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DEPARTMENT OF BIOTECHNOLOGY

BT 47L

Enzyme Technology and Biokinetics Lab Manual

Experiment - 1

Determination of alkalinity in the given water sample

AIM: To Determine the Alkalinity in a Given Water Sample

THEORY: Alkalinity is a measure of the capability of water to absorb H+ ions without significant change of pH. In other words, alkalinity is a measure of the acid buffering capacity of water. The determination of alkalinity of water is necessary for controlling the corrosion, to calculate the amount of lime and soda needed for water softening; in conditioning the boiler feed water, etc. Alkalinity of a sample of water is due to the presence of OH– (hydroxide ion), HCO3 – (bicarbonate ion) and CO3 2– (carbonate ion) or the mixture of two ions present in water. The possibility of OH– and HCO3 – ions together is not possible since they combine together to form CO3 2– ions. OH– + HCO3 – CO3 2– + H2O The alkalinity due to different ions can be estimated separately by titration against standard acid solution, using selective indicators like phenolphthalein and methyl orange. i) OH– + H+ H2O ii) CO3 2– + H+ HCO3 – iii) HCO3 – + H+ H2O + CO2 The neutralization reaction upto phenolphthalein end point shows the completion of reactions (i) and (ii) (OH– and CO3 2–) and (CO3 2– and HCO3 –) only. The amount of acid used thus corresponds to complete neutralization of OH– plus half neutralization of CO3 2– . The titration of water sample using methyl orange indicator marks the completion of the reactions (i), (ii) and (iii). The amount of acid used after phenolphthalein end point corresponds to one half of normal carbonate and all the bicarbonates. Total amount of acid used represent the total alkalinity due to all ions present in water sample.

APPARATUS: Burette, pipette, conical flask, beakers, burette stand and clamp

CHEMICALS: Dry Na_2CO_3, concentrated 10(N) HCl, phenolphthalein and methyl orange indicator

PROCEDURE:

1. Prepare Primary standard solution of Na_2CO_3 (0.1N).

2. Take Secondary standard solution of HCl and the water sample.

3. Standardization of HCl solution by primary standard Na_2CO_3 solution– Pipette out 10 mL of Na_2CO_3 solution in a conical flask, add 2 drops of methyl orange indicator, fill up the burette with (10N) HCl solution and titrate till the color of the solution changes from yellow to red.

4. Analysis of water sample

i) Pipette 20 mL of the sample of water into a 100 mL conical flask and add2 drops of phenolphthalein indicator and titrate against HCl till the color of the solution changes from pink to colorless. Corresponding burette reading indicates the phenolphthalein end point (V1).

ii) Again pipette out 20 mL of the water sample in a conical flask, add 2 drops of methyl orange indicator. Color of the solution becomes yellow. Continue the titration against the HCl solution till the color changes to red. This burette reading corresponds to the methyl orange end point (V2).

Analysis of water using phenolphthalein indicator

Strength of HCl solution = ____ (N)

Strength of water=$(N_{HCl}*V_{HCl})/20$

Strength in terms of $CaCO_3$ equivalent=Strength of water * equivalent weight of $CaCO_3$

Phenolphthalein alkalinity(P) =A g/L =A*1000mg/L

Slno	Amount of water taken	Volume of HCl rundown	Mean volume of HCl
1			
2			

Analysis of water using Methyl orange indicator

Strength of HCl solution = ____ (N)

Strength of water=(N_{HCl}*V_{HCl})/20

Strength in terms of $CaCO_3$ equivalent=Strength of water * equivalent weight of $CaCO_3$

Methyl orange alkalinity(M) =B g/L =B*1000mg/L

Slno	Amount of water taken	Volume of HCl rundown	Mean volume of HCl
1			
2			

CONCLUSION:

If P > ½M Both OH^- and CO_3^{2-} ions are present in the water sample.

If P=0 Both OH^- and CO_3^{2-} ions are not present in the water sample

Alkalinity is present due to HCO_3^- ion only which can be determined using methyl orange indicator and called methyl orange alkalinity (M).

If P = ½ M; indicates that only CO3 2– ions are present.

If P < ½ M; indicates that beside CO_3^{2-} ions HCO_3^- ions are also present.

If P = M; indicates only OH^- ions are present.

Experiment 2

Identification of enzymes in different sources

Aim: To identify the enzymes present in different solutions

Principle: Different enzymes will identify using following reactions

Starch $\xrightarrow{Amylase}$ Simple sugars

Sucrose $\xrightarrow{Invertase}$ Glucose +fructose

H_2O_2 $\xrightarrow{Catalase}$ water +oxygen

Sources: Dry leaves, fresh leaves, raw potatoes, Boiled potatoes, Sprouted seeds, apple, banana and yeast.

Apparatus required: Glass wares, Pestle and Mortar, Water bath, Centrifuge, Testubes, Beaker.

Reagents Required: 2% solution of Glucose, Maltose , Starch and Sucrose, Benedicts reagent and Iodine solution

Control preparation

- Take 3 test tubes
- Add 2.5ml of 2% solution of starch to 1 test tube maltose to the second and glucose to third test tube
- Add 1 ml of 2% iodine solution to each test tube.
- Add 1 ml of Benedicts reagent to all test tubes and vertex
- Use this as reference for the color change in the sample.

Procedure

Sample preparation for Amylase and Catalase Assay

- Take 5 g of each sample and homogenize using distilled water in Pestle and Mortar
- Transfer homogenate to centrifuge tubes and centrifuge for 3 min.
- Collect the supernatant in test tubes

- Mark the test tubes accordingly to the sources.

Amylase assay

- Take 2.5 ml of different samples in different test tubes.
- Add 2.5ml of 2% solution of starch,1 ml of 2% iodine solution and 1 ml of Benedicts reagent to all test tubes and vertex
- Place the test tubes in boiling water bath for few minutes.
- Take out the test tubes from water bath and compare the color with control.

Invertase assay

- Take 2.5 ml of different samples in different test tubes.
- Add 2.5ml of 2% solution of sucrose and add 1 ml of Benedicts reagent to all test tubes and vertex.
- Place the test tubes in boiling water bath for few minutes.
- Take out the test tubes from water bath and compare the color with control.

Catalase assay

- Take 5 ml of H_2O_2 in different test tubes
- Crush different samples and add it to the different test tubes
- Observe the test tubes for effervescence

Observation table

Source	**Amylase**	**Invertase**	**Catalase**

Experiment 3

Isolation of œ Amylase from different sources

Aim: To isolate œ Amylase from different sources

Sources: Sweet Potato and Potato

Reagents required: 10mM $CaCl_2$ solution

Apparatus required: Beaker, Pestle & Mortar, Measuring cylinder, Centrifuge and its tubes

Procedure:

1. Take 2 g of given source and homogenize using 10ml 10mM $CaCl_2$ solution.
2. Incubate the homogenate for 24 h at 4^o C
3. Centrifuge the homogenate at 10000 RPM for 20 min.
4. Collect the supernatant and discard the pellets.
5. Measure the volume of supernatant obtained.

Result: the volume of crude enzyme collected from the source is ……

Experiment 4

Determination of œ Amylase Enzyme activity

Aim: To determine the enzyme activity of œ Amylase

Principle: The reducing sugars produced by the action of œ Amylase react with dinitrosalcylic acid giving a brown colored product.

Materials and reagents:

1. **0.1 M Sodium acetate buffer: pH 4.7:** Dissolve 3.4g of sodium acetate in 200ml of distilled water and adjust the pH to 4.7 by adding glacial acetic acid and make the volume up to 250ml.
2. **1% starch solution:** Dissolve 1 g of starch in 100ml of distilled water and heat up to 50^{o} C to get clear solution.
3. **Dinitrosalicylic acid:** Solution A: Dissolve 1 g of DNS in 20ml of 2N NaOH solution.

   Solution B: Dissolve 30g of sodium Potassium tartarate in60mi of distilled water.

   Mix solutions A and B and heat up to 80-90^{o} C.The resulting solution is clear DNS
4. **Standard glucose solution:** Stock solution :1g/100ml, working solution0.4,0.8,1.2,1.6,2 mg/ml.
5. **Enzyme dilution:** Dilute crude enzyme in the ratio 1:25

Procedure:

1. Take 9 test tubes.
2. Pipette standard glucose solutions to test tubes and make the volume 2 ml by adding distilled water and name the test tubes from 1-6.
3. Add 1 ml of starch solutions to 7, 8 and 9 test tubes respectively.

4. Add 1, 0.5 and 0 ml Sodium acetate buffer to 7,8 and 9 test tubes respectively.
5. Add 0, 0.5 and 1ml of diluted enzyme to 7,8 and 9 test tubes respectively.
6. Vertex the test tubes and incubate at 37^{o} for 15 min.
7. Arrest the enzyme substrate reaction by adding 1ml of DNS solutions to all test tubes.
8. Boil the test tubes in water bath for 5 min.
9. Add 5 ml of distilled water to all test tubes and vertex
10. Check the OD of solutions for all testubes.

Observation and Calculation

Standard Glucose curve

Sl no	Volume of glucose (ml)	Concentration(µg)	Volume of water (ml)	Volume of DNS	Incubation in hot water bath 5 min	Volume of distilled water	OD at 540nm
1	0	0	2	1ml		5ml	
2	0.4	400	1.6				
3	0.8	800	1.2				
4	1.2	1200	0.8				
5	1.6	1600	0.4				
6	2	2000	0				

Enzyme activity

Test tubes	Vol of substrate ml	Vol of buffer ml	Vol of enzyme ml	Incubate for 15 min at room temperature	Volume of DNS ml	Incubation in hot water bath 5 min	Vol of distilled water	OD at 540nm
7	1	1	0		1ml		5	
8	1	0.5	0.5				5	
9	1	0	1				5	

Calculation:

Enzyme activity =……..µg/15min/ml of diluted enzyme

=……..µg/min/ml of diluted enzyme

Enzyme activity for undiluted enzyme=…….*25 =µg/min/ml of undiluted enzyme

Total volume of enzyme extract =……..

Total enzyme activity for ……x…….ml of enzyme taken from …y…….g of source= ……*x/y

Total œ Amylase Enzyme activity in y g of source= ……./1000 = …….mg/min/ml

Result : Total œ Amylase Enzyme activity=…….mg/min/ml

Experiment 5

Specific activity of œ Amylase

Aim: To determine Specific activity of œ Amylase from different source

Principle: Specific activity is calculated by determining amount of proteins present in I mg in 1 ml of enzyme source and dividing it by the enzyme activity.

Materials and Reagents: Lowry's reagent, Folin's reagent, BSA standard solution

Procedure

1. Take 7 test tubes.
2. Pipette 0,0.2,0.4,0.6,0.8 and 1ml of working BSA solution to 6 test tubes and number it from 1-6.
3. Make the volume as 1 ml in each test tube by adding water
4. Add 1ml of diluted enzyme to 7 th test tube.
5. Add 5 ml of Lowry's reagent to all test tubes.
6. Incubate the test tubes at room temperature for 15 min.
7. After incubation add 0.5 ml of FC reagent to all test tubes
8. Keep the test tubes in dark at room temperature for 30 minutes.
9. Measure the OD and calculate the concentration of protein in 1ml of enzyme.

Tabular column

Sl no	Volume of BSA ml	Volume of water ml	Concentration of protein in µg	Lowry's reagent	Incubation at room temperature for 15min	FC reagent	Incubation at room temperature for 15min	OD at 660nm
1				5ml		0.5ml		
2								
3								
4								
5								
6								
7								

Calculations:

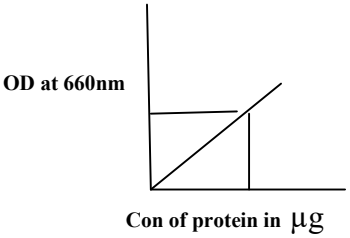
Enzyme activity of œ Amylase =…….mmole/min

1ml of 1:25ml diluted enzyme consists of…… µg of protein

1ml of undiluted enzyme has ……..mg of protein

Specific activity=enzyme activity/mg of proteins= ……..µmol/min/mg

Nature of graph:



Result: Specific activity of enzyme is …….

Experiment 6

Determination of K_m and V_{max} of œ Amylase

Aim: to determine K_m and V_{max} of œ Amylase

Reagents required :Citrate buffer (pH 5.3),Enzyme extract, Starch solution, DNS reagent.

Procedure:

1. Clean and dry 10 testubes
2. Mark test tubes as C_1 T_1 to C_{10} T_{10} depending on substrate concentration.(c- control without enzyme).
3. Add o.5 ml of diluted (1:5) enzyme to test tubes marked as T
4. Add substrate (in the range of 0.1-1) to different test tubes
5. Add buffer to make the volume as 2ml
6. Vertex the test tubes and incubate at room temperature for 15 min
7. Add 1ml of DNS and keep it in boiling water bath for 5 min.
8. Cool all test tubes and add 4 ml of distilled water to all test tubes.
9. Vertex the contents in test tube and read the absorbance at 540nm.
10. Calculate the activity for each test tube.
11. Plot a graph and determine the constants by using Michalis-Menton plot and Line-weaver Burk plot.

Tabular column:

Sl.no	Test tube	Vol of enzyme (ml)	Vol of substrate (ml)	Vol of buffer(ml)	Incubate at room temperature for 15 min	Vol of DNS	Keep it in boiling water bath for 5 min .Add 4 ml of distilled water after 5 min	OD at 540nm	Activity	1/ V	[S]	1/[S]
1	C1 T1	- 0.5	0.1 0.1	1.9 1.4								
2	C1 T1	- 0.5	0.2 0.2	1.8 1.3								
3	C1 T1	- 0.5	0.3 0.3	1.7 1.2								
4	C1 T1	- 0.5	0.4 0.4	1.6 1.1								
5	C1 T1	- 0.5	0.5 0.5	1.5 1.0								
6	C1 T1	- 0.5	0.6 0.6	1.4 0.9								
7	C1 T1	- 0.5	0.7 0.7	1.3 0.8								
8	C1 T1	- 0.5	0.8 0.8	1.2 0.7								
9	C1 T1	- 0.5	0.9 0.9	1.1 0.6								
10	C1 T1	- 0.5	1 1	1 0.5								

Experiment 7

Mixed Flow Reactor

Aim: To find out order and rate constant of the reaction between no-equimolar quantities of NaOH and ethyl acetate in a mixed flow reactor.

Principle:
Continuous stirred tank reactors (CSTR) or mixed flow reactor (MFR) is configured much like a batch reactor except reactants and products continuously from in and out of the reactor. Steady state flow reactors are ideal for industrial purpose when large quantities of material are to be processed and when the rate of reaction is fairly high to extremely high. Flow reactors need more supporting equipments like pump. But good product quality can be obtained space item (τ) is the time required to process the reactor volume of feed at specified column.

$$\tau = \frac{volume\ of\ reactor}{volumetric\ flow\ rate\ of\ feed} = \frac{V}{VF}$$

Theory:
The SCTR also known as backmix reactor is a common ideal reactor type in chemical engineering. Perfect mixing is assumed in care of ideal CSTR. In a perfectly mixed reactors, the outfeed composition material inside the reactor which is a function is residence time and rate of reaction. The ideal CSTR model is used to simplify engineering claculations and can be used to describe research reactors. In practise, it only to be approached in particular in in industrial size reactors. Design equation for mixed flow reactor in steady state is

$$\tau = \frac{CA0}{-rA} = \frac{V}{FA0}$$

Plot, $\ln\frac{\mu - XA}{\mu(1-XA)}$ v/s τ

If the points fits well into a straight line then the assumption of second order, kinetics is true. The rate constant may be calculated from the slope of the line.

Apparatus:
Setup the vessels as shown in the figure with necessary connections, conical flask, burette, measuring cylinder.

Reagents required:
0.04N NaOH, 0.04N ethyl acetate, 0.1N HCl, 0.04N oxalic acid.

Procedure:
- Volume of the MFR was noted down.
- The volumetric flow rate of NaOH and ethyl acetate was adjusted.
- The space time was calculated using the formula $\tau = \frac{VR}{VT}$ V_R – volume reactor; V_T –volumetric flow.

- The reactants were allowed to flow through the reactor for τ minutes.
- The rection was arrested by adding 0.1N HCl.
- It was titrated against NaOH by adding 2-3 drops of phenolphthalein indicator till the color changes from colourless to pale pink.
- The above procedure was repeated for different reactant flow rates.
- The order and rate constant was determined by plotting a graph of $\ln\frac{(\mu - XA)}{\mu(1-XA)}$ v/s τ.

Result:
Rate constant for the given reaction is k = _____ $(mol/L)^{-1}$ min^{-1}

Experiment 8

Determination of Rate Constant and Order for Heating of Water

Aim: To find order and rate constant for heating of water.

Principle:
If the ration follows

- Zero order:

Then $T-T_0 = kt$
The graph of $T-T_0$ v/s time should pass through the origin with slope k or if it is

- First order:

Then $\ln T/T_0 = kt$
The graph of $\ln(T/T_0)$ v/s time should pass through the origin with slope k, and if it is

- Second order:

$\frac{1}{T0} - \frac{1}{T} = kt$

The graph of $\frac{1}{T0} - \frac{1}{T}$ v/s time pass through origin with slope.

Theory:
Changing the concentration of substances taking part in a reaction usually changes the rate of the reaction.
Order: It the power to which concentration is raised.
Zero order reaction:
A zero order reaction has a constant rate that is independent of the reactants concentration. The rate law is as follows
rate = k
Where k has unit of ms^{-1}. In other words, a zero order reaction has a rate law in which the sum of the exponents is equal to zero. A reaction is zero order if concentration data are plotted versus time and the result is a straight line.

First order reaction:
A first order reaction has a rate proportional to the concentration of one reactant.
rate = kC_a^1
First order rate constants have units of sec^{-1}. In other words, a first order reaction has a rate law in which the sum of the exponent is equal to 1.

Second order reaction:
A second order reaction has a rate proportional to the product of the concentration of a reactants, or to the square of the concentration of a single reactant.
rate = kc_a^2

Apparatus:
Heating mantle, stop watch, beaker.

Procedure:

- 1000 mL of water was collected in a beker.
- The initial temperature of water was noted.
- The beaker was then placed on a heating mantle (Bunsen burner).
- The temperature raise was noted for every 1 minute.
- The order of reaction and rate constant was checked for zero order, first order and second order.
- Based on the correct and straight line fir from the graph order was found.

Result:

From the graph it is clear that the reaction is zero order and rate constant is _____ mol/L/sec.

Experiment 9

Determination of Kinetics for Esterification

Aim: To find the order and rate constant for non-equimolar concentration reaction.

Principle: A batch reactor has neither inflow nor outflow of reactants or products while the reaction is being carried out. This is an unsteady state operation where composition changes with time; however, at any instant the composition throughout the reactor is uniform. As the concentration of the reactant species in the reactor decreases, conversion increases.

Theory: The reaction of ester hydrolysis is chosen for this experiment for particular reasons. First, it takes place with rather low rates so that changes can be observed on time scale of minutes. Second, it requires an acid as catalyst. The amount of products can thus be followed as a function of time by extracting small amounts from the reacting solution at certain time intervals. Third, the product itself is an acid. So that its concentration can be easily and precisely determined by titration.

The batch reactor is the generic term for a type of vessel widely used in the process industries. In a batch reactor, the reactants and the catalyst are placed in the reactor and the reaction is allowed to proceed for a given time where upon the mixture of unreacted material together with the product is withdrawn. In an ideal batch reactor, the concentration and temperature are assumed to be spatially uniform. In practise, the condition can be approximately reduced by vigorous agitation or stiring.

Order and the rate constant of the reaction can be obtained by experiments. Mainly two types of analysis may be used for rate law determination. a) Integral Method of Analysis. b) Differential Method of Analysis.

Plot:

$$\ln \frac{\mu - X_a}{\mu(1 - X_a)} = C_{ao}(\mu - 1)kt$$

Where t = time

$$\mu = \frac{C_{bo}}{C_{ao}}$$

x_a is conversion of a at given time. If the data points fit well into a straight line then the assumption of second order kinetics is true. The rate constant can be calculated from the slope of the line $kC_{ao}(\mu - 1)$.

Apparatus:
Conical flask, beakers, burette, burette stand, measuring cylinder, reactor, magnetic pellet and magnetic stirrer.

Reagents required:

- 0.08N 1 L sodium hydroxide solution.
- 0.05N 1 L ethyl acetate solution.
- 0.1N 250 mL hydrochloric acid.
- 0.1n 100 mL oxalic acid.

Procedure:

- Batch reactor of capacity 1 L was taken and 400 mL of prepared NaOH solution was added.
- The stirrer was switched on and 400 mL of ethyl acetate was added immediately after addition, the stop watch was started.
- 10 mL of the reaction mixture was withdrawn from the reactor after 5 mins. This was done after every 5 mins intervals simultaneously, the reaction was arrested by adding 10 mL of 0.1N HCl each time.
- The excess HCl in this mixture was titrated against standardized NaOH solution using phenolphthalein as indicator and the volume of NaOH comnsumed was noted.
- The above procedure was repeated for all the reaction mixtures withdrawn at different time intervals from the reactors.
- From the experimental data, C_a value was found and hence X_a value was calculated.
- A graph of $\ln \frac{\mu - Xa}{\mu(1-Xa)}$ v/s time was plotted and checked for a straight lune fit.
- The rate constant was determined from the slope of the graph.

Result:
Rate constant for given reaction k = _____ $(mol/L)^{-1}$ min^{-1}

Observation:

Time	Volume of NaOH

Time	$\ln(\frac{\mu - xa}{\mu(1-xa)})$