Model Answer

BSc IV semester

Paper: LBC-401 (General Microbiology)

Section-A

Ans.1.

i. (d) All of the above

ii. (a) Discovery of pox vaccine

iii. (b) Thermoplasma

iv. (b) Rhizobacterium

v. (a) Agrobacterium

vi. (c) Heat resistant endospore forming bacteria

vii. (a) Christian Gram

viii. (d) The organism should not be able to be isolated from the newly infected animal

ix. (a) Mycoplasma

x. (c) Both of the above

Section-B

Ans. 2.

Robert Hooke (1635–1703), an English mathematician and natural historian, was also an excellent microscopist. In his famous book Micrographia (1665), the first book devoted to microscopic observations, Hooke illustrated, among many other things, the fruiting structures of molds. This was the first known description of microorganisms**.**

Antoni van Leeuwenhoek (1632–1723) He discovered bacteria in 1676 while studying pepper–water infusions. He reported his observations in a series of letters to the prestigious Royal Society of London, which published them in 1684. Leeuwenhoek, who was well aware of the work of Hooke, used extremely simple microscopes of his own construction to examine the microbial content of natural substances.

Ferdinand Cohn (1828–1898) was born in Breslau (now in Poland). He was trained as a botanist and became an excellent microscopist. His interests in microscopy led him to the study

of unicellular algae and later to bacteria, including the large sulfur bacterium *Beggiatoa*. Cohn was particularly interested in heat resistance in bacteria, which led to his discovery that some bacteria form endospores. Cohn described the life cycle of the endospore-forming bacterium Bacillus (vegetative cell endospore vegetative cell) and showed that vegetative cells but not endospores were killed by boiling. He laid the groundwork for a system of bacterial classification. Cohn devised simple but effective methods for preventing the contamination of culture media, such as the use of cotton for closing flasks and tubes.

Louis Pasteur (1822–1895) Pasteur studied the mechanism of the alcoholic fermentation at that time thought to be a strictly chemical process From his work on tartrate metabolism this suggested to Pasteur that the beet fermentation was a biological process. Food science also owes a debt to Pasteur, as his principles are applied today in the preservation of milk and many other foods by heat treatment (pasteurization). Pasteur's work on rabies was his most famous success, culminating in July 1885 with the first administration of a rabies vaccine to a human. In 1864 Pasteur gave deadly blow to theory of 'Spontaneous Generation' simply and brilliantly by constructing a swannecked flask, now called a Pasteur flask. Pasteur's fame from his rabies research was legendary and led the French government to establish the Pasteur Institute in Paris in 1888.

Ans 3.

Batch culture: A batch culture is that in which growth of microbes occurs in a limited volume of liquid medium. It is typically a closed system in which limited amount of nutrients are present. In this culture after inoculums no any additional growth substance is added. Batch culture is the most common laboratory growth method in which bacterial growth is studied.

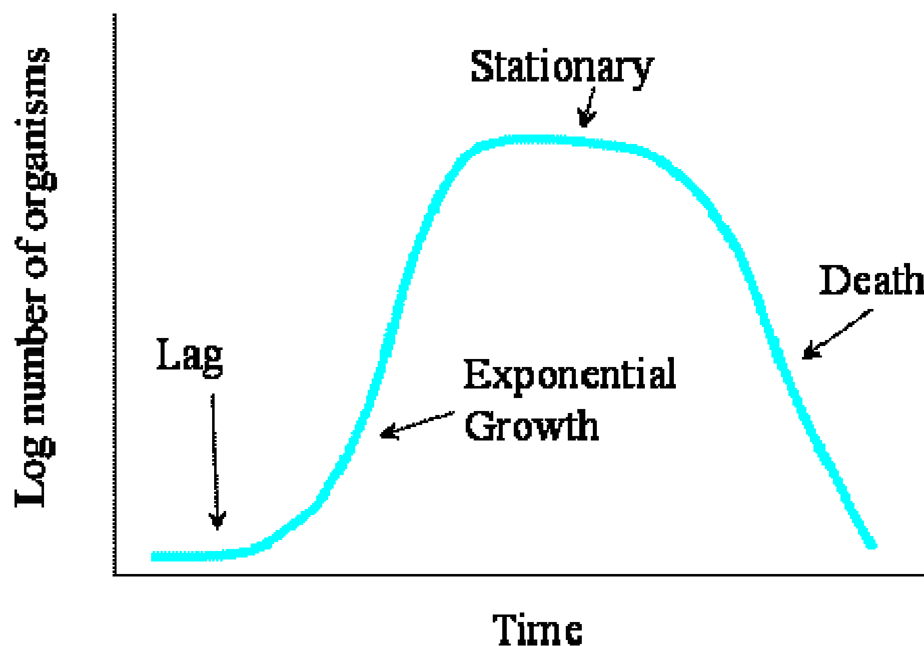
In a typical batch culture, sigmoid growth curve is obtained which depicts four major growth phases as follows:

1. Lag phase: It is the beginning of the batch culture. In this phase bacteria are incubated in the culture medium. At first, the bacteria adjust themselves in the medium or new culture environment. They start synthesizing different types of compounds for their metabolism and growth like synthesis of ATP, RNA, new enzymes, and co-factors etc. In this phase the growth rate of microorganism is very slow. This phase is long, if the inoculum cells are old, damaged, or grown previously in different medium.

2. Log phase/ Exponential phase: Once the cell have synthesized their requirements, they start growing at a constant rate. The rate of growth in log phase is expressed in generation time (doubling time) of the bacterial population which is constant for a particular species. The cells grow in a geometric fashion. At this phase the growth of the bacteria is at maximum rate. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

3. Stationary phase: The "stationary phase" is often due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of an inhibitory product such as an organic acid or accumulation of waste products. Stationary phase results from a situation in which growth rate and death rate are equal. The number of new cells created is limited by the growth factor and as a result the rate of cell growth matches the rate of cell death. The result is a "smooth," horizontal linear part of the curve during the stationary phase. This is a phase of cryptic growth.

4. Death phase: If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. This is the last phase of growth in a batch culture and the growth curve goes down. At this stage the death rate of bacteria exceeds the rate of formation of new cells.



Bacterial growth in batch culture

Ans.4.

Transformation in bacteria: Transformation is a genetic process by which free DNA is incorporated into a recipient cell and brings about genetic changes. Several prokaryotes are naturally transformable, including certain species of both gram-positive and gram-negative bacteria and also some species of Archaea.

Transformation process in bacteria was first discovered by Griffith while working on *Streptococcus pneumoniae*. The wild varieties of these bacteria (S strain = smooth colony forming) synthesize a polysaccharide capsule and cause disease in mouse leading to death. The mutant varieties of same bacteria (R strain = Rough colony forming) do not produce

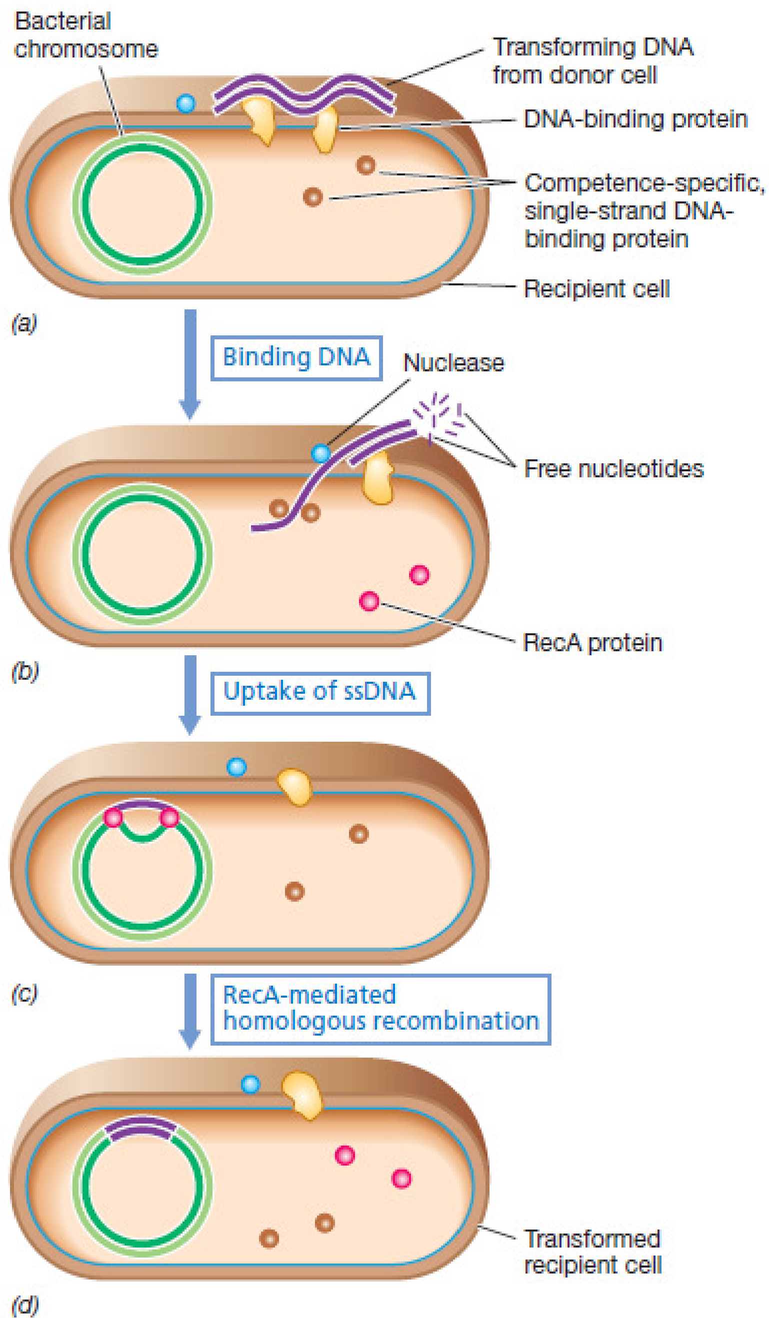
polysaccharide and thus cannot cause disease. When Griffith injected heat killed S strains of bacteria along with R strain into the mouse, they developed fatal infection and the bacteria isolated from the dead mouse were S type. The Griffith concluded that R cells had been transformed into a new type.

The bacterial competence for transformation is very limited confined to certain strains or species. A cell that is able to take up the DNA and become transformed is called Competent and this capacity is genetically determined. Competence in bacteria is regulated and special DNA binding proteins present on cell wall play a role in the uptake and processing of DNA. Some naturally competent bacteria are *Bacillus, Streptococcus, Haemophilus, Thermus, Neisseria* etc.

During transformation, competent bacteria reversibly bind DNA which later becomes non-reversible. The size of transforming DNA fragments are much smaller than that of the whole genome and fragments are further degraded during the uptake process. As these fragments are taken up, they are converted into single-stranded pieces of about 8 kb, with the complementary strand degraded. The DNA fragments in the mixture compete with each other for uptake. The maximum frequency of transformation that has so far been obtained is about 20% of the population; the usual values are between 0.1% and 1.0%. But when recipient population sizes arevery high, even this low frequency is easy to detect. The minimum concentration of DNA yielding detectable transformants is about 0.01 ng/ml, which is so low that it is chemically undetectable.

Integration of transforming DNA

Transforming DNA is bound at the cell surface by a DNA-binding protein. Next, either the entire double-stranded fragment is taken up, or a nuclease degrades one strand and the remaining strand is taken up, depending on the organism. After uptake, the DNA is bound by a competence-specific protein. This protects the DNA from nuclease attack until it reaches the chromosome, where the RecA protein takes over. The DNA is integrated into the genome of the recipient by recombination. If single-stranded DNA is integrated, a heteroduplex DNA is formed. During the next round of chromosomal replication, one parental and one recombinant DNA molecule are generated. On segregation at cell division, the recombinant molecule is present in the transformed cell, which is now genetically altered compared to its parent. The preceding applies only to small pieces of linear DNA. Many naturally transformable bacteria are transformed only poorly by plasmid DNA because the plasmid must remain double-stranded and circular in order to replicate.



Transformation in bacteria

Ans. 5.

Fermentation technology for production of Acetic acid:

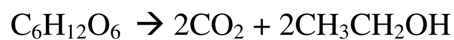
Acetic acid (CH3COOH) is the principal constituent of vinegar. The first vinegar was probably a result of spoiled wine, considering that the Latin word *acetum* means sour or sharp wine. Thus, it

has been produced as long as wine making has been practiced and therefore dates back to at least 10 000 BC. For most of human history, acetic acid was produced by fermentation of sugar to ethyl alcohol and its subsequent oxidation to acetic acid by microorganisms. This process was supplemented in the nineteenth century by wood distillation. In 1916, the first dedicated plant for the production of acetic acid by chemical rather than biological means became commercial. This method was based on acetylene-derived acetaldehyde, and it marked the advent of inexpensive, industrial-grade acetic acid and the birth of a viable industry based on its use.

Biologically, by fermentation acetic acid can be produced by either an anaerobic or aerobic route.

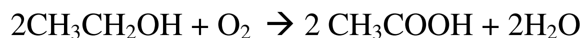
Aerobic Process

Food-grade acetic acid is produced by the two-step vinegar process. The first step is the production of ethanol from a carbohydrate source such as glucose. This is carried out at 30–32 -C using the anaerobic yeast *Saccharomyces cerevisiae.*



$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2CH_3CH_2OH$$

The second step is the oxidation of ethanol to acetic acid. Although a variety of bacteria can produce acetic acid, only members of *Acetobacter* are used commercially, typically the aerobic bacterium *Acetobacter aceti* at 27–37 _C. This fermentation is an incomplete oxidation because the reducing equivalents generated are transferred to oxygen and not to carbon dioxide. The overall theoretical yield is 0.67 g acetic acid per gram glucose.



$$2CH_3CH_2OH + O_2 \rightarrow 2\ CH_3COOH + 2H_2O$$

Anaerobic Process

In the 1980s, another process for production of acetic acid emerged based on anaerobic fermentation using *Clostridia.* These organisms can convert glucose, xylose, and some other hexoses and pentoses almost quantitatively into acetate according to the following reaction:

$$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$$

Clostridium thermoaceticum is also able to utilize five-carbon sugars.

A variety of substrates, including fructose, xylose, lactate, formate, and pyruvate, have been used as carbon sources in an effort to lower substrate costs. This factor is also important if cellulosic renewable resources are to be used as raw materials. Typical acidogenic bacteria are *Clostridium aceticum, C. thermoaceticum, Clostridium formicoaceticum,* and *Acetobacterium woodii.* Many can also reduce carbon dioxide and other one-carbon compounds to acetate.

‘Let Alone-method’: Industrial fermentation processes have evolved from the simple ‘let-alone’ method involving a partially filled open container of wine exposed to air to the ‘field’ fermentation in which a series of casks are filled with wine and inoculated in series by the vinegar produced in the previous casks.

‘Orleans’ method: In this method the holes are bored into the casks and a glass tube is inserted to allow the addition and removal of vinegar. The ‘trickling’ or ‘German’ process is a surface fermentation in which the microbial population is attached to an appropriate support (usually beechwood shavings) and the wine is trickled down while a large volume of air is sparged up through the bottom of the tank. This process was the basis for the manufacture of the trickling generator that incorporates forced aeration and temperature control. The partially converted solution collects at the bottom and is cooled, pumped back up to the top, and allowed to trickle down until the reaction is complete. Ethanol conversion into acetic acid is 88–90%; the rest of the substrate is used in biomass production or lost by volatilization. Advantages of this process include low costs, ease of control, high acetic acid concentrations, and lower space requirements. The costs of the wood shavings, long startup time, loss of ethanol by volatilization, and production of slime-like material by the *Acetobacter* (e.g., A. *xylinum*) are some of the disadvantages. Furthermore, there are often local zones of overoxidation, uneven aeration, and heat development.

Downstream Processing

Downstream processing refers to the series of unit operations used to isolate, purify, and concentrate the product. Downstream processing often determines the economic feasibility of the process. The first operation is cell separation, which can be done by cross-flow microfiltration. When a microfilter or ultrafilter is combined in a semi-closed loop configuration to the bioreactor or fermenter, it becomes a powerful tool to dramatically improve the productivity of the fermentation while simultaneously providing a cell-free broth for subsequent downstream processing. Other membrane technologies, such as nanofiltration and electrodialysis, are useful in subsequent stages of downstream processing to separate and perhaps concentrate the acid.

Ans. 6.

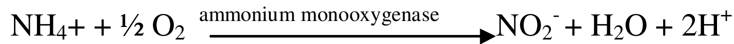
Ammonification: It is the process of microbial decomposition in which the organic nitrogen (amino acids, nucleotides, proteins etc.) is converted into ammonia by a variety of microorganism. This process is called ‘ammonification’. Some of the ammonia may be volatilize and re-enter the atmosphere but most of it is recycled to nitrate by soil bacteria. Upon proteolysis of proteins, amino acids are produced which are subjected to various pathways for microbial decomposition. During oxidative deamination amino acids are removed and the end product comes as ammonia.

$CH_3CHNH_2COOH + {}^1/_2\, O_2 \rightarrow \text{Pyruvic acid} + NH_3$

Another process contributing to generation of ammonia is respiratory reduction of nitrate to ammonia which is also known as Dissimilative Reduction of Nitrate to Ammonia (DRNA). This process dominates in anoxic environment such as highly organic marshy sediments.

The ammonifying microbes are *Pseudomonas*, *Bacillus*, *Closteridium*, *Micrococcus*, *Mucor*, *Rhizopus*, *Penicillium*, and actinomycetes. These organisms synthesize extra-cellular proteolytic enzymes for decomposition of proteins.

Nitrification: The process in which microorganisms convert ammonia to nitrate is called nitrification and such microorganisms are called nitrifying bacteria. It is a 2 step aerobic process in which some species oxidize ammonia to nitrite and then another species oxidize nitrite to nitrate. No any bacteria is known today which can convert ammonia in to nitrate directly.



$$NH_4+ + ½ O_2 \xrightarrow{\text{ammonium monooxygenase}} NO_2^- + H_2O + 2H^+$$

This process is carried out by *Nitrosomonas, Nitrosococcus, Nitrosolobus, Nitrosospira* etc.

This nitrite is further oxidized by another set of bacteria in to nitrate like *Nitrobacter, Nitrococcus, Nitrospira* etc.

$$NO_2^- + H_2O \xrightarrow{\text{nitrite oxidoreductase}} H_2O.\ NO_2^- \longrightarrow NO_3^- + 2H^+$$

The nitrifying bacteria are chemoautotrophic bacteria which obtain energy by oxidizing inorganic substances such as ammonium and nitrite which is used to convert CO_2 to organic carbon. The nitrifying bacteria are widespread in soil and water but present in highest number in habitats where ammonia is abundant like sites with extensive protein decomposition and sewage treatment facilities. Some archebacteria in ocean can also oxidize ammonia as chemolithotrophic substrate.

Denitrification: In this process some bacteria reduce nitrate to nitrogen which is then returned to the atomosphere. Thus they complete with the plants in taking up the nitrate from the soil. About 93 million to 190 million metric tons of nitrogen is lost by denitrification annually. The denitrifying bacteria use nitrate as electron acceptors in anaerobic respiration which is reduced to nitrous oxide, nitric oxide and dinitrogen. Because, these products are all gaseous they can easily be lost from the environment by process of denitrification. Denitrifying bacteria are metabolically diverse, they are primarily aerobic when oxygen is available but can reduce other electron acceptors like ferric ions and certain other organic electron acceptors besides nitrate. Therefore, denitrification is also termed as Nitrate Respiration. There is only one eukaryote Protozoan which is a foraminiferan *Globobulimina pseudospinescens* which can act as denitrifier to survive in anoxic marine sediments.

Ans. 7.

Use of microorganisms in genetic engineering

Microorganisms have played enormous role in growth and development of genetic engineering. The genetic engineering relies on the ability to manipulate molecules in vitro. Most biomolecules exist in low concentrations and as complex, mixed populations which it is not possible to work

with effectively. Bacteria, particularly, Escherichia coli solved this problem by acting as a vector molecule. By inserting a piece of DNA of interest into vector molecule a large number of identical copies are produced. Together with rapid growth of bacterial colonies all derived fom a single original cell bearing the recombinant vector, in a short time a huge amount of DNA of interest is produced. Most of the vector molecules are derived from two sources:

1. Plasmid: these are small, autonomously replicating circular pieces of bacterial DNA, which often carry antibiotic resistant genes

2. Bacteriophages – are viruses which infect the bacteria.

Agrobacterium act as source of Ti-plasmid which is used to insert the cloned genes into the dicots.

Bacteria are the source of restriction endonuclease enzymes which are known as molecular scissors. It cut the DNA at specific places. The endonuclease enzyme which produce staggered cut having a tail at both the ends are most commonly used in genetic engineering. Eg. EcoRI enzyme is derived from *Escherichia coli*, BamHI form *Bacillus amyloliquefaciens*.

Bacteria are the source of enzyme DNA ligase which provides the ability to rejoin cut fragments of DNA and form artificial recombinant molecules. The ligase encoded by bacteriophage T4 is particularly valuable. The source of reverse-transcriptase enzyme is also the bacteria. Bacteria and yeast are used as store house for keeping the desired genes in gene bank and genome bank.

DNA polymerase enzymes are used for replication of DNA and for Polymerase Chain Reactions to synthesize DNA from pre-existing template in vitro. Most commonly used enzyme Taq DNA Polymerase has been derived from bacterium *Thermus auaticus*, *Pyrococcus furiosus*. This enzyme has the capacity to withstand the high temperature of the PCR without compromising its ability. This technique has speed up the process of cloning enormously.

Ans. 8.

Methanococcus: This bacteria belong to group 'The Methanogens' and have been placed in to group 3 subgroup 2 according Bergey's Mannual of Systematic Bacteriology. All methanogens produce methane as a major catabolic product.

These bacteria have cells without muvein, without outer membrane to cell wall. Their membrane is composed of ether-linked isoprenoid. Methanococcus are very strict anaerobic, chemoautotrophic or chemoheterotrophic. Hydrogen, carbon di oxide, formate, acetate, methanol etc. serve as carbon source and energy source. Ammonia serve as nitrogen source.

Methanogens have been divided into 3 taxonomic subgroups and Methanococcus have been included into 2nd sub group. These bacteria are irregular, coccoid in shape. They grow on H_2 +

CO, formate or alcohol + CO_2, pseudomurein is absent from cell wall and cells are lysed by detergents and hypotonic sock. Cells are non-motile and strictly anaerobe.

Size of these bacteria is about 1-2 µm in diameter, and they are non-endospore forming bacteria. These bacteria are mesophilic grow mainly at temperature between 35-40° C, some are thermophilic which can tolerate up to 65° C. They use H_2+ CO_2 and usually formate are catabolic susbstrate. These bacteria are found in anoxic, salt, marsh, marine, esturine environment.

Halobacterium: These bacteria have been placed in group 33 according to Bergey's Mannual of Systematic Bacteriology which includes extremely halophilic, aerobic archebacteria. These bacteria are found in neutral, highly saline environment like salt-lake, marine salterns and are associated with spoilage of salt fish and hides.

Halobacterium is irregular rod-shaped 0.5-1.2 x 1.0-6.0µm in diameter. They form characteristic flat-cell morphology and exhibit pleomorphic forms from regular rods to irregular ribbon shaped cells. These bacteria lyse when suspended in distilled water. Inside cell gas-vacuoles may be present. Colonies are various shades of red because of presence of carotenoid pigments and may become pink or white when gas-vacuoles are present. Purple Halobacterium species have a light sensitive protein 'bacteriorhodopsin' which provides chemical energy fro the cell by suing sunlight to pump protons out of cell membrane. This protein is very much similar to rhodopsin pigment found in vertebrate retina.

They are mainly aerobic; some are able to grow anaerobically in presence of nitrate. They are chemo-heterotrophic in nature, carbohydrate, alcohol, carboxylic acid, amino acids serve as carbon and energy source. These bacteria require atleast 1.5 M NaCl for growth but optimally grow at 2-4 M NaCl. Some members are alkalophilic growing at pH 8.5. The lipid content in plasma membrane is characterized by presence of diphytanyl derivatives of phosphatidyl glycerol sulphate.