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Appendix A

Agar Plate Inoculation Techniques

The two normal media used in bacteriology are a clear soup-like liquid nutrient broth, usually in tubes, and nutrient agar, which is set into a jelly by the addition of a seaweed extract called agar, and when melted poured into glass or plastic Petri dishes - also known as "plates".

A standard carbon source is glucose, and nitrogen is often provided by peptones (partially digested proteins), or inorganic salts. Minerals and vitamins may also be provided, according to the growth requirements of the bacteria. Combinations of chemicals (buffers) may be used to keep the pH stable. Measured amounts of the concentrates are added to water, and dissolved to reconstitute the media. These media must then be sterilized by heating in an autoclave at 121°C (pressure 1 bar) for 15 minutes, which kills all living organisms, including spores. All apparatus used after this point onwards must be sterilized by heat (glassware - 160 °C for 2 hrs) or exposure to radiation.

Aseptic techniques must be used to reduce the likelihood of bacterial contamination. This usually involves disinfection of working areas, minimizing possible access by bacteria from the air to exposed media, and use of flames to kill bacteria which might enter vessels as they are opened. Bacteria may be introduced to the media (inoculated) by various means. Usually the bacteria e.g. from a drop in a heat-sterilized loop are spread on the surface of (ready set) agar. A similar technique is used with broth cultures.

Then the Petri dishes containing agar or tubes containing broth are incubated, i.e. put in a special apparatus at a fixed temperature (usually 37°C - human body temperature, for possible pathogens - or 25°C for bacteria from the environment). When growing bacteria, it is usual to invert the Petri dishes, so as to prevent condensation droplets from falling onto the surface of the agar. Petri dishes are often "sealed" at this stage to prevent people who handle them from contamination by bacteria, which will multiply greatly. It is normal to use 2 strips of adhesive tape

from base to lid rather than attempt seal the circular edge of the Petri dish. This is to guard against the possibility of anaerobic organisms growing due to lack of air. Cultures are usually examined after 24 hrs incubation.

Liquid media such as broth become cloudy if bacteria are present. This could be the result of only one bacterial cell originally entering the medium, then dividing repeatedly to produce millions! Bacteria on agar "plates" become visible as distinct circular colonies; each colony should represent an individual bacterial cell (or group) which has divided repeatedly but, being kept in one place, the resulting cells have accumulated to form a visible patch

A.1 Streak Method for Agar Plates

The streak plate is used primarily for isolating microorganisms in pure culture from specimens or samples containing mixed flora. Obtaining isolated colonies on plates allows colonial morphology and hemolytic reactions to be examined, and biochemical/serological testing to be performed.

1. With a sterile inoculating loop, streak a loopful of the sample across the surface of an agar plate. The four-quadrant streak is the most common, and accomplished by streaking and rotating the plate in four sections, one quarter at time, slightly overlapping the original streak area. The fourth quadrant contains the greatest dilution of microorganisms, and usually provides isolated colonies for further testing.
2. Incubate plates under favorable growth conditions.
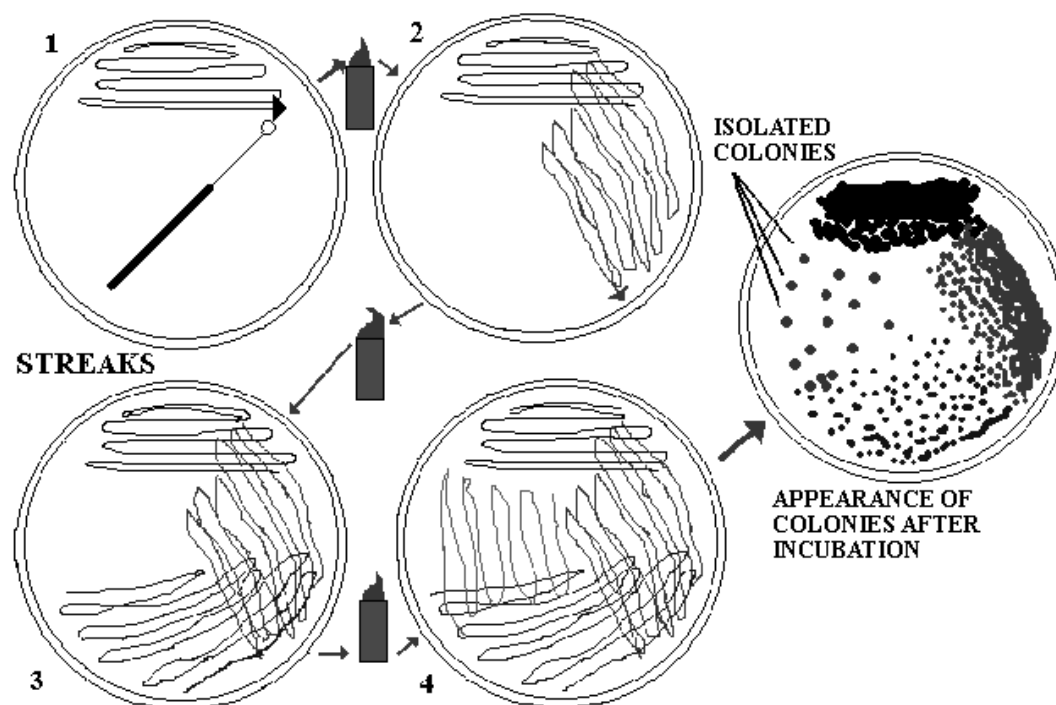3. Examine plates for isolated colonies.

Figure A1-Streak method for agar plates

A.2 Spread Plate Technique

The spread plate technique is used for enumerating microorganisms.

1. Drop 0.1 mL aliquots from serial dilutions onto the surface of an agar plate.

2. Aseptically spread inoculum across the surface using a bent glass rod or sterile inoculating loop. By spreading the suspension over the plate, a dilution gradient is established to provide isolated colonies.

3. Incubate plates agar inverted in appropriate conditions.

4. Count colonies and calculate the number of microorganisms in the original suspension.

(a)   (b)
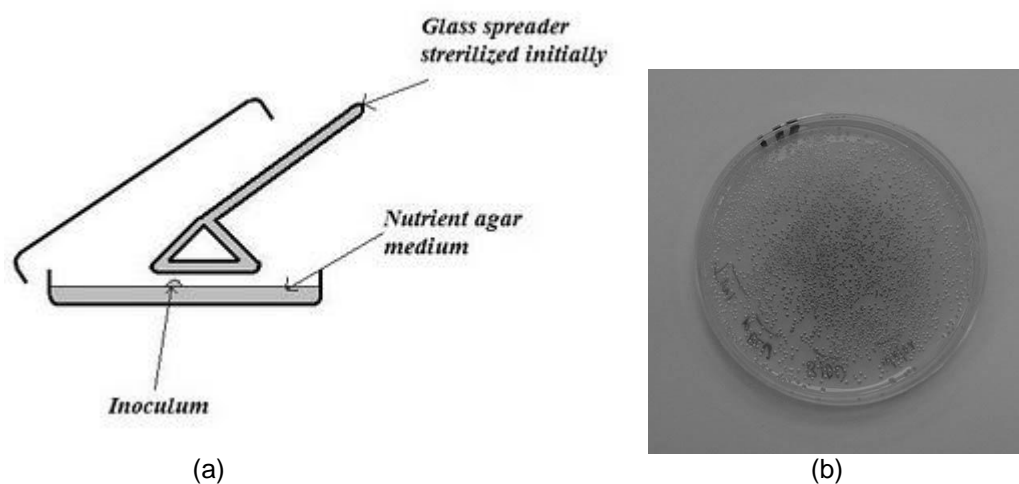
Figure A2-Spread plate technique (a- performing spread plated inoculation, b-
bacterial growth after streak plate inoculation)

A.3 Pour Plate Technique

The pour plate technique is also used for enumeration of microorganisms in a
particular sample. In this technique, test samples or suspensions of microorganisms
are mixed with molten agar (45-50°C). The agar is allowed to solidify, trapping the
bacteria at separate discrete positions within the matrix of the medium. While the
medium holds bacteria in place, it is soft enough to permit growth of bacteria and the
formation of discrete isolated colonies.

1. Perform serial dilution of sample.
2. Aseptically pipette microorganism dilutions into labeled petri dishes.
3. Add melted agar that has been cooled to approximately 44–45°C.
4. Mix well by slightly rotating plate with bacteria and agar mixture.
5. Allow the agar to solidify, trapping bacteria at separate discrete positions
   within the medium.
6. Incubate plates in a favorable environment.
7. Count the number of colonies and calculate the number of microorganisms in
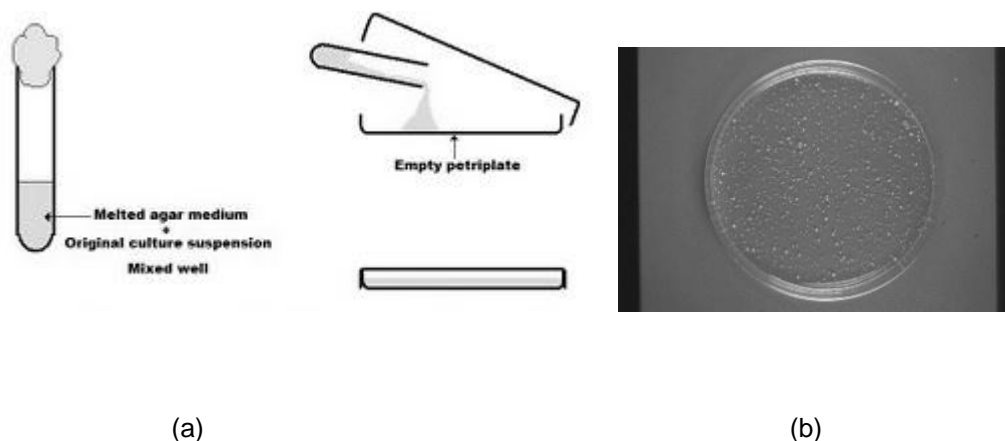   the original sample

(a)   (b)

Figure A3-Pour plate technique (a- performing pour plated inoculation, b- bacterial growth after pour plate inoculation)

A.4 Inoculation of Broth Media

Broth media are generally used as enrichments, general cultivation and sterility testing.

1. Aseptically inoculate appropriate broth media with the sample or specimen using sterile pipette, syringes or forceps.

2. Incubate inoculated broth at the appropriate atmospheric conditions, temperature, and time.

3. Examine broth for any signs of growth including, turbidity with or without gas bubbles, "puff-ball" appearance, hemolysis (in blood cultures), pellicle formation and precipitate on the bottom of the tube or bottle.

Appendix B

Gram Staining

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet.

Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolorization, and counterstaining, usually with safanin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria, Gram positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolorization process, while Gram negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process. The process involves three steps:
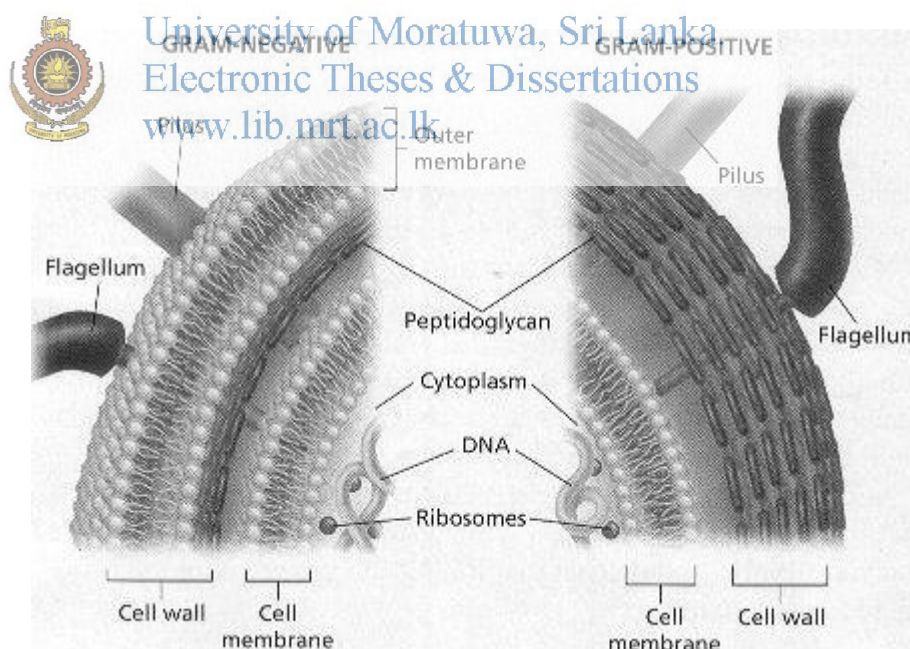

Figure B1-Comparison between gram positive and negative cell wall

1. Cells are stained with crystal violet dye. Next, a Gram's iodine solution (iodine and potassium iodide) is added to form a complex between the crystal violet and iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water.

2. A decolorizer such as ethyl alcohol or acetone is added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. The large crystal violet-iodine complex is not able to penetrate this tightened peptidoglycan layer, and is thus trapped in the cell in Gram positive bacteria. Conversely, the the outer membrane of Gram negative bacteria is degraded and the thinner peptidoglycan layer of Gram negative cells is unable to retain the crystal violet-iodine complex and the color is lost.

3. A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram positive cells. However, the decolorized Gram negative cells are stained red.
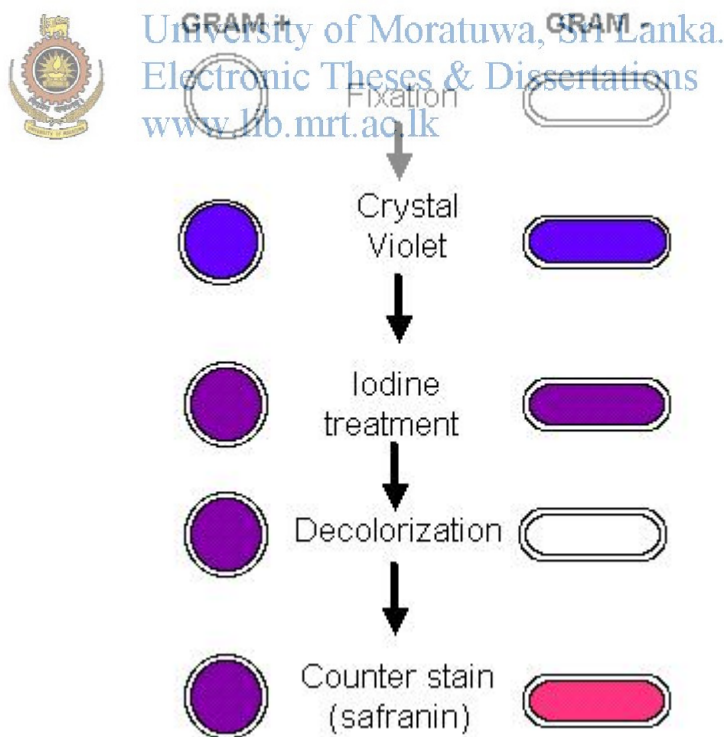

Figure B2-Comparison between gram positive and negative cell gram staining

Appendix C

Agar Types

Chemically, agar is a polymer made up of subunits of the sugar galactose, and is a component of the cell walls of several species of red algae that are usually harvested in eastern Asia and California. Dissolved in boiling water and cooled, laboratory agar looks gelatinous.

C.1 Nutrient Agar

Nutrient agar will grow the largest number of different types of microbes - fungi and bacteria. Yet, not all bacteria can grow on these. Some find it too rich, and others find it deficient. The nutrient in this is beef broth, and some extracts from yeast. It is suitable for many study level experiments since it does not selectively grow pathogenic bacteria.

Figure C1 – Nutrient agar plate

C.2 Blood Agar

Blood agar is a solid growth medium that contains red blood cells. The medium is used to detect bacteria that produce enzymes to break apart the blood cells. This process is also termed hemolysis. The degree to which the blood cells are hemolyzed is used to distinguish bacteria from one another.

Hemolysis is the break down of the membrane of red blood cells by a bacterial protein known as hemolysin, which causes the release of hemoglobin from the red blood cell. Many types of bacterial posses hemolytic proteins. These proteins are thought to act by integrating into the membrane of the red blood cell and either punching a hole through the membrane or disrupting the structure of the membrane in some other way.

Figure C2 – Alpha, beta and gamma hemolysis on a blood agar plate

There are three types of hemolysis, designated alpha, beta and gamma. Alpha hemolysis is a greenish discoloration that surrounds a bacterial colony growing on the agar. This type of hemolysis represents a partial decomposition of the hemoglobin of the red blood cells. Alpha hemolysis is characteristic of *Streptococcus pneumonia* and so can be used as a diagnostic feature in the identification of the bacterial strain.

Beta hemolysis represents a complete breakdown of the hemoglobin of the red blood cells in the vicinity of a bacterial colony. There is a clearing of the agar around a colony. Beta hemolysis is characteristic of *Streptococcus pyogenes* and some strains of *Staphylococcus aureus*.

The third type of hemolysis is actually no hemolysis at all. Gamma hemolysis is a lack of hemolysis in the area around a bacterial colony. A blood agar plate displaying gamma hemolysis actually appears brownish. This is a normal reaction of the blood

to the growth conditions used (37° C in the presence of carbon dioxide). Gamma hemolysis is a characteristic of *Enterococcus faecalis*.

The determination of hemolysis and of the hemolytic reactions is useful in distinguishing different types of bacteria. Subsequent biochemical testing can narrow down the identification even further. For example, a beta hemolytic reaction is indicative of a Streptococcus. Testing of the Streptococcus organisms with bacitracin is often the next step. Bacitracin is an antimicrobial that is produced by the bacterium *Bacillus subtilis*. *Streptococcus pyogenes* strains are almost uniformly sensitive to bacitracin. But other antigenic groups of Streptococcus are not bacitracin sensitive.

C.3 MacConkey Agar

This is an agar upon which only Gram-negative bacteria can grow. Acting as a visual pH indicator, the agar distinguishes those Gram-negative bacteria that can ferment the sugar lactose (Lac+) from those that cannot (Lac-).

By utilizing the lactose available in the medium, Lac+ bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid, which lowers the pH of the agar below 6.8 and results in the appearance of red/pink colonies. The bile salts precipitate in the immediate neighborhood of the colony, causing the medium surrounding the colony to become hazy.

Non-Lactose fermenting bacteria such as *Salmonella*, *Proteus species, Pseudomonas aeruginosa* and *Shigella* cannot utilize lactose, and will use peptone instead, exception (shigella sonie is a late lactose fermentor). This forms ammonia, which raises the pH of the agar, and leads to the formation of white/colorless colonies formed in the plate. But they can also look golden to brown with dark centers. They are circular colonies and arranged randomly.
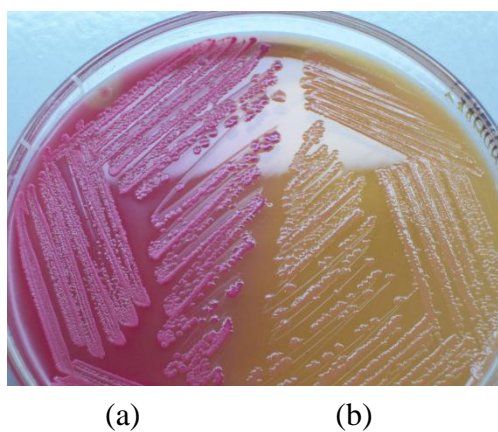
(a)　　　　　　　　　(b)

Figure C3 – MacConkey agar plate (a- Lactose acid producing bacteria, b- Lactose acid non-producing bacteria)

Appendix D

FTIR (Fourier Transform Infrared Spectrometry)

Infrared spectroscopy is a useful method for matching unknown substances to known substances in order to identify them. As a characterization tool, IR spectroscopy can provide certain structural clues to the overall molecular structure of the unknown substance. However, other methods must be used in conjunction with IR analysis in order to fully characterize the new substance.

The mid-infrared region is a common region used in IR spectroscopy. This region spans the wavenumbers from about 400 cm^{-1} to 4000 cm^{-1}. Infrared photons have energies similar to the vibrational energies of molecular bonds. Since vibrational energy transitions are quantized, a bond can be caused to vibrate if it absorbs a photon with a frequency equal to its natural vibrational frequency. This absorption of IR photons forms the basis of IR spectroscopy.

The older version of an IR spectrometer works by shining light through a sample chamber and through a solvent reference chamber then measuring the amount of radiation absorbed by the sample as compared to that absorbed by the reference. A detector plots the absorbance (or % transmittance) as a function of wavenumber. This process gives a spectrum for the sample which may be used to learn information about the sample.

This older style was replaced by the FTIR or Fourier Transform Infrared Spectrometer. Fourier transform is a mathematical function that allows the entire IR spectrum to be analyzed at once. Instead of passing through a monochromator, the beam passes into an interferometer where the mathematical calculation is performed to get a spectrum. This type of spectrometer works much more quickly than the older style because the analysis does not have to be performed in steps. Additionally, there is no reference chamber, so a blank sample is run and stored in the memory of the computer to correct for air or solvents

Any bond in a molecule can undergo several types of motion. Stretching, Scissoring, Rocking, Wagging, Twisting are the common types of bond motions for bonds

around an sp^3 central atom. Both stretching and bending motions can absorb IR radiation.

In order for a molecule to absorb IR radiation, the electric component of the radiation must interact with the bond. This can only occur if the bond has a change in dipole moment as a result of the vibration. The oscillating electric field of the radiation would cause alternating stretching and compressing of a polar bond because the field exerts a force on the positive end in an opposite way to the negative end. As the electric field oscillates, the bond would vibrate. If the vibration caused by the oscillating electric field is equal in frequency to the natural vibration, the bond can absorb the energy. One can consider the vibrational energy level of a molecule. If the IR photon has energy equal to the difference between two energy states, then the molecule can absorb the photon and jump to the higher of the two vibrational states. A bond that has a zero net dipole moment can still absorb IR radiation because it has small portions of time when it is unsymmetrical due to dispersion forces and molecular collisions. These absorbances are so weak, that they are not usually considered when analyzing the IR spectrum

Even within different molecules, the vibrational frequencies of certain types of bonds are not highly affected by the structural environment around the bond. These bonds produce characteristic absorption bands within a specific range on the IR spectrum. Though many molecules share similar types of bonds, it is unlikely that any two molecules would produce exactly the same absorption spectra.
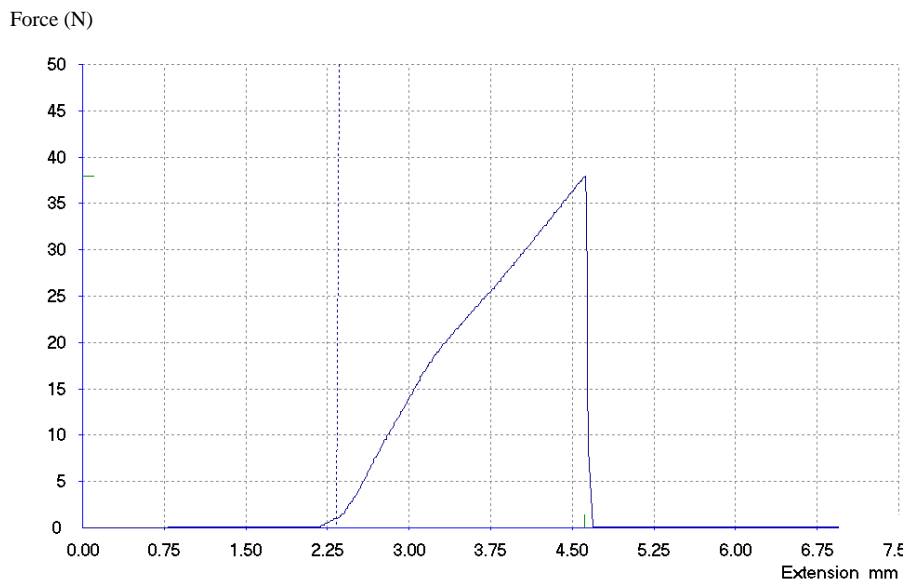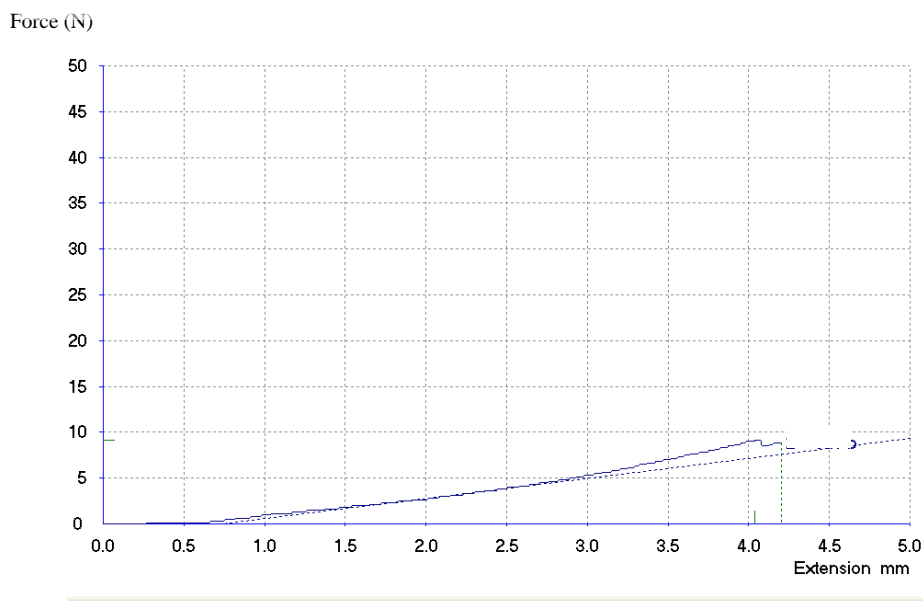
Appendix E

Tensile Test Results

Figure E1 – Force vs Extension graph of side a with 1% moisture content specimen

Figure E2 – Force vs Extension graph of side b with 1% moisture content specimen
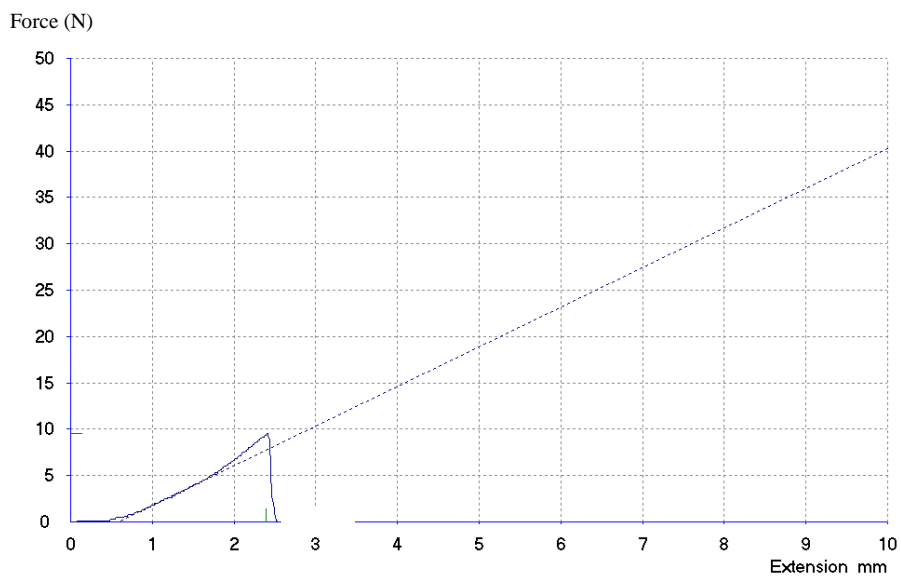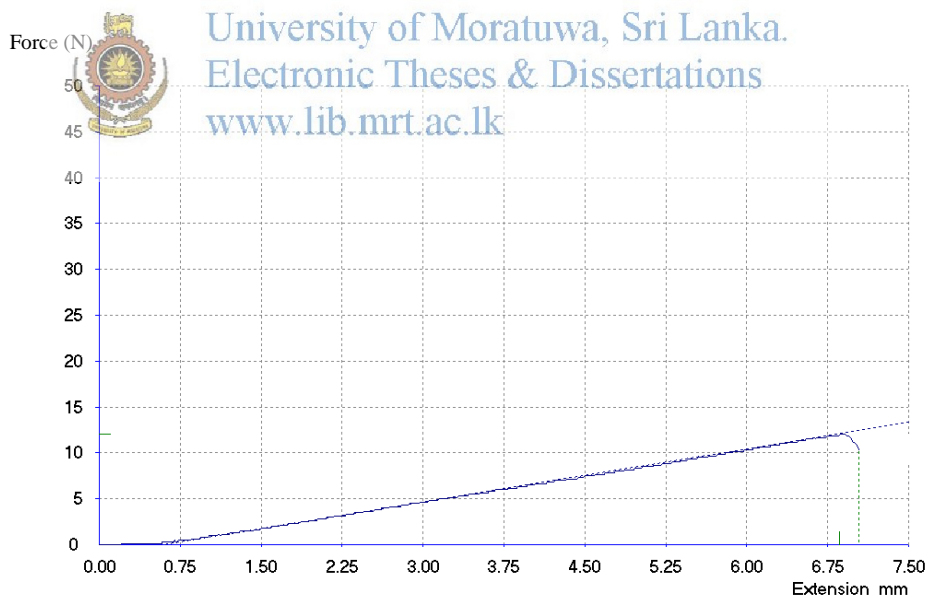
Force (N)

Figure E3 – Force vs Extension graph of side a with 15% moisture content specimen

Figure E4 – Force vs Extension graph of side b with 15% moisture content specimen

Appendix F

Tensile Test

Stress-Strain Diagram expresses a relationship between a load applied to a material and the deformation of the material, caused by the load. Stress-Strain Diagram is determined by tensile test. Tensile tests are conducted in tensile test machines, providing controlled uniformly increasing tension force, applied to the specimen.

The specimen's ends are gripped and fixed in the machine and its gauge length L_0 (a calibrated distance between two marks on the specimen surface) is continuously measured until the rupture. Test specimen may be round or flat in the cross-section. In the round specimens it is accepted, that $L_0 = 5 *$ diameter. The specimen deformation (strain) is the ratio of the increase of the specimen gauge length to its original gauge length:

$\delta = (L - L_0) / L_0$

Tensile stress is the ratio of the tensile load F applied to the specimen to its original cross-sectional area S_0:
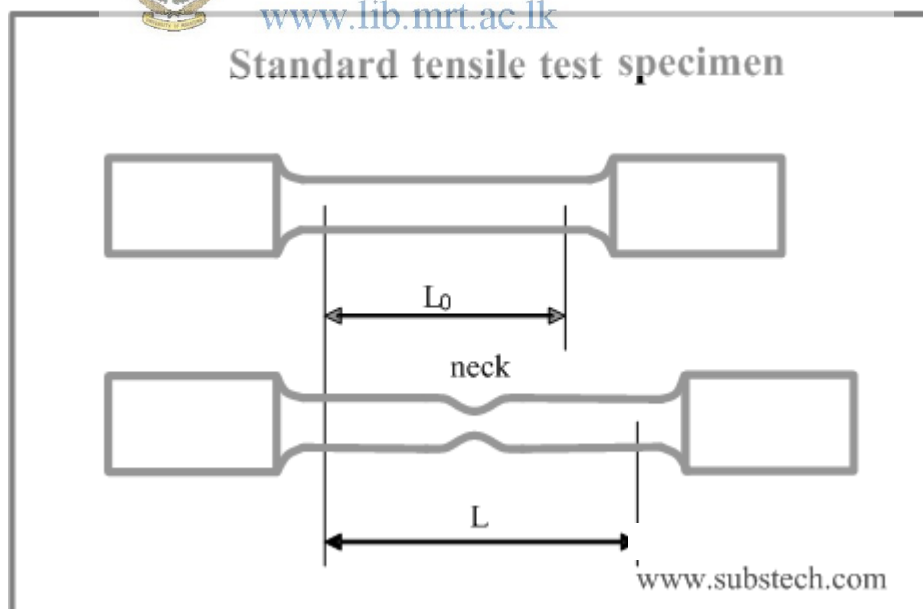
$\sigma = F / S_0$

Figure F1 – Standard tensile test specimen

The initial straight line (0P)of the curve characterizes proportional relationship between the stress and the deformation (strain).

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The stress value at the point P is called the limit of proportionality:

$\sigma_p = F_P / S_0$

This behavior conforms to the Hook's Law:

$\sigma = E * \delta$

Where E is a constant, known as Young's Modulus or Modulus of Elasticity. The value of Young's Modulus is determined mainly by the nature of the material and is nearly insensitive to the heat treatment and composition. Modulus of elasticity determines stiffness - resistance of a body to elastic deformation caused by an applied force. The line 0E in the Stress-Strain curve indicates the range of elastic deformation – removal of the load at any point of this part of the curve results in return of the specimen length to its original value. The elastic behavior is characterized by the elasticity limit (stress value at the point E):

$\sigma_{el} = F_E / S_0$

For the most materials the points P and E coincide and therefore $\sigma_{el} = \sigma_p$.
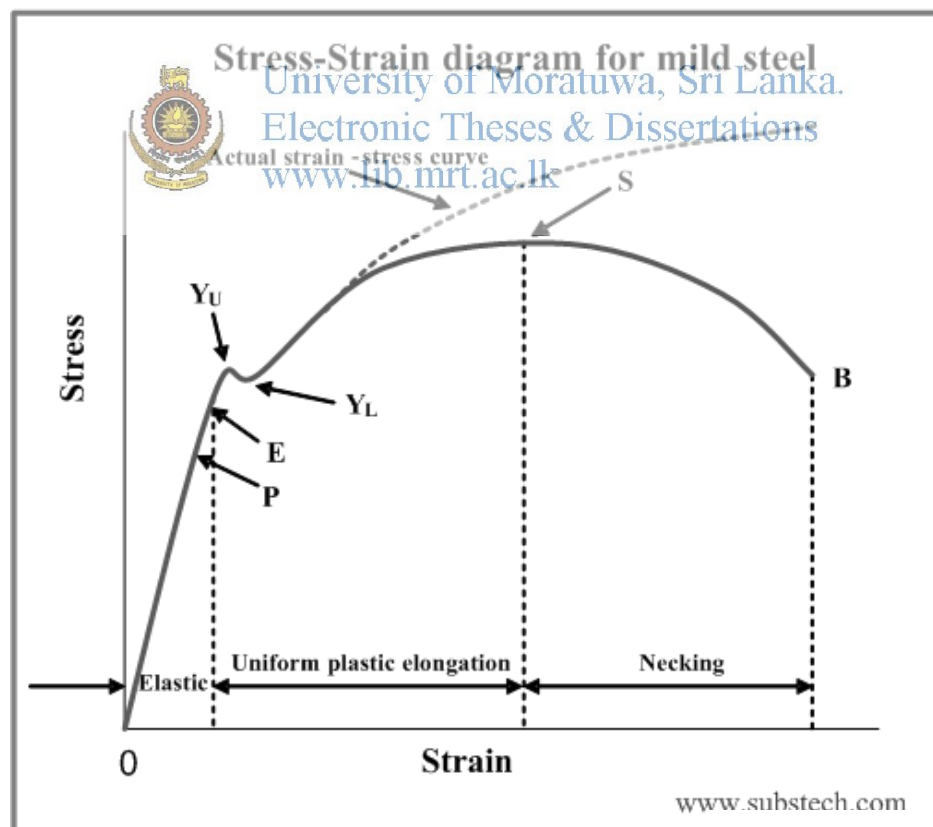

Figure F1 – Stress-strain diagram for mild steel

A point where the stress causes sudden deformation without any increase in the force is called yield limit (yield stress, yield strength):

σ$_y$= F$_Y$ / S$_0$

The highest stress (point Y$_U$) , occurring before the sudden deformation is called upper yield limit . The lower stress value, causing the sudden deformation (point Y$_L$) is called lower yield limit. The commonly used parameter of yield limit is actually lower yield limit. If the load reaches the yield point the specimen undergoes plastic deformation – which can also be called as strain hardening region it does not return to its original length after removal of the load.
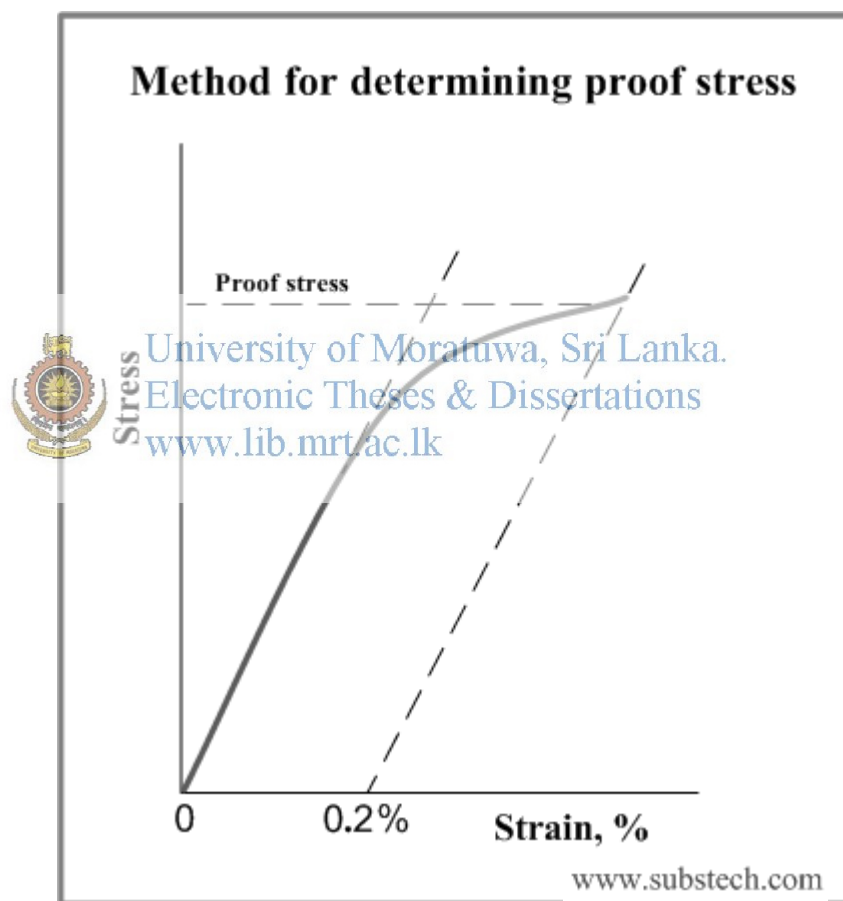

Figure F1 – Method for determining proof stress

Hard steels and non-ferrous metals do not have defined yield limit, therefore a stress, corresponding to a definite deformation (0.1% or 0.2%) is commonly used instead of yield limit. This stress is called proof stress or offset yield limit (offset yield strength):

$$\sigma_{0.2\%} = F_{0.2\%} / S_0$$

The method of obtaining the proof stress is shown in the picture.

As the load increase, the specimen continues to undergo plastic deformation and at a certain stress value its cross-section decreases due to "necking" (point **S** in the Stress-Strain Diagram). At this point the stress reaches the maximum value, which is called ultimate tensile strength (tensile strength):

$$\sigma_t = F_S / S_0$$

Continuation of the deformation results in breaking the specimen - the point B in the diagram. The actual Stress-Strain curve is obtained by taking into account the true specimen cross-section instead of the original value. Other important characteristic of metals is ductility - ability of a material to deform under tension without rupture. Two ductility parameters may be obtain from the tensile test: Relative elongation - ratio between the increase of the specimen length before its rupture and its original length:

$$\delta = (L_m - L_0) / L_0$$

Where L_m – maximum specimen length.

Relative reduction of area **-** ratio between the decrease of the specimen cross-section area before its rupture and its original cross-section area**:**

$$\psi = (S_0 - S_{min}) / S_0$$

Where S_{min} – minimum specimen cross-section area.