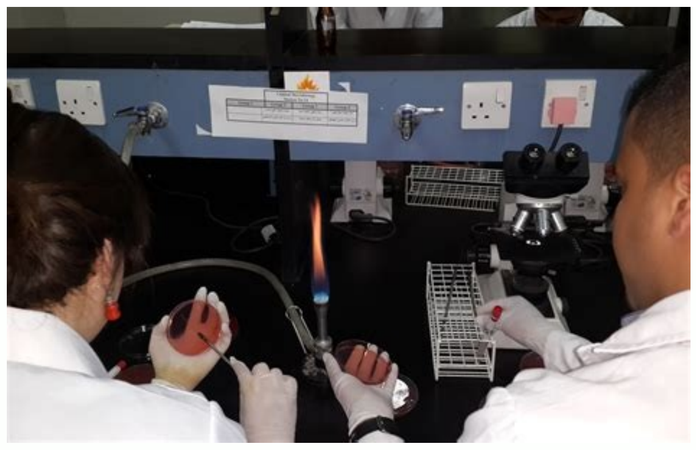
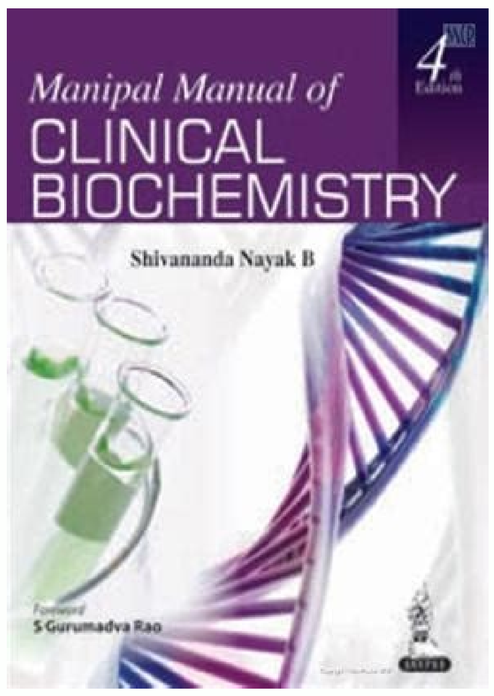
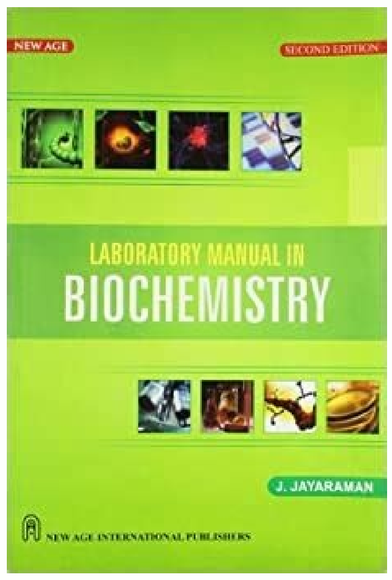
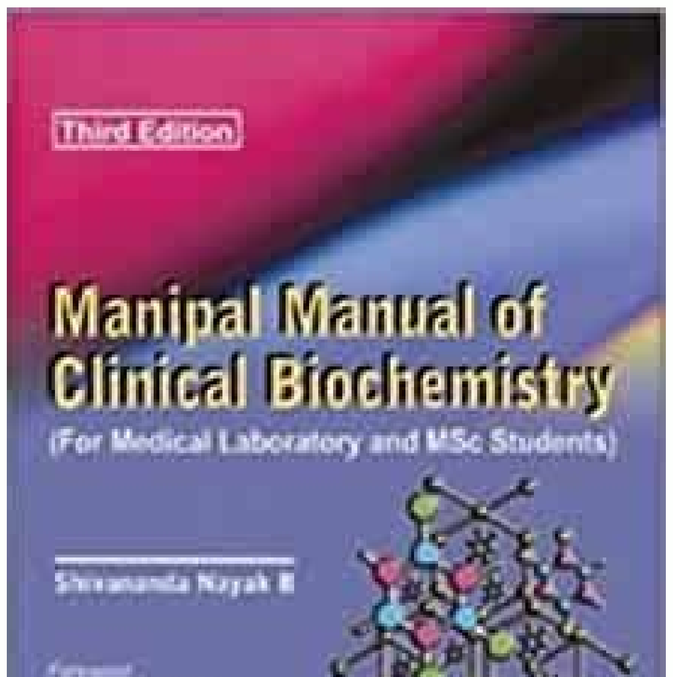
Clinical biochemistry laboratory manual









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Flipkart Internet Private Limited, Buildings Alyssa, Begonia & Clove Embassy Tech Village, Outer Ring Road, Devarabeesanahalli Village, Bengaluru, 560103, Karnataka, India CIN : U51109KA2012PTC066107 Telephone: 044-45614700 For Writers by Javin Bishnu Gogoi This simplified practical manual focuses on biochemistry. In medical sciences, biochemistry serves as the Nucleus of Modern Medicine, and plays the role of being the foundation to all other subjects related to its fraternity. The biochemical parameters that it conducts help to understand, diagnose, provide treatment, prognosis and also help in the monitoring of diseases in the modern practice of medicine. This practical manual consists of 33 chapters, which include basic instruments used in a biochemistry laboratory, types of reagents or solutions, sample collection, use of preservatives and anticoagulants, tests for detection of carbohydrates, color reactions of proteins, precipitation reactions of proteins, qualitative analysis of lipids, qualitative reactions of urea, normal urine analysis, automation and quality control, analysis of cerebrospinal fluid, estimation of serum creatinine, urinary creatinine and creatinine clearance test, estimation of uric acid, estimation of transaminases, and estimation of serum alkaline phosphatase. The contents of the book are based on the MCI syllabus for the MBBS course. This manual book is useful for the MBBS students to understand the practical importance of biochemistry. Select Section SECTION I INTRODUCTION SECTION II QUALITATIVE ANALYSIS SECTION III DEMONSTRATION SECTION IV QUANTITATIVE ANALYSIS SECTION V REFERENCE VALUES [Please select above dropdown to change Section] Chapters of the selected section will be displayed in the chapter section below. ADD TO FAVOURITES 1. 1/1/2018 BIOCHEMISTRY MANUAL For B.Sc. and M.Sc. students of Biological sciences Sardar Hussain & Komal K.P. [COMPANY NAME] 2. Biotechnology practical 2-Biochemistry 2016-17 SH 1 contents SL No. Experiment Page no. 1 Preparation of Molar, Normal Solutions and Buffers 1-6 2 Determination of pH and use of pH meter 7-8 3 Qualitative analysis of carbohydrates 9-20 4 Estimation of glucose by DNS method. 21-22 5 Estimation of protein by Biuret method. 23-24 6 Construction of maltose calibration curve 25-26 7 Determination of activity of human salivary α-amylase 25-26 8 Effect of temperature on activity of human salivary α-amylase 29-30 9 Effect of pH on the activity of human salivary α-amylase 31-32 10 Determination of Km value of human salivary α-amylase 33-34 11 Separation of amino acids by thin layer chromatography. 35-37 12 Circular Paper Chromatography 38-39 13 Ascending Paper Chromatography 40-41 14 Separation of plant pigment by paper chromatography 42-43 3. Biotechnology practical 2-Biochemistry 2016-17 SH 2 Expt 1. Preparation of Molar, Normal Solutions and buffers 1. Molar solutions Molarity (M) means the number of moles of solute per liter of solution. To prepare a 1 M solution, slowly add 1 gram formula weight of compound to a clean 1-L volumetric flask half filled with distilled or deionized water. Allow the compound to dissolve completely, swirling the flask gently if necessary. Once the solute is completely dissolved and the solution is at room temperature, dilute to the mark with water. Invert the flask several times to mix. To make a 1 M solution of sodium hydroxide, slowly add 40 g sodium hydroxide to 500 mL distilled or deionized water in a 1000mL volumetric flask. When all the solid is dissolved and the solution is at room temperature, dilute to the mark and invert the flask several times to mix. To make a 1 M solution acetic acid, dissolve 60.05 g acetic acid in 500 mL distilled or deionized water in a 1000-mL volumetric flask. Because acetic acid is a liquid, the acid may also be measured by volume. Divide the mass of acid by its density (1.049 g/mL) to determine the volume (57.24 mL). Use either 60.05 g or 57.24 mL acetic acid to make the solution. Swirl the flask gently to mix the solution. When the solution is at room temperature, dilute to the mark and invert the flask several times to mix. 2. Percent solutions: a. Mass percent means the number of grams of solute per 100 g of solution. For example, 10 g sodium chloride in 90 g water is a 10% by mass solution. Mass percent = mass of solute/mass of solution = 10 g/ (10 g + 90 g) × 100% = 10% b. Volume percent means the number of milliliters of solute per 100mL of solution. The volume percent of a solution cannot be calculated directly from the volumes of its components because the final volume may not equal the sum of the components' volumes. To prepare volume percent solutions, first determine the final volume and concentration of solution desired and then determine the amount of solute. Dilute the solute in sufficient solvent to produce the final volume of solution desired. For example, to prepare 100 mL of a 10% by volume solution of acetic acid, dilute 10 mL acetic acid with distilled or deionized water to make 100 mL of solution. Note: Solutions of concentrated reagents, such as 37% hydrochloric and 85% phosphoric acids, are percent solutions by mass. In general, percent solutions are by mass. 3. Normal solutions: Normality (N) means the number of equivalents of solute per liter of solution. An equivalent is defined separately for acid-base and reduction oxidation (redox) chemistry. In acid-base chemistry, an equivalent is the mass of chemical that donates or accepts one mole of protons. For example, sulfuric acid is a diprotic acid. One-half mole of sulfuric acid therefore provides one mole of protons. To prepare a 1 N solution of sulfuric acid, slowly add one-half gram formula weight of sulfuric acid to a clean 1-L volumetric flask and fill to the mark with distilled or deionized water. In 4. Biotechnology practical 2-Biochemistry 2016-17 SH 3 redox chemistry, an equivalent is the mass of chemical that donates or accepts one mole of electrons. Determine the number of electrons a chemical donates or accepts from its half-reaction. Normal solution of HCL Relative molecular mass of HCL is 36.5. In 36.5 g of HCL, there is 1gm of replaceable hydrogen. Therefore the equivalent of HCL is 36.5. Normal solution of H2SO4 The relative mass of Sulphuric acid is 98. In 98 gm of H2SO4, there is 2 gm replaceable hydrogen. In 49 Gms of H2SO4, there is 1gm of replaceable hydrogen. Therefore the equivalent of H2SO4 is 49. Since equivalents of compounds react with one another, the figures obtained above can be used to obtain the equivalents of bases and salts. This is done by working from the equation for the reaction between the compound needed and one whose equivalent is known. How to prepare a normal solution? After calculating the equivalent of the compound, the next step is to determine how many gms of the compound is required for making a given volume of solution of known normality. 4 N NaOH Solution Required quantity of 4N NaOH 5 Litres (Equivalent is 40) Therefore 1 litre of IN NaOH contains 40gm NaOH. Therefore 1 litre of 4N NaOH contains 4 x 40 gm NaOH = 160 gm NaOH. Therefore 5 litres of 4N NaOH contains 5 x 160gm = 800 gm NaOH. So 5 Litres of 4N NaOH solution can be prepared by dissolving 800 Gms NaOH in 5 litres of distilled water. Practice Problems 1. Molar solutions a. Describe how you would prepare 1 L of a 1 M solution of sodium chloride? The gram formula weight of sodium chloride is 58.44 g/mol. Answer: To make a 1 M solution sodium chloride, dissolve 58.44 g sodium chloride in 500 mL water in

a 1000-mL volumetric flask. When all the solid is dissolved and the solution is at room temperature, dilute to the mark and then invert the flask several times to mix. b. Describe how you would prepare 1 L of a 2 M solution of acetic acid? The gram formula weight of acetic acid is 60.05 g/mol, and its density is 1.049 g/mL. Answer: To make a 2 M solution of acetic acid, dissolve 120.1 g acetic acid in 500 mL distilled or deionized water in a 1000-mL volumetric flask. Since acetic acid is a liquid, the acid may also be measured by volume. Divide the mass of acid by its density (1.049 g/mL) to determine the volume (114 mL). Use either 120.1 g or 114 mL acetic acid to make the solution. Swirl the flask gently to mix the solution. Once the solution is at room temperature, dilute to the mark and then invert the flask several times to mix. 5. Biotechnology practical 2-Biochemistry 2016-17 SH 4 2. Percent solutions a. Describe how you would prepare 100 g of a solution that is 0.5% phenolphthalein by mass? Answer: Mass percent means the number of grams of solute per 100 g of solution. Mass percent = (mass of solute/mass of solution) × 100% mass of solute = mass percent × mass of solution/100% = 0.5% × 100 g/100% = 0.5 g Since the total mass of the solution equals 100 g, the remaining 99.5 g of the solution is water. To prepare the solution, dissolve 0.5 g phenolphthalein in 99.5 g distilled or deionized water. b. Describe how you would prepare 100 mL of a solution that is 22% acetic acid by volume. Answer: Volume percent means the number of milliliters of solute per 100 mL of solution. Dilute 22 mL acetic acid with distilled or deionized water to make 100 mL of solution. 3. Dilutions a. Describe how you would prepare 1.0 L of a 0.10 M solution of sulfuric acid from a 3.0 M solution of sulfuric acid? Answer: Calculate the volume of 3.0 M sulfuric acid needed to prepare the dilution. Mreagent × Vreagent = Mdilution × Vdilution 3.0 M × Vreagent = 0.10 M × 1.0 L Vreagent = 0.033 L = 33 mL Slowly add 33 mL of 3.0 M sulfuric acid to a 1000-mL volumetric flask half filled with distilled or deionized water and swirl the flask to mix. Once the solution is at room temperature, dilute to the mark with water and then invert the flask several times to mix. b. Describe how you would prepare 500 mL of a 0.25 M solution of sodium hydroxide from a 5.0 M solution of sodium hydroxide? Answer: Calculate the volume of 5.0 M sodium hydroxide needed to prepare the dilution. Mreagent × Vreagent = Mdilution × Vdilution 5.0 M × Vreagent = 0.25 M × 0.500 L Vreagent = 0.025 L = 25 mL Slowly add 25 mL of 5.0 M sodium hydroxide to a 500-mL volumetric flask half-filled with distilled or deionized water and swirl the flask to mix. Once the solution is at room temperature, dilute to the mark with water and then invert the flask several times to mix. 4. Special cases a. Describe how you would prepare 500 mL of a 1.0 M solution of potassium chloride that is 93.0% pure? The gram formula weight of potassium chloride is 74.56 g/mol. Answer: Calculate the mass of impure potassium chloride needed. Mass of impure potassium chloride = M pure × V pure × gram formula weight/percent purity = 1.0 M × 0.500 L × 74.56 g/mol 0.930 = 40 g Slowly add 40 g of 93% potassium chloride to a 500-mL volumetric flask half filled with distilled or deionized water and swirl the flask to mix. When all the solid is dissolved and the solution is at room 6. Biotechnology practical 2-Biochemistry 2016-17 SH 5 temperature, dilute to the mark and invert the flask several times to mix. b. Describe how you would prepare 500 mL of a 1.0 M solution of phosphoric acid from phosphoric acid that is 85.0% pure. The gram formula weight of phosphoric acid is 98.00 g/mol, and the density of 85.0% phosphoric acid is 1.685 g/mL. Answer: Calculate the volume of 85.0% phosphoric acid needed. Volume of impure = M pure × V pure × gram formula weight/ phosphoric acid (percent purity × density) = (1.0 M × 0.500 L × 98.00 g/mol) 0.850 × 1.685 g/mL = 34 mL Slowly add 34 mL of 85.0% phosphoric acid to a 500-mL volumetric flask half filled with distilled or deionized water and swirl the flask to mix. Once the solution is at room temperature, dilute to the mark with water and invert the flask several times to mix. Preparation of buffers A buffer solution is an aqueous solution consisting of a mixture of a weak acid and its conjugate base, or vice versa. Its pH changes very little when a small or moderate amount of strong acid or base is added to it and thus it is used to prevent changes in the pH of a solution. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications. Many life forms thrive only in a relatively small pH range so they utilize a buffer solution to maintain a constant pH. In nature, the bicarbonate buffering system is used to regulate the pH of blood. Applications Buffer solutions are necessary to keep the correct pH for enzymes in many organisms to work. Many enzymes work only under very precise conditions; if the pH moves outside of a narrow range, the enzymes slow or stop working and can denature. In many cases denaturation can permanently disable their catalytic activity. A buffer of carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) is present in blood plasma, to maintain a pH between 7.35 and 7.45. Industrially, buffer solutions are used in fermentation processes and in setting the correct conditions for dyes used in coloring fabrics. They are also used in chemical analysis and calibration of pH meters. The majority of biological samples that are used in research are made in buffers, especially phosphate buffered saline (PBS) at pH 7.4. Simple buffering agents Buffering agent pKa Useful pH range Citric acid 3.13, 4.76, 6.40 2.1–7.4 Acetic acid 4.8 3.8–5.8 KH_2PO_4 7.2 6.2–8.2 CHES 9.3 8.3–10.3 Borate 9.24 8.25–10.25 7. Biotechnology practical 2-Biochemistry 2016-17 SH 6 Citrate Buffer Stock Solutions A: 0.1 M solution of citric acid (21.01 g in 1000 ml of distilled water) B: 0.1 M solution of sodium citrate (29.41 g of $C_6H_5O_7Na_3 \cdot 2H_2O$ in 1000 ml of distilled water) x ml of A + y ml of B, diluted to a total of 100 ml with distilled water: X Y pH X Y pH 46.5 3.5 3 23 27 4.13 43.7. 6.3 3.2 20.5 29.5 5 40.0 10 3.4 18 32 5.2 37 13 3.6 16 34 5.4 35 15 3.8 13.7 36.3 5.6 33 17 4 11.8 38.2 5.8 31.5 18.5 4.2 9.5 41.5 6 28 22 4.4 9.5 41.5 6.0 25.5 24.5 4.6 Phosphate Buffer Stock Solutions A: 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml of distilled water) B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4 \cdot 7H_2O$ or 71.7 g of $Na_2HPO_4 \cdot 12H_2O$ in 1000 ml of distilled water) x ml of A + y ml of B, diluted to a total of 200 ml with distilled water: X Y pH X Y pH 93.5 6.5 5.7 45 55 6.9 92 8 5.8 39 61 7.0 90 10 5.9 33 67 7.1 87.7 12.3 6.0 28 72 7.2 85 15 6.1 23 77 7.3 81.5 18.5 6.2 19 81 7.4 77.5 22.5 6.3 16 84 7.5 73.5 26.5 6.4 13 87 7.6 68.5 31.5 6.5 10.5 89.5 7.7 62.5 37.5 6.6 8.5 91.5 7.8 56.5 43.5 6.7 7 93 7.9 51 49 6.8 5.3 94.7 8.0 Results: ....pH citrate and ....pH phosphate buffer is prepared. 8. Biotechnology practical 2-Biochemistry 2016-17 SH 7 Practical 2. Determination of pH and use of pH meter Principles of operation of a pH meter A pH meter is essentially a voltmeter with high input impedance which measures the voltage of an electrode sensitive to the hydrogen ion concentration, relative to another electrode which exhibits a constant voltage. The key feature of the pH-sensitive electrode is a thin glass membrane who's outside surface contacts the solution to be tested. The inside surface of the glass membrane is exposed to a constant concentration of hydrogen ions (0.1 M HCl). Inside the glass electrode assembly, a silver wire, coated with silver chloride and immersed in the HCl solution, is called an Ag/AgCl electrode. This electrode carries current through the half-cell reaction. The potential between the electrode and the solution depends on the chloride ion concentration, but, since this is constant (0.1 M), the electrode potential is also constant. A reference electrode is needed to complete the electrical circuit. A common choice is to use another Ag/AgCl electrode as the reference. The Ag/AgCl electrode is immersed in a 0.1 M KCl solution which makes contact with the test solution through a porous fiber which allows a small flow of ions back and forth to conduct the current. The potential created at this junction between the KCl solution and the test solution is nearly zero and nearly unaffected by anything in the solution, including hydrogen ions. Using the pH Meter: Allow the meter a few minutes to stabilize after you plug it in. When you are not using the meter, keep the electrode immersed in pH 7.0 buffer to a depth of about one inch. The meter must be calibrated by using standards of known pH before an unknown is measured. Since the unknowns are acidic, the pH 4.00 and pH 7.00 standards should be used. An accurate pH reading depends on standardization, the degree of static charge, and the temperature of the solution. Glass electrode Reference electrode Combined electrode 9. Biotechnology practical 2-Biochemistry 2016-17 SH 8 The pH meter should be standardized each time it is used with a buffer of known pH, preferably one closest to the desired final pH. To calibrate the pH meter, expose the hole in the electrode, rinse the electrode with deionized water, and place the electrode in a standard solution, e.g., pH 7. Turn the selector to "pH". Adjust the pH meter to the appropriate pH. Rinse electrode with deionized water and place in a second standard buffer solution. The choice of the second standard depends on the final he standard pH buffers used should be 7 and 10. If the final pH desired, for example, if the final pH desired is 8.5, t pH desired is 5.5, the standard pH buffers used should be 4 and 7. Turn the selector to "pH". Adjust the temperature knob to the second standard pH. Rinse the electrode with deionized water, and return the electrode to the soaking solution. 1. When rinsing the electrode, never wipe the end, but blot gently since wiping can create a static electric charge, which can cause erroneous readings, 2. Make sure the solution you are measuring is at room temperature since the pH can change with a change in temperature. 3. The pH-sensitive glass membrane is very thin and very easily broken. Do not touch the membrane with anything harder than a Kim-Wipe and do that very gently. Do not drop the electrode or bump it on the bottom of the beaker when immersing it in a solution. 4. The glass membrane must be thoroughly hydrated to work properly. Do not allow the electrode to remain out of water any longer than necessary. When the electrode is not in use, keep it immersed in the pH 7.00 buffer. Do not put the electrode down on the desk. 10. Biotechnology practical 2-Biochemistry 2016-17 SH 9 Practical 3. Qualitative analysis of carbohydrates Introduction: Carbohydrates are polyhydroxy aldehydes and ketones or substances that hydrolyze to yield polyhydroxy aldehydes and ketones. Aldehydes (–CHO) and ketones (= CO) constitute the major groups in carbohydrates. Carbohydrates are mainly divided into monosaccharides, disaccharides and polysaccharides. The commonly occurring monosaccharides includes glucose, fructose, galactose, ribose, etc. The two monosaccharides combine together to form disaccharides which include sucrose, lactose and maltose. Starch and cellulose fall into the category of polysaccharides, which consist of many monosaccharide residues. Molisch's Test: This is a common test for all carbohydrates larger than tetroses. The test is on the basis that pentoses and hexoses are dehydrated by conc. Sulphuric acid to form furfural or hydroxymethylfurfural, respectively. These products condense with α-naphthol to form purple condensation product. Fehling's Test: This forms the reduction test of carbohydrates. Fehling's solution contains blue alkaline cupric hydroxide solution, heated with reducing sugars gets reduced to yellow or red cuprous oxide and is precipitated. Hence, formation of the yellow or brownish-red colored precipitate helps in the detection of reducing sugars in the test solution. 11. Biotechnology practical 2-Biochemistry 2016-17 SH 10 Benedict's test: As in Fehling's test, free aldehyde or keto group in the reducing sugars reduce cupric hydroxide in alkaline medium to red colored cuprous oxide. Depending on the concentration of sugars, yellow to green color is developed. All monosaccharides are reducing sugars as they all have a free reactive carbonyl group. Some disaccharides, like maltose, have exposed carbonyl groups and are also reducing sugars, but less reactive than monosaccharides Barfoed's Test: Barfoed's test is used to detect the presence of monosaccharide (reducing) sugars in solution. Barfoed's reagent, a mixture of ethanoic (acetic) acid and copper (II) acetate, is combined with the test solution and boiled. A red copper (II) oxide precipitate is formed will indicates the presence of reducing sugar. The reaction will be negative in the presence of disaccharide sugars because they are weaker reducing agents. This test is specific for monosaccharides. Due to the weakly acidic nature of Barfoed's reagent, it is reduced only by monosaccharides. Seliwanoff's Test: It is a color reaction specific for ketoses. When conc. HCl is added. Ketoses undergo dehydration to yield furfural derivatives more rapidly than aldoses. These derivatives form complexes with resorcinol to yield deep red color. The test reagent causes the dehydration of ketohexoses to form 5-hydroxymethylfurfural. 5-hydroxymethylfurfural reacts with resorcinol present in the test reagent to produce a red product within two minutes (reaction not shown). Aldohexoses reacts so more slowly to form the same product. Bial's Test: Bial's test is used to distinguish between pentoses and hexoses. They react with Bial's reagent and are converted to furfural. Orcinol and furfural condense in the presence of ferric ion to 12. Biotechnology practical 2-Biochemistry 2016-17 SH 11 form a colored product. Appearance of green color or precipitate indicates the presence of pentoses and formation of muddy brown precipitate shows the presence of hexoses. Iodine Test: This test is used for the detection of starch in the solution. The blue-black color is due to the formation of starch-iodine complex. Starch contain polymer of α-amylose and amylopectin which forms a complex with iodine to give the blue black color. Osazone Test: The ketoses and aldoses react with phenyl hydrazine to produce a phenylhydrazone which further reacts with another two molecules of phenyl hydrazine to yield osazone. Needle-shaped yellow osazone crystals are produced by glucose, fructose and mannose, whereas lactosazone produces mushroom shaped crystals. Crystals of different shapes will be shown by different osazones. Flower-shaped crystals are produced by maltose. Materials Required: 1) Glassware 2) Test tubes 3) Test tube holder 4) Water bath 5) Spatula 6) Dropper Reagents Required: 1) Molisch's Reagent 2) Iodine solution 3) Fehling's reagent A 4) Fehling's reagent B 5) Benedict's qualitative reagent 6) Barfoed's reagent 7) Seliwanoff 's reagent 8) Bial's reagent 9) Phenyl hydrazine hydrochloride 10) Sodium acetate 11) Glacial acetic acid 13. Biotechnology practical 2-Biochemistry 2016-17 SH 12 Overview No. Test Observation Inference Reaction 1 Molisch's Test 2-3 drops of alpha -naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test tube. A deep violet coloration is produced at the junction of two layers. Presence Of carbohydrates This is due to the formation of an unstable condensation product of beta-naphthol with furfural (produced by the dehydration of the carbohydrate). 2 Iodine test 4-5 drops of iodine solution are added to 1ml of the test solution and contents are mixed gently. Blue color is observed. Presence of polysaccharide . Iodine forms colored adsorption complexes with polysaccharides. 3 Fehling's test About 2 ml of sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min A red precipitate is formed Presence of reducing sugar This is due to the formation of cuprous oxide by the reducing action of the sugar. 4 Benedict's test To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool. Formation of a green, red, or yellow precipitate Presence of reducing sugars If the saccharide is a reducing sugar it will reduce Copper [Cu] (11) ions to Cu(1) oxide, a red precipitate 5 Barfoed's test To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool. A deep blue color is formed with a red ppt. settling down at the bottom or sides of the test tube. Presence of reducing sugars. If the saccharide is a reducing sugar it will reduce Cu (11) ions to Cu(1) oxide 6 Seliwanoff test To 3ml of Seliwanoff reagent, add 1ml of the test solution. Boil in water bath for 2 minutes. A cherry red colored precipitate within 5 minutes is obtained. A faint red color produced Presence of ketoses [Sucrose gives a positive ketohexose test] Presence of aldoses When reacted with Seliwanoff reagent, ketoses react within 2 minutes forming a cherry red condensation product Aldopentoses react slowly, forming the colored condensation product. 14. Biotechnology practical 2-Biochemistry 2016-17 SH 13 Result: the given unknown carbohydrate is................. 7 Bial's test Add 3ml of Bial's reagent to 0.2ml of the test solution. Heat the solution in a boiling water bath for 2 minutes. A blue-green product. A muddy brown to gray product Presence of pentoses. Presence of hexoses. The furfurals formed produces condensation products with specific color. 8 Osazone Test To two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope. Formation of beautiful yellow crystals of osazone Needle shaped crystals Hedgehog crystals Sunflower shaped crystals Glucose/fructo se Presence of lactose Presence of maltose Reducing sugars forms osazone on treating with phenyl hydrazine 15. Biotechnology practical 2-Biochemistry 2016-17 SH 14 Qualitative analysis of unknown carbohydrates 16. Biotechnology practical 2-Biochemistry 2016-17 SH 15 Unknown sample solution 1. Molisch's test Reddish violet ring 2. Iodine test Blue color No color (May be starch) (Mono or di saccharide) 3. Benedict's test/4.Fehling's test No reduction (may be sucrose) Red ppt. (may be reducing sugar) 5. Barfoed's test Brick red color (monosaccharide) No color 6. Selvinoffs test 7.osazone test No color Red color powder puff sunflower Glucose Fructose Lactose Maltose 17. Biotechnology practical 2-Biochemistry 2016-17 SH 16 SAMPLE: A SL NO TEST OBSERVATION INFERENCE 1 Molisch's Test 2-3 drops of alpha -naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test-tube. 2 Iodine test 4-5 drops of iodine solution are added to1ml of the test solution and contents are mixed gently. 3 Fehling's test About 2 ml of sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min 4 Benedict's test To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool. 5 Barfoed's test To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool. 6 Seliwanoff test To 3ml of Seliwanoff reagent, add 1ml of the test solution. Boil in water bath for 2 minutes. 7 Bial's test Add 3ml of Bial's reagent to 0.2ml of the test solution. Heat the solution in a boiling water bath for 2 minutes. 8 Osazone Test To two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope STRUCTURE: RESULT: The given sugar is identified as .... 18. Biotechnology practical 2-Biochemistry 2016-17 SH 17 SAMPLE: B SL NO TEST OBSERVATION INFERENCE 1 Molisch's Test 2-3 drops of alpha -naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test-tube. 2 Iodine test 4-5 drops of iodine solution are added to1ml of the test solution and contents are mixed gently. 3 Fehling's test About 2 ml of sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min 4 Benedict's test To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool. 5 Barfoed's test To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool. 6 Seliwanoff test To 3ml of Seliwanoff reagent, add 1ml of the test solution. Boil in water bath for 2 minutes. 7 Bial's test Add 3ml of Bial's reagent to 0.2ml of the test solution. Heat the solution in a boiling water bath for 2 minutes. 8 Osazone Test To two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope STRUCTURE: RESULT: The given sugar is identified as .... 19. Biotechnology practical 2-Biochemistry 2016-17 SH 18 SAMPLE: C SL NO TEST OBSERVATION INFERENCE 1 Molisch's Test 2-3 drops of alpha -naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test-tube. 2 Iodine test 4-5 drops of iodine solution are added to1ml of the test solution and contents are mixed gently. 3 Fehling's test About 2 ml of sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min 4 Benedict's test To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool. 5 Barfoed's test To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool. 6 Osazone Test To two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope STRUCTURE: RESULT: The given sugar is identified as .... 20. Biotechnology practical 2-Biochemistry 2016-17 SH 19 SAMPLE: D SL NO TEST OBSERVATION INFERENCE 1 Molisch's Test 2-3 drops of alpha -naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test-tube. 2 Iodine test 4-5 drops of iodine solution are added to1ml of the test solution and contents are mixed gently. 3 Fehling's test About 2 ml of sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min 4 Benedict's test To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool. 5 Barfoed's test To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool. 6 Osazone Test To two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope STRUCTURE: RESULT: The given sugar is identified as .... 21. Biotechnology practical 2-Biochemistry 2016-17 SH 20 SAMPLE: E SL NO TEST OBSERVATION INFERENCE 1 Molisch's Test 2-3 drops of alpha -naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test tube.. 2 Iodine test 4-5 drops of iodine solution are added to1ml of the test solution and contents are mixed gently. 3 Hydrolysis of sample To about 5ml of sample add Conc. HCl or Conc. H_2SO_4 about 8-10 drops, heat it. Allow to cool to room temperature gradually. With this sample perform Benedict's, Fehling's and Osazone test. 4 Fehling's test About 2 ml of hydrolyzed sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min. 5 Benedict's test To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool. 6. Seliwanoff test To 3ml of Seliwanoff reagent, add 1ml of the test solution. Boil in water bath for 2 minutes. 7 Osazone Test To two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope STRUCTURE: 22. Biotechnology practical 2-Biochemistry 2016-17 SH 21 RESULT: The given sugar is identified as .... Expt no 4: Estimation of glucose by DNS method. Aim: To estimate the given sugar sample by DNS method. Principle: several reagents have been employed which assay sugars by their reducing properties. One such compound is 3,5-Dinitrosalicyclic acid, which in presence of alkaline solution reduces to the 3- Amino-5- nitrosalicyclic acid. Reaction: Reagents required: 1) Dinitrosalicyclic acid (DNS) reagent: a) 30% sodium potassium tartarate: Dissolve 300gm of Rochelle's salt in 1000ml of water. b) 3,5-Dinitrosalicyclic acid: Dissolve10 gm of DNS in 200ml of 2N NaOH. Mix both the solutions and make up to 1000ml with dis. H_2O. 2) Standard glucose solution: 1mg/1ml 3) Working standard solution: 100µg/ml Take 10ml from the standard solution to another 100 ml volumetric flask and make up to the mark. Method: 1) Take the seven test tubes and mark as 1(blank),2,....7. 2) Pipette out the working standard glucose solution in the range of 0, 0.5,1.0,....3.0 (0-300 µg . the tube containing 0ml of sugar solution is used as blank. 3) Make up the volumes of all the test tubes to 3ml by adding dis. H_2O. 4) Add 1ml of DNS reagent to all the tubes. 5) Cover each test tube with marble and keep in the boiling water bath for 5min. 6) All the tubes cool at room temperature by keeping under running tap water or in a beaker containing tap water. 7) Adjust the colorimeter at 540nm to zero by keeping blank test tube. Then take the OD values of all tubes. 8) Plot a graph of concentration of glucose solution v/s OD at 540nm. Then estimate the concentration of the unknown. 23. Biotechnology practical 2-Biochemistry 2016-17 SH 22 Observation Sl.n o. Vol. Of standard Glucose solution Vol. Of dis. H_2O. In ml Conc. Of glucose solution in mg DNS reagent in ml Keep the test tubes in BWB For 5 min OD at 540 nm 1 0.0 3.0 00 1 2 0.5 2.5 50 1 3 1.0 2.0 100 1 4 1.5 1.5 150 1 5 2.0 1.0 200 1 6 2.5 0.5 250 1 7 3.0 0.0 300 1 8 UNKNOWN - 1 Report: The given unknown solution contains ....of sugar. Reference: 1) An introduction to practical Biochemistry----by D.T.Plummer 2) Standard method of Biochemical analysis ----by S.K.Thimmaiah 24. Biotechnology practical 2-Biochemistry 2016-17 SH 23 Expt 5: Estimation of protein by Biuret method. Aim: To estimate the amount of protein present in the given sample by biuret method. Principle: It is based on the fact that the –Co-NH- groups (peptide group) of protein form a purple complex with copper ions in an alkaline solution. The intensity of the purple complex is measured at 520 nm colorimetrically. This method is commonly used to estimate the protein in the range of 0.5 – 5 mg. Reaction: Reagents required: 1) Biuret reagent: Dissolve 3gm of $CuSO_4.5H_2O$ and 9 gm of sodium potassium tartarate in 500ml of 0.2 N NaOH solution. To this solution add 5 gm of potassium iodide (KI) and make upto 1 lit with 0.2 N NaOH 2) Standard protein solution: 5mg/1ml of Bovine serum albumin. Method: 1) Take the seven test tubes and mark as 1(blank),2,....7. 2) Pipette out the standard protein solution in the range of 0.2, 0.4, 0.6, 0.8 and 1.0 (0-5mg concentration) . The tube containing 0ml of protein solution is used as blank. 3) Make up the volumes of all the test tubes to 1ml by adding dis. H_2O. 4) Add 6ml of Biuret reagent to all the tubes. 5) Incubate in the room temperature for 10min. 6) Immediate after 10 min the OD values are taken in the colorimeter at 510nm to zero by keeping blank test tube. Then take the OD values of all tubes. 7) Plot a graph of concentration of protein solution v/s OD at 510nm. Then estimate the concentration of the unknown. Report: The given unknown solution contains ....of protein. Reference: 1) An introduction to practical Biochemistry----by D.T.Plummer 2) Standard method of Biochemical analysis ----by S.K.Thimmaiah 25. Biotechnology practical 2-Biochemistry 2016-17 SH 24 Observation and calculations: Sl.n o. Vol. Of standard protein solution Vol. Of dis. H_2O. In ml Conc. Of protein solution in mg Biuret reagent in ml Keep the test tubes in room temp . For 10 min OD at 510 nm 1 0.0 1.0 0 6 2 0.2 0.8 1 6 3 0.4 0.6 2 6 4 0.6 0.4 3 6 5 0.8 0.2 4 6 6 1.0 0.0 5 6 7 UNKNOWN - 6 Calculations: 1ml of sample contains........................ mg of protein Therefore 100ml contains= 100X ..../1 26. Biotechnology practical 2-Biochemistry 2016-17 SH 25 Expt 6: Construction of maltose calibration curve Aim: To construct the maltose calibration curve Principle: Maltose is a reducing disaccharide, which reduces 3, 5-dinitro salicylic acid in alkaline medium into 3-amino, 5-nitro salicylic acid, which is an orange red colored complex. The intensity of color depends upon the concentration of maltose and its optical density is measured at 540nm. 3,5-dinitrosalicylic acid 3-amino, 5-nitro salicylic acid Requirements: 3, 5-dinitrosalicylic acid: 1g of DNS was dissolved in 20ml of 2N NaOH, 30g of sodium potassium tartarate in 50ml of dist.H_2O. The 2 mixtures were mixed and the final volume was made upto 100ml with dist.H_2O. Standard maltose solution: 100mg of maltose dissolved in 100ml of dist.H_2O. Test tubes, pipettes, colorimeter, etc. Procedure: Standard maltose solution ranging from 0.0, 0.2, 0.4,........1.4ml was pipette out with the concentration range 0-1400µg into the eight different test tubes. The volume of all the tubes was made up to 2ml with dist.H_2O. 1ml of DNS reagent was added to each test tube and all the tubes were kept in boiling water bath for 5mins. Then these tubes were cooled at room temperature and 10ml of distilled H2Owas added to each test tubes. The optical density was read at 540m. Result: OH NO2 NO2 COOH OH NO2 COOH NH2 Maltose Alkaline condition 27. Biotechnology practical 2-Biochemistry 2016-17 SH 26 Observation and calculations: Sl.No Vol. of maltose (ml) Vol. of dist.H_2O (ml) Vol. of DNS (ml) Vol. of dist.H_2O (ml) Concentration of maltose (µg) OD at 540nm 1 0 2.0 1 Incubate all the tubes in boiling water bath for 10min 10 2 0.2 1.8 1 10 3 0.4 1.6 1 10 4 0.6 1.4 1 10 5 0.8 1.2 1 10 6 1.0 1.0 1 10 7 1.2 0.8 1 10 8 1.4 0.6 1 10 28. Biotechnology practical 2-Biochemistry 2016-17 SH 27 Expt 7: Determination of activity of human salivary α-amylase Aim: To determine the activity of human salivary α-amylase. Introduction: Although amylase is found in many tissues, it is most prominent in pancreatic juice and saliva which have their own isoform of human salivary α-amylase. They behave differently on isoelectric focusing and can also be separated in testing by using specific monoclonal antibodies. It is found in saliva and breaks starch into maltose and dextrin. This form of amylase is also called ptylin. It will break large, insoluble starch into soluble starch producing smaller starch and ultimately maltose. Principle: The enzyme activity is expressed in terms of International unit. It is defined as the amount of enzyme required to convert one micro mole of substrate into product per minute under optimum pH and temperature. α-amylase catalyses the hydrolysis of α-1 4 linkage of starch to produce maltose. Maltose reduces 3, 5-dinitro salicylic acid into 3-amino, 5-nitro salicylic acid, which is an orange red colored complex its optical density is measured at 540nm. Requirements: Sodium phosphate buffer: 0.2 molar (pH-6.9 to 7.2). Solution A: Sodium dihydrogen phosphate (0.2M) – monobasic Solution B: Disodium hydrogen phosphate (0.2M) – dibasic Substrate: 1% soluble starch DNS reagent Enzyme: Salivary α-amylase (crude): Collected saliva is filtered and centrifuged at 3000rpm for 20mins; supernatant is taken which serves as enzyme source. Working enzyme solution: Saliva which is centrifuged is diluted in 1:10 ration with phosphate buffer. 29. Biotechnology practical 2-Biochemistry 2016-17 SH 28 Procedure: Three test tubes were taken. 1.5 ml of buffer and 0.5ml of 1% starch were added to the first tube which served as blank. Other two tubes were added with 0.5ml of substrate, 0.5ml of enzyme and volume was made up to 2 ml with buffer. All the tubes were kept for incubation at room temperature for 5mins. 1ml of DNS reagent was added to all the tubes to stop the reaction. Then all the tubes were kept in boiling water bath for 10mins. Then 10ml of distilled water was added to all the tubes, after cooling optical density was read at 540nm. By using maltose calibration curve, the amount of maltose liberated was determined and enzyme activity was calculated by using the formula, Amount of maltose liberated × volume Activity = Molecular weight of maltose × incubation time Result: Observation and calculations: Sl. No Vol. of substr ate (ml) Vol. of enzym e (ml) Vol. of buffer (ml) Vol. of DNS (ml) Vol. of dist.H_2O (ml) OD at 540nm Activity µmol/ml /min 1 0.5 0 1.5 Incubat e all the tubes in room temp. for 5min 1 Incubat e all the tubes in boiling water bath for 10min 10 2 0.5 0.5 1 1 10 3 0.5 0.5 1 1 10 30. Biotechnology practical 2-Biochemistry 2016-17 SH 29 Expt 8: Effect of temperature on activity of human salivary α-amylase Aim: To determine the effect of temperature on the activity of human salivary α-amylase. Principle: α-amylase catalyses the hydrolysis of α-1 4 linkage of starch and produces the reducing sugar maltose. Maltose reduces 3, 5-dinitro salicylic acid into 3-amino, 5-nitro salicylic acid, which is an orange red colored complex. Its optical density is measured at 540nm. As the temperature rises, the rate of a chemical reaction increases due to the increase in activation energy. But when the temperature rises above a certain limit i.e., more than 50°C, the enzyme loses its activity due to the denaturation and loss of its tertiary structure. Further rise in temperature leads to coagulation which is an irreversible change. The optimum temperature for most of the enzymes is between 40-50°C. However few enzymes are active even at 100°C. Requirements: Substrate (1% starch in phosphate buffer) Phosphate buffer (pH – 7.0) Enzyme (Human salivary α-amylase) DNS reagent Procedure: Seven clean and dry test tubes were taken and 0.5ml of substrate was added to all the tubes. 0.5 ml of dilute enzyme was pipette out into all the test tubes except for the first tube which served as blank. The volume of all the tubes was made up to 2 ml with buffer. The tubes were incubated for 5mins at respective temperature (0°C, 10°C, 27°C, 37°C, 50°C and 100°C). (Before adding enzyme to the substrate, the substrates were pre incubated at respective temperature for 10mins) Then 1ml of DNS reagent was added to all the tubes and kept in boiling water bath for 5mins and were cooled. 31. Biotechnology practical 2-Biochemistry 2016-17 SH 30 10ml of distilled water was added to all the test tubes. Optical density was read at 540nm. Activity was calculated and the graph was plotted by taking temperature on x-axis and activity on y-axis. Result: Observation and calculations: Sl. No Vol. of buffer (ml) Vol. of substrat e (ml) Vol. of enzym e (ml) Temp. in °C Vol. of DNS (ml) Vol. of dist.H_2O (ml) OD at 540 nm Activity µmole/ ml/min 1 1.5 0.5 0 Incubat e all the tubes for 5mins at respecti ve temp. 27 1 Incub ate all the tubes in boilin g water bath for 10min 10 2 1 0.5 0.5 0 1 10 3 1 0.5 0.5 10 1 10 4 1 0.5 0.5 27 1 10 5 1 0.5 0.5 37 1 10 6 1 0.5 0.5 50 1 10 7 1 0.5 0.5 100 1 10 32. Biotechnology practical 2-Biochemistry 2016-17 SH 31 Expt: 9 Effect of pH on the activity of human salivary α-amylase Aim: To determine the effect of pH on the activity of human salivary α-amylase. Principle: α-amylase catalyses the hydrolysis of α-1 4 linkage of starch and produces the reducing sugar maltose. Maltose reduces 3, 5-dinitro salicylic acid into 3-amino, 5-nitro salicylic acid, which is an orange red colored complex. Its optical density is measured at 540nm. Enzymes are active over a limited pH range only. A plot of activity against pH usually gives a bell shaped curve. The pH value of maximum activity is known as optimum pH, and this is a characteristic of the enzyme. The variation of the activity with pH is due to the change in the state of ionization of the enzyme and other components of reaction mixture. Requirements: Substrate (1% starch) – 1g of starch is dissolved in buffers of different pH (3.0, 4.5, 7.0, 9.0 and 11.0) Enzyme (Human salivary α-amylase) DNS reagent Buffer solutions 1. Acetate buffer (pH-4.5 and 3.0) Solution A – 0.2M acetic acid Solution B – 0.2M sodium acetate Using solution B as base, solution A is added till the pH 3.0 and 4.5 are obtained. 2. Phosphate buffer (pH-7.0) Solution A – 0.2M disodium hydrogen phosphate Solution B – 0.2M sodium dihydrogen phosphate Using solution B as base, solution A is added till pH 7.0 is obtained. 3. Tris HCl buffer (pH-9.0 and 11.0) Solution A – 0.2M tris HCl Solution B – 0.2M hydrochloric acid 33. Biotechnology practical 2-Biochemistry 2016-17 SH 32 Using solution A as base, solution B is added till pH 9.0 and 11.0 is obtained. Procedure: 0.5ml of diluted enzyme was pipette out to each test tube, except for the blank. 0.5ml of substrate prepared in different buffers having pH 3.0, 4.5, 7.0, 9.0 and 11.0 was added to the respective test tubes. 1ml of substrate and buffer of any pH was added to the blank. To each test tube 1ml of their respective buffer was added. The test tubes were incubated for exactly 5mins. 1ml of DNS reagent was added to all the tubes to stop the reaction and the tubes were kept in boiling water bath for 10mins. The tubes were cooled at room temperature and 10ml of distilled water was added to all the tubes. Optical density was read at 540nm. Activity was calculated and a graph was plotted by taking pH on x-axis and activity on y-axis. Result: Observation and calculations: Sl.N o Vol. of enzyme (ml) Vol. of substrate (ml) Vol. of buffer (ml) pH Vol. of DNS (ml) Vol. of dist.H_2O (ml) OD at 540 nm Activity µmole/ml /min 1 0 1.0 1 Incubate all the tubes at room tempera- ture for 5mins. 7 1 Incubate all the tubes in boiling water bath for 10min 10 2 0.5 0.5 1 3 1 10 3 0.5 0.5 1 4.5 1 10 4 0.5 0.5 1 7 1 10 5 0.5 0.5 1 9 1 10 6 0.5 0.5 1 11 1 10 34. Biotechnology practical 2-Biochemistry 2016-17 SH 33 Expt 10.Determination of Km value of human salivary α-amylase Aim: To determine the Km value of human salivary α-amylase. Principle: α-amylase catalyses the hydrolysis of α-1 4 linkage of starch and produces the reducing sugar maltose. Maltose reduces 3, 5-dinitro salicylic acid into 3-amino, 5-nitro salicylic acid, which is an orange red colored complex. Its optical density is measured at 540nm. If the activity of enzyme is determined over a range of substrate concentration, rectangular hyperbola is obtained. An equation relating enzyme activity and substrate concentration can be obtained for the whole curve and was first derived by Michaleis and Menten in 1913. The basic assumption was that the enzyme and substrate form a complex which then breaks down to give enzyme and product. If S = Km, V = v/2, so that the Michalei's constant (Km) is the concentration which gives half of the maximum velocity, large Km has low enzyme substrate affinity. Line Weaver and Burke modified the equation, as per mathematical manipulation has converted a hyperbolic curve into straight line and thus V maximum velocity and Km can be easily calculated. Requirements: Substrate (1% starch in phosphate buffer) Enzyme (Diluted salivary α-amylase) DNS reagent Phosphate buffer (pH-7.0) Procedure: 6 clean dry test tubes were taken, first one served as blank. Substrate volume ranging from 0.1, 0.2........... 0.5 ml was pipette out to all test tubes except for the blank. 0.5ml of enzyme was added to all the test tubes. The volume in all the tubes was made to 2ml with phosphate buffer. 35. Biotechnology practical 2-Biochemistry 2016-17 SH 34 The tubes were kept for incubation at room temperature for 5mins. Then 1ml of DNS reagent was added to all the tubes and optical density was read at 540nm. With the help of maltose calibration, µg of maltose liberated was determined and enzyme activity (v) was calculated. 1/v and 1/s was calculated using enzyme activity (v) and substrate concentration (s) respectively. A graph was plotted by taking 1/s on x-axis and 1/v on y-axis, and then Km was determined by using the plot. Result: Observation and calculations: Sl. N o Vol. of buffer (ml) Vol. of enzym e (ml) Vol. of substrat e (ml) Vol. of DNS (ml) Vol. of dist. H_2O (ml) OD at 540 nm Activity (V) µmole/ ml/min 1/V Concent r-ation of (S) (µg) 1/(S) 1 1.5 0.5 0 Incub ate all the tubes at room tempe ra- ture for 5mins. 1 Incub ate all the tubes in boilin g water bath for 10min 10 2 1.4 0.5 0.1 1 10 3 1.3 0.5 0.2 1 10 4 1.2 0.5 0.3 1 10 5 1.1 0.5 0.4 1 10 6 1.0 0.5 0.5 1 10 36. Biotechnology practical 2-Biochemistry 2016-17 SH 35 Experiment 11: separation of amino acids by thin layer chromatography. Theory: Chromatography is by far the most useful general group of techniques available for the separation of closely related compounds in a mixture. Here the separation is effected by differences in the equilibrium distribution of the components between two immiscible phases, viz., the stationary and the mobile phases. These differences in the equilibrium distribution are a result of nature and degree of interaction of the components with these two phases. The stationary phase is a porous medium like silica or alumina, through which the sample mixture percolates under the influence of a moving solvent (the mobile phase). There are a number of interactions between the sample and the stationary phase and these have been well exploited to effect the separation of compounds. Thin layer chromatography [TLC]: Thin layer chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data. Thin layer chromatography is a technique used to separate and identify compounds of interest. A TLC plate is made up of a thin layer of silica adhered to glass or aluminum for support. The silica gel acts as the stationary phase and the solvent mixture acts as the mobile phase. In the ideal solvent system the compounds of interest are soluble to different degrees. Separation results from the partition equilibrium of the components in the mixture. In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is applied near one end of the TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is called as the development of TLC plates. The separation depends on several factors; (a) solubility: the more soluble a compound is in a solvent, the faster it will move up the plate. (b) attractions between the compound and the silica, the more the compound interacts with silica, the lesser it moves, (c) size of the compound, the larger the compound the slower it moves up the plate. The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value. The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value. 37. Biotechnology practical 2-Biochemistry 2016-17 SH 36 Chromatographic Separation of Amino acids: The present experiment employs the technique of thin layer chromatography to separate the amino acids in a given mixture. All 20 of the common amino acids [standard amino acids] are a-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α- carbon). They differ from each other in their side chains, or R groups, which vary in structure, size, and electric charge. The interaction of the amino acids with the stationary phase like silica varies depending on their 'R' groups. The amino acid that interacts strongly with silica will be carried by the solvent to a small distance, whereas the one with less interaction will be moved further. By running controls [known compounds] alongside, it is possible to identify the components of the mixture. Since amino acids are colourless compounds, ninhydrin is used for detecting them. To identify this, after development, the TLC plate is sprayed with ninhydrin reagent and dried in an oven, at 105°C for about 5 minutes. Ninhydrin reacts with α- amino acids that results in purple coloured spots [due to the formation of the complex - Rheuman's purple values can be calculated and compared with the reference values to identify the amino acids. [The RF value for each known compound should remain the same provided the development of plate is done with the same solvent, type of TLC plates, method of spotting and in exactly the same conditions]. Materials Required: 38. Biotechnology practical 2-Biochemistry 2016-17 SH 37 Reagents: 1. 2% solution of individual amino acids. 2. Solvent mixture of normal butanol, acetic acid and water in the ratio 12:3:5 by volume. 3. Ninhydrin reagent. Requirements: 1. TLC plate. 2. TLC chamber. 3. Capillary tubes. 4. Reagent spray bottle. 5. Conical flasks. 6. Beakers. Procedure: 1. Pour the solvent mixture in to the TLC chamber and close the chamber. 2. The chamber should not be disturbed for about 30 minutes so that the atmosphere in the jar becomes saturated with the solvent. 3. Cut the plate to the correct size and using a pencil (never ever use a pen) gently draw a straight line across the plate approximately 2 cm from the bottom. 4. Using a capillary tube, a minute drop of amino acid is spotted on the line. 5. Allow the spot to dry. 6. Spot the second amino acid on the plate [enough space should be provided between the spots]. 7. Repeat the above step for spotting the unknown acid. 8. Place the plate in the TLC chamber as evenly as possible and lean it against the side(immerse the plate such that the line is above the solvent). Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end. 9. Remove the plate and immediately draw a pencil line across the solvent top. 10. Under a hood dry the plate with the aid of a blow dryer. 11. Spray the dry plate with ninhydrin reagent. 12. Dry the plates in hot air oven at 105°C for 5 min. [Ninhydrin will react with the faded spots of amino acids and make them visible as purple colored spots.] 13. After some time, mark the center of the spots, then measure the distance of the center of the spots from the origin and calculate the Rf values. Rf value can be calculated using the formula: 39. Biotechnology practical 2-Biochemistry 2016-17 SH 38 The Rf values with butanol-acetic acid- water solvent are as follows: alanine 0.24, glutamic acid 0.25, glycine 0.2, leucine 0.58, valine 0.4, lysine 0.58, tyrosine 0.42. Expt: 12 Circular Paper Chromatography Aim: To separate the Amino acids by Circular Paper Chromatography. Principle: Amino acids in a given mixture or sample aliquot are separated on the basis of differences in their solubilities and hence differential partitioning coefficient in a binary solvent system. The amino acids with higher solubilities in stationary phase move slowly as compared with those with higher solubilities in the mobile phase. The separated amino acids are detected by spraying the air dried chromatogram with ninhydrin reagent. All amino acids give purple color or bluish purple colour on reaction with ninhydrin while proline and hydroxyl-proline gives yellow. Reaction: Materials required: 1) Whattmann no. 1 filter paper (circular disc) 2) Microsyringe or capillary tube 3) Hair dryer 4) Sprayer 5) Chromatographic chamber 6) Developing solvent : Butanol: glacial acetic acid:water(4:1:1) 7) Ninhydrin reagent: 0.1 % in acetone 8) Standard amino acids: 1mg/ml in 10% isopropanol 0r 0.1 N HCl 9) Unknown sample 10) Oven set at 1000C Procedure: 1) Take a circular whattmann no. 1 filter paper of dia.10cm. 40. Biotechnology practical 2-Biochemistry 2016-17 SH 39 2) Draw two perpendicular lines at the centre in such a way that filter paper is divided into 4 equal parts. 3) Draw a small circle at the centre of the paper with the use of coin. 4) Spot amino acid sample as small as possible in the mid of the part on the circle drawn. 5) Dry the paper and insert the paper wig in the centre. 6) Place it in the petridish containing the developing solvent for about 30-40 min to develop a chromatogram. 7) Take out the paper mark the solvent front with the help of pencil and dry it in an oven at 1000C 8) Spray the paper with ninhydrin then repeat the drying process in oven. 9) Measure the distance of amino acids in cm and find out the Rf value by using formula, Rf = Distance travelled by the solute from the origin Distance travelled by the solvent from the origin Compare the Rf values of standard and unknown amino acids. Report: The unknown amino acid is, (Name with structure) Reference: 1) Introductory practical Biochemistry by S.K.Sawhney and Randhir Singh 2) Standard methods of biochemical analysis by S.K.Thimmaiah 41. Biotechnology practical 2-Biochemistry 2016-17 SH 40 Expt: 13 Ascending Paper Chromatography Aim: To separate the Amino acids by Ascending Paper Chromatography. Principle: Amino acids in a given mixture or sample aliquot are separated on the basis of differences in their solubilities and hence differential partitioning cieffcient in a binary solvent system. The amino acids with higher solubilities in stationary phase move slowly as compared with those with higher solubilities in the mobile phase. The separated amino acids are detected by spraying the air dried chromatogram with ninhydrin reagent. All amino acids give purple colour or bluish purple colour on reaction with ninhydrin while proline and hydroxyproline gives yellow. Reaction: Materials required: 1) Whattmann no. 1 filter paper (40 X20cm) 2) Microsyringe or capillary tube 3) Hair dryer 4) Sprayer 5) Chromatographic chamber 6) Developing solvent : Butanol: glacial acetic acid: water(4:1:1) 7) Ninhydrin reagent: 0.1 % in acetone 8) Standard amino acids: 1mg/ml in 10% isopropanol 0r 0.1 N HCl 9) Unknown sample 10) Oven set at 1000C Procedure: 1) Take a whattmann no. 1 filter paper of 40X20cm. 2) Draw a line at the end of one side by leaving 2cm from the end. 3) Spot amino acid sample as small as possible at equal distance. 4) Dry the paper. Keep it in ascending manner in the chromatographic chamber. 42. Biotechnology practical 2-Biochemistry 2016-17 SH 41 5) Place it in the chromatographic chamber containing the developing solvent for about 30-40 min to develop a chromatogram. 6) Take out the paper mark the solvent front with the help of pencil and dry it in an oven at 1000C 7) Spray the paper with ninhydrin then repeat the drying process in oven. 8) Measure the distance of amino acids in cm and find out the Rf value by using formula, Distance travelled by the solute from the origin Rf = Distance travelled by the solvent from the origin Compare the Rf values of standard and unknown amino acids. Report: The unknown amino acid is, (Name with structure) Reference: 1) Introductory practical Biochemistry by S.K.Sawhney and Randhir Singh 2) Standard methods of biochemical analysis by S.K.Thimmaiah 43. Biotechnology practical 2-Biochemistry 2016-17 SH 42 Experiment 14: Separation of plant pigment by paper chromatography INTRODUCTION: Chlorophyll often hides the other pigments present in leaves. In autumn, chlorophyll breaks down, allowing xanthophyll and carotene, and newly made anthocyanin, to show their colors. The mix of pigments in a leaf may be separated into bands of color by the technique of paper chromatography. Chromatography involves the separation of mixtures into individual components. Chromatography means "color writing." With this technique the components of a mixture in a liquid medium are separated. The separation takes place by absorption and capillarity. The paper holds the substances by absorption; capillarity pulls the substances up the paper at different rates. Pigments are separated on the paper and show up as colored streaks. The pattern of separated components on the paper is called a chromatogram. MATERIALS: Chromatography solvent (92 parts Petroleum ether to 8 parts acetone) Chromatography paper (or filter paper) about 1 cm x 15 cm Ethyl alcohol Fresh spinach Test tube Test tube rack Fresh leaves of plants Mortar and pestle Sand (optional) PROCEDURE: 1. Cut a strip of filter paper or chromatography paper so that it just fits inside a 15-cm (or larger) test tube. Cut a point at one end. Draw a faint pencil line as shown in figure 1. The sides of the strip should not touch the glass. 2. Tear a spinach leaf into pieces about the size of a postage stamp. Put them into a mortar along with a pinch or two of sand to help with grinding. Add about 5 ml ethyl alcohol to the leaf pieces. Crush leaves with the pestle, using a circular motion, until the mixture is finely ground. The liquid in which the leaf pigments are now for paper chromatography dissolved is called the pigment extract. 44. Biotechnology practical 2-Biochemistry 2016-17 SH 43 3. Use a glass rod to touch a drop of the pigment extract to the center of the pencil line on the paper strip. Let it dry. Repeat as many as 20 times, to build up the pigment spot. NOTE: You must let the dot dry after each drop is added. The drying keeps the pigment dot from spreading out too much. 4. Pour 5 ml chromatography solvent into the test tube. Fit the paper and cork assembly inside. Adjust it so that the paper point just touches the solvent (but not the sides of the tube). The pigment dot must be above the level of the solvent. Watch the solvent rise up the paper, carrying and separating the pigments as it goes. At the instant the solvent reaches the top, remove the paper and let it dry. Observe the bands of pigment. The order, from the top, should be carotenes (orange), xanthophylls (yellow), chlorophyll a (yellow-green), chlorophyll b (blue-green), and anthocyanin (red). Identify and label the pigment bands on the dry strip. Write the species of leaf on the strip as well. 5. Each pigment has an Rf value, the speed at which it moves over the paper compared with the speed of the solvent. Rf = Distance moved by the pigment / Distance moved by the solvent DATA TABLE: Chromatography Data Leaf Type (species) External color Chromatogram Pigments Colors from the Top Pigment Names Rf Values 45. Biotechnology practical 2-Biochemistry 2016-17 SH 44

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