ligands. The protocol is for the protein mixture to be passed over the column with a suitable specific segment of the molecule which is attached by a covalent bond to a solid support. The protein which has affinity to the ligand of interest will be the only protein to bind to the column while the other proteins will be washed away. Affinity chromatography is very precise and enables the isolation of a single protein in a single step.

10.3.9 Separation of proteins using electrophoresis

Separation of proteins using electrophoresis is dependent on the charge distribution of the protein molecules which are being separated. Electrophoresis of proteins is usually carried out in solution as the capacity to split these molecules is dependent on their ability to diffuse. Polyacrylamide gels or agarose gels are good media for achieving great resolutions. However, polyacrylamide is the choice gel for protein electrophoresis while agarose gel is more commonly used for nucleic acids. This is because agarose gels have larger pore size than acrylamide gels thus more effective for bigger macromolecules. Both types of gels may however be used for either of proteins or nucleic acids.

10.3.10 Polyacrylamide gel

Sodium dodecyl sulfate (SDS) is a detergent which can be added to proteins. The proteins become negatively charged when they interact with the SDS anions. SDS will totally affect the protein-protein interaction thus denature virtually all proteins present in the test substance which will reveal the entire proteins present. A situation whereby these proteins are separated on a polyacrylamide gel is referred to as Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis or SDS--PAGE. It is the protocol used for the determination of molecular weight. The following is a hypothetical procedure for SDS-PAGE.

10.3.10.1 Materials required:

• SDS-polyacrylamide gel

• Protein markers

• 2X-SDS Sample Buffer

• 1X-SDS Electrophoresis Running Buffer (*i.e.*Tris-Glycine + SDS)

• 0.001% (w/v) Bromophenol Blue

- Micropipettes with disposable tips
- 0.25% (w/v) Coomasie Brilliant Blue R 250 in methanol-water-glacial acetic acid (5-5-1), filtered immediately before use.
- 7% (v/v) acetic acid

10.3.10.2 Procedure

1. Pour the separating gel which should contain about 6-20 percentcrylamide. Note that the choice of the acrylamide concentration is dependent on the weight of proteins being separated, the desired resolution and the quantity of sample

2. Pour stacking gel after the separating gel has polymerized and just prior to electrophoresis. This will minimise diffusion between the two gels.

3. Load test samples. The proteins to be analyzed should first be solubilized in a sample buffer (contains 2% SDS and 5% β-mercaptoethanol) and then boiled. Load the [rotein marker on the first and last wells of the gel. Avoid overloading the wells as this result in the pores in the gels results becoming plugged. A tracking dye (bromophenol blue) should also be included in each sample. The function of the dye is that when it reaches the
bottom of the separating gel, the power can be turned off and the proteins detected

4.An electric field field id applied after loading the samples into the wells of the gel to run across the gel (20 mA constant current per 1.5 mm gel). If excessive heat is applied, this may result in the proteins running off the gel.

5. Stain or process gel. To detect proteins after electrophoresis, the gel is processed by staining with Coomassie blue (a dye that binds proteins). The gels is then 'fixed' before staining with an acetic acid and methanol solution which precipitates proteins into the acrylamide matrix. Use at least 10 volumes of Coomasie Blue staining solution for 2-4 hours, tilting gently to aid distribution of the dye evenly over the gel.

6. Destaining and washing of the gel. At the conclusion of the staining, Wash the gels while changing the water until the blue colouration stops. Place the gel in a 7% acetic acid solution for at least 1 hour and move the gel into fresh 7% acetic acid as required (until the blue background is no longer visible). Place the gel into a container and cover gel with 7% acetic acid fix proteins.

7. Photograph the gels.

10.3.11 Agarose gel

Agarose gels are formed by heating the appropriate concentration of agarose in an aqueous buffer. When the agarose has dissolves, the solution is allowed to cool down to enable the gel to

form. The protein samples are then loaded into the wells while the protein markers are put in the wells on the first and last wells.

10.4 Conclusion

Despite the many techniques for the separation and characterisation of proteins, electrophoresis and chromatography are the techniques of choice. A knowledge of the physical (molecule size) and chemical properties determine the technique of choice for the separation of proteins into the different components.

10.5 Summary

- The choice of technique for separation of protein requires knowledge of the physical and chemical properties of protein molecules in the mixture.
- Proteins with similar physical and chemical properties can be separated using chromatography technique to purify the mixture.
- Electrophoresis is a major technique for laboratory protein separation as it is very easy and cheap to carry out.
- There are seven (7) methods in the use of chromatography technique for protein separation and characterisation.
- There are two (20 methods in the use of electrophoresis for protein separation and characterisation.

10.6 Tutor marked assignment

1. What technique do you think is the most suitable method for separating protein in a solution?

2. Describe the principle behind liquid chromatography technique.
3. How would you use electrophoresis to separate proteins into it components?
4. Make a list of the materials you will require to separate protein in an electrophoresis assignment.

10.7 References/ Further Reading

- Wiser M.F. Lecture notes for methods in cell biology (TRMD 623). Pp 73-101 http://www.tulane.edu/~wiser/methods/notes.pdf

Unit 11: Techniques involved in separation and characterisation of components involving the use of radio-isotopy and manometry

11.1 Introduction

11.2 Objectives

To understand the principle/application of radioisotopy and manometry to biological experiments.

11.3 Main content:

11.3.1 Principles and application of radioisotopy

11.3.2 Principles and application of manometry

11.4 Conclusion

11.5 Summary

11.6 Tutor marked assignment

11.7 References/ Further Reading

11.1 Introduction

Radioactivity is employed for biological research as radioisotopes have been used to label the test molecules resulting in effective and safe means of monitoring molecular interactions. It is the only technique which gives accuracy and specificity of radioactive isotopes/tracers. The

properties of radioisotopes which favours the use in biological analysis is that individual radioactive decay or emission have its own unique and specific energy.

11.2 Objectives

You will be able to:

Define isotopes

To understand the principle/application of radioisotopy to biological experiments

Identify isotopes that are useful in agricultural research

Identify key areas of agriculture where isotopes are used

To understand the principle/application of manometry to biological experiments.

11.3 Main content

11.3.1 **What is radioisotope?**

Radioisotopes are elements that emit some extraordinary types of energy in form of rays (alpha, beta and gamma), which are very helpful to agricultural activities among other usefulness for human beings, in minute quantity. The rays emitted by radioisotopes are not visible to human eyes but can penetrate solid materials such as skins and eyes. An instrument such as Geiger Muller and Scintillation Counters are normally used for detecting such invisible energy. These instruments are designed to detect the minutest quantity of this element.

11.3.2 **The use of radioisotopy in Agriculture**

It is commonly used in the field of agriculture than any other science field.

Nutritional Studies

In agriculture, radioisotopes are used in the nutritional studies of major and minor elements, milk production, mechanism of photosynthesis studies. Radioisotopes are also important to the study and research in plant protection, plant pathology, action of insecticides, uptake of fertilizers, ions mobility in soil, and plants and food preservation. It is also used in order to determine the correct nutrition for a plant we need to know the exact soil plant relationship and the factors involved therein.

11.3.3 Advantages of radioisotopes to agriculture

i) With the help of radioisotopes we can easily locate the presence of a single atom and molecule and their movement. Hence, they give research workers the opportunity to follow up step by step all kinds of processes that are related to the nutrition of plant from germination to maturity.

ii) Very small quantities of labeled nutrients can be accurately measured in presence of large quantities of other nutrients.

iii) The location of materials can be identified by radio-autography.

iv) Tracer technique enables one in tracing those elements taken by the plants accurately and precisely.

v) It also helps to study accurately the effect of one element upon the absorption of another and their interaction by plants and now it has become very easy to study properly the phenomenon of interaction among the mineral nutrients.

They are used for soil analysis especially in detecting P content of the soil.

Radioactive phosphorus are normally used by researcher to distinguish between soil phosphorus and the fertilizer phosphorus, taken up by the plants.

Many elements are leached through the soil. Radioisotopes like Fe, Mn, K, Ca, N, Rb, C, Cs, Si and Sr etc. and other macro and micro-elements have also been used by researchers in order to find out how this elements moves in different types of soils and also their position in different clay fractions of the soils.

The radioisotope method is a very reliable and helpful method in the determination of soil fertility.

Radioisotopes technique is used to determine the amount of nutrient that are taken by plants. It is also help to know the movement and the places of accumulation of various elements in the plant.

Radioisotopes are very useful in genetic studies. This is because they are used for mutation induction. Mutation is a sudden heritable changes of the hereditary factors located in the DNA found in the chromosomes of the organisms, plant or animal.

11.3.4 Tracer Technique

It is technique in which one or more atoms of a chemical compound have been replaced by a radio isotope to trace the metabolic pathways. A radioactive tracer can also be used to track the distribution of a substance within a natural system such as a cell or tissue. Radioactive tracers form the basis of a variety of imaging systems, such as, PET scans, SPECT scans and technetium scans.

It is based on the principle that a stable isotope is replaced by a radioisotope. The radioisotope is capable of emitting radiations that can be detected and analysed. It is powerful than chemical reactions as they can be detected even in lower concentration as seen inside a cell. In general, isotopes of hydrogen, carbon, phosphorus, sulphur, and iodine have been used extensively to trace the path of biochemical reactions. In metabolism research, Tritium(^{3}H) and ^{14}C-labeled glucose are commonly used to measure rates of glucose uptake, fatty acid synthesis, and other metabolic processes.

11.3.5 Autoradiography

The common application of radioisotopy is the uses of autoradiography in combination with gel electrophoresis or with microscopy. Autoradiography of gels is used to identify and quantify specific proteins or nucleic acids. Radioactive markers could be localised to particular cells in specific parts of the tissues or within cells using autoradiography.

Protocol for autoradiography is as follows;

1. Fix tissue or cells to slide. This is done after radioactive isotopes have been incorporated into cellular molecules

2. Cover with a photographic emulsion. Ionizing radiations should be emitted during radioactive decay as the silver ions in the photographic emulsion become reduced to metallic silver grains.
3. Develop the emulsion after sufficient exposure
4. Examine the slide under the microscope. It will be observed that dark grains (or spots) will appear over the cells or structures that contain the radioactive marker. These grains do not only detect radioactivity but provide information with respect to the quantity and cellular distribution of radioactive label (due to their number and distribution)

11.3.6 Principles and application of manometry

Manometry: technique of measuring the pressure variation due to gas production/consumption caused by biochemical reactions or physical changes. The manometer is the name of the instrument that measures and compares the pressure of fluid.

Manometry is applicable to any bioprocess that involve the production/consumption of a poorly soluble gas.

The main applications of manometry include:

aerobic processes (oxygen consumption)

anoxic processes (N2 production)

anaerobic processes (biogas production)

Manometer

The instrument consists of a glass U-tube such that if pressure is exerted on one end of the U-shaped tube (partially filled with liquid), the liquid will be displaced upwards on the other side of

the tube. The difference in height or distance between the levels of these two arms is proportional to the difference in pressure difference on each side of the tube.

11.3.7 Microcalorimetry

Calorimetry is the technique of measuring the heat produced by chemical reactions or physical changes.

Microcalorimeter is the instrument that is able to measure really small heat exchange (5-10 mW L^{-1}).

It is sensitivity up to 0.001°C which is equivalent to 3-5 mg BCOD L^{-1}.

It can measure any temperature change associated with biochemical reactions. It is the universal tool for the study, optimisation and modelling of bioprocesses (substrates consumption as well as biomass adaptation, inhibition, growth).

11.4 Conclusion

There are safe and effective means of quantifying and identifying specific proteins in cells/tissues of animals such as the use of radioisotopy. Pressure in a fluids can be effectively determined using the manometry technique.

11.5 Summary

- Radioisotopy is an effective and safe way of monitoring molecule interaction
- Specific proteins can be identified and quantified by radioisotopy technique
- Manometry measures pressure
- Claorimetry measures heat

11.6 Tutor marked assignment

1. Radioisotopes are very important in genetic studies. Explain how.

2. Radioisotopes are very important in soil fertility studies. Justify this statement.

3. List four advantages of radioisotopes to agriculture.

4. List 5 isotopic elements that used in agriclutre

11.7 References/ Further Reading

- Wiser M.F. Lecture notes for methods in cell biology (TRMD 623). Pp 33-35 http://www.tulane.edu/~wiser/methods/notes.pdf

  Importance of Radioisotopes in Agriculture http://www.pakistaneconomist.com/issue2003/issue49/i&e3.asp

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Unit 12: Techniques involved in separation and characterisation of components involving the use of spectrometry and colorimetry

12.1 Introduction

12.2 Objectives

12.3 Main content:

13.3.1 Principles and application of spectrometry

12.3.2 Principles and application of colorimetry

12.4 Conclusion

12.5 Summary

12.6 Tutor marked assignment

12.7 References/ Further Reading

12.1 Introduction

There are various techniques which utilise the light wave to determine some characteristics of interest in a known solution. They include colorimetry and spectrometry. These techniques, colorimetry and spectrometry, have devices or apparatus that can be used to determine the values of interest in the laboratory. The colorimeter is the device used for taking colorimetric measurements while the spectrometer, spectrophotometer or spectrograph are the devices used in spectrometry.

12.2 Objectives

To understand the principle/application of spectrometry and colorimetry to biological experiments.

12.3 Main content

12.3.1 **Principles and application of spectrometry**

Spectrometry is a method/technique used to obtain a quantitative measure of a spectrum. It is a spectroscopic technique which can assess the quantity or concentration of a given chemical (atomic, molecular or ionic) species.

The main forms of spectrometry; Rutherford backscattering spectrometry and neutron triple spectrometry, ion-mobility spectrometry and mass spectrometry. However, the most common form used is the mass spectrometry. The spectrometer is the device used in spectrometry technique, however, the type of spectrometer depends on either of the four forms of spectrometry.

Mass spectrometry is the plot between intensity (*i.e.* number of incident particles) and the mass of the particle.

A mass spectrometer creates charged particles (known as ions) from molecules of a substance. The analyses of these ions by the mass spectrometer will provide information about the molecular weight and chemical structure of the substance. All mass spectrometers have three distinct components;

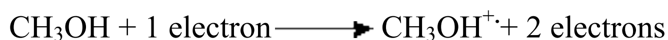
i. Ionizer
ii. Ion Analyser

iii. Detector

Ionizer

In the ionizer, the charged particles (ions) required for mass analysis are created by Electron Impact (EI) Ionization. EI Ionization normally produces single charged ions which has one unpaired electron. Energy imparted by the electron impact causes instability in a molecular ion which will make that ion to fragment (break into small pieces)

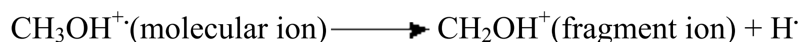
E.g, The reaction of methanol in the ionizing region:



CH_3OH + 1 electron $\longrightarrow$ $CH_3OH^{+\cdot}$ + 2 electrons

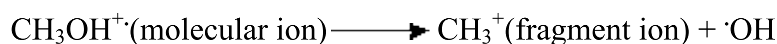
(note: the symbol + is an· indication that a radical cation has been formed)

The methanol ion may fragment in different ways, where one fragment will be the charged and the other fragment remain uncharged. For example:



$CH_3OH^{+\cdot}$(molecular ion) $\longrightarrow$ CH_2OH^{+}(fragment ion) + $H^{\cdot}$

(or)



$CH_3OH^{+\cdot}$(molecular ion) $\longrightarrow$ CH_3^{+}(fragment ion) + $^{\cdot}OH$

Ion Analyzer

The acceleration of molecular ions and fragment ions through the mass spectrometer is by manipulation of the charged particles. The uncharged molecules and fragments will be pumped away. Ions travel down the path based on their mass to charge ratio (m/z). EI ionization produces singly charged particles, so the charge (z) is one, hence, an ion's path will depend on its mass.

Detector

Detectors in mass spectrometer work by producing an electronic signal when struck by an ion. Timing mechanisms incorporate these signals with the scanning voltages and allow the device report which m/z struck detector. The mass analyzer will then arrange the ions according to the m/z and the detector records the abundance of each m/z.

12.3.2 Principles and application of colorimetry

Colorimetry is a technique which is used to measure the quantity or concentration of a known solute in a particular solution by analyzing the intensity of light. The technique is based on the application of Beer-Lambert law which states that 'the concentration of a solute is proportional to the absorbance'.

The colorimeter is a device that measures the absorbance of particular wavelength of light by a specific solution. This is to determine the concentration of the specific solution by analysing its color intensity.

The protocol for measuring the concentration of a specific solution with the aid of a colorimeter is as follows;

1. The calorimeter should be calibrated by placing a blank in the cuvette slot and the lid of the colorimeter closed. The blank is a cuvette is usually distilled water. The blank acts as a control, which is used to measure the small amount of light which is absorbed by the solvent and by the sides of the cuvette.
2. Fill the cuvette up to the 2.2 to 3.5 ml mark with the sample solution.
3. Place the standard disposable cuvettes (1cm x 1cm cuvettes) containing the sample solution in the colorimeter .Ensure to place the cuvette appropriately in the colorimeter.

This is two opposite sides of the cuvette are ribbed and do not transmit light from the LED while the two smooth surfaces are transmit lights.

4. Monochromatic light from the LED light source will pass through the cuvette containing the sample solution to the photodiode
5. The monochromatic light will be absorbed by the solution. Light which has a lower intensity than was beamed will strike a photodiode.
6. Calculating absorbance;

i. The amount of light that penetrates a solution is called transmittance *T* and is expressed as that ration of the intensity of the transmitted light *It,* and the initial intensity of the light beam *I0* as expressed by the formula:

$T = I t / I0$

ii. The transmittance of the sample is determined by the cuvette width *d* and the concentration of the solution *c*. The relation between *T*, *d*, *c* is:

*T = e-a*d*c (*where *a is an constant that depends on the solution and used the light wavelength)*

iii. The formular for absorbance derived from equations (i and ii) is;

$A =- {}^{-10}\log(T) = {}^{10}\log(1/T) = {}^{10}\log(e) * a * d * c = e * d * c$

*(*where e *is the molar absorptivity of the solution)*

iv. For a given solution contained in a cuvette with a constant cell width, the Absorbance is proportional to the concentration: *(Beer's law)*

$A = k * c$

where *k* is the rate of reaction (k)

12.4 Conclusion

Colorimetry and spectrometry use light waves to measure concentration of particular substances in a solution.

12.5 Summary

- Spectrometry can measure the molecular weight and chemical structure of a substance in solution using light wave technology
- There are four (4) main forms of spectrometry; Rutherford backscattering spectrometry and neutron triple spectrometry, ion-mobility spectrometry and mass spectrometry
- The instrument use for spectrometry is spectrometer but the type of spectrometer depends on which form of spectrometry is used for analysis
- A typical spectrometer has three (3) basic components; ionizer, ion analyser and detector
- Colorimetry determine concentration of a particular solute in a solution by analyzing the intensity of light

12.6 Tutor marked assignment

1. What is colorimeter?
2. Describe the principle of colorimetry
3. List the protocols for colorimetry.

References/ Further Reading

- Wiser M.F. Lecture notes for methods in cell biology (TRMD 623). http://www.tulane.edu/~wiser/methods/notes.pdf

Unit 13: Determination, preparation and purification of enzymes

13.1 Introduction

13.2 Objective

13.3 Main content:

13.3.1 Properties of enzymes

13.3.2 Mode of action of enzymes

13.4Conclusion

14 Summary

15 References/ Further Reading

13.1 Introduction

All enzymes are proteins. The activities of enzymes enable all cells (plants and animals) to function. This activities result in constant changes in cells which are regulated by catalytic reactions and these reactions are also regulated by enzymes. An important property of enzymes is that they act as catalysts to a reaction and that they affect only the reaction rate. A good knowledge of the activities of enzymes will help define the properties of cells. It is therefore necessary to study enzymes *in vitro* after extraction from cells.

13.2 Objective

- To understand the properties and activities of enzymes

- To understand the process of extracting enzymes from tissues
- To understand the process of purifying enzymes from crude extract.

13.3 Main Content

13.3.1 Properties of enzymes

Like all proteins, the enzymes are characterised by peculiar properties. They include the following;

a. Enzymes only act as catalysts to a reaction and that they affect only the reaction rate and do not change chemical composition of the reaction.
b. Enzymes have differing solubility in salt solutions
c. Minute changes in the temperature of a reaction will significantly alter the reaction rate. Extremely high temperature can denature the enzymes.
d. Enzymes are highly sensitive to changes in pH
e. Salts cause denaturation of enzymes especially heavy metal salts and irreversibly alter the structure of the enzymes except the effects of ammonium sulfate which are may be reversible.

13.3.2 Mode of action of enzymes

An enzyme works by binding to a given substrate such that the substrate undergoes the reaction more rapidly. This reaction is a perfect fit. It means that there is a specific part of the enzyme structure that actually binds in a lock and key mode to a substrate. The enzyme makes the substrate be in an accurate alignment for it to react spontaneously or together with other substance(s). Every reaction goes on usually by a random kinetic action of molecules running into each other and whenever these molecules align, they react faster. In ideal conditions (when

the pH, temperature are optimal) and the right concentration of enzyme, the reaction will proceed fastest provided the substrate concentration is adequate.

13.3.3 **Preparation of enzymes**

The success of the purification of enzymes begins at the extraction stage. Each stage in the extraction procedure should be closely monitored for enzyme activity. Monitoring during the extraction process can be achieved in several manners which usually entail a measurement of the decrease in substrate, or the increase in product specific to the enzyme. The product of the process of enzyme extraction is not a pure form of the target enzyme but a cocktail of the enzyme and numerous other proteins.

13.3.3.1 Extraction of enzymes

The protocol for the extraction of enzyme is as follows;

a. Chose the tissue from which the enzyme will be extracted from
b. Homogenize the tissue in a buffer(*example* phosphate buffer) that will preserve the enzyme in its soluble state
c. Centrifuge and collect the supernatant This process will allow the enzyme to be separated from all insoluble proteins
d. Repeat the step c (combining the supernatant and cold buffer)
e. Use the supernatant from step d as the source of enzyme. The enzyme will be impure in the supernatant because any other proteins are present in solution.

13.3.4 Purification of enzymes

Pure enzyme can be obtained from the impure enzyme extract. This is by the purification of the impure extract by salt precipitation. The sequential addition of varied salt concentrations such as

ammonium sulfate solution to the extract will cause individual proteins to precipitate depending on their solubility. However, the precipitate containing the target enzyme can be subjected to addition processing if absolute purity of the enzyme is required. This is in the form of dialysis, gel filtration, electrophoresis and column chromatography, ion exchange chromatography in order to obtain the enzyme in the purest form possible. The pure enzyme could then be utilised for further analysis.

13.3.4.1 Protocol for purification of enzymes

Material required:

i. Ice bath
ii. Ammonium sulphate (finely ground)
iii. Weighing scale
iv. Centrifuge
v. Buffer(Potassium phosphate)

Protocol:

1. Weigh the ammonium sulfate powder
2. Add the ammonium sulfate powder slowly and carefully to the impure extract, constant stirring to ensure complete solubility
3. Centrifuge the solution at required speed (in rpm) for a period of time (in minutes) at a cold temperature(°C)
4. Different precipitation steps will need to be carried out for different enzymes by varying the salt concentration (45–80%) and precipitates will be collected.

An example is the purification of tyrosinase enzyme as stated below;

1. Weigh the finely grounded ammonium sulfate powder
2. Add the ammonium sulfate powder slowly and carefully to the impure extract, constant stirring to ensure complete solubility
3. Centrifuge the solution at 5000 rpm for 30 minutes at 4°C
4. Vary the salt concentration between 45-80 percent and collect precipitate
5. Dialyse the precipitate against 100 mM potassium phosphate buffer (pH 7.0) for 24 hours by changing the buffer thrice.
6. The enzyme is now ready for use for further studies

12.5 Conclusion

The properties of enzymes allow them to be utilise in fast tracking a reaction under ideal conditions. The ability of the enzymes to speed up reaction rate can only be possible if the right concentration of enzyme is in the mixture, ideal temperature and pH are maintained. A successful enzyme recovery process depends on proper monitoring of the process at the extraction stage. It is also worthy to note that the product of the enzyme extraction process is not a pure form of the target enzyme but a cocktail of enzymes and other proteins.

A successful enzyme recovery process depends on proper monitoring of the process at the extraction stage. It is also worthy to note that the product of the enzyme extraction process is not a pure form of the target enzyme but a cocktail of enzymes and other proteins.

12.6 Summary

Enzymes are proteins

They influence the rate of reaction and not the final product of the reaction

Enzymes are highly sensitive to pH, temperature, salt concentration

Reactions proceed more rapidly if the right concentration of enzyme is present in the reaction mixture and optimum temperature, pH are maintained for the specific reaction

Purification of enzyme is the next step in the process after extracting the impure enzyme.

Varying the salt concentration added to the impure extract will cause different proteins in the cocktail to precipitate.

13.6 Tutor Marked Assignment

1. Define enzyme? Describe the protocol you will employ for the extraction of enzyme
2. Describe the protocol for the purification of enzyme
3. List the materials you will require for the purification of enzyme

13.7 References/ Further Reading

Heidcamp W.H. Cell Biology Laboratory Manual. Pp 90-111

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