Answer Keys/ Model Answer
AS-2245
M. Sc. (3rd Semester) Examination, 2013
Biotechnology

Section A **10 X 2 = 20**

Objective type questions

1. Choose the correct alternative

i) (a) $dp / dt = q_p X - D p$
ii) (b) $dp / dt = 0$
iii) (a) $\tau = V/F$
iv) (c) chemostat
v) (c) Liquid shear
vi) (a) Liquid-liquid extraction
vii) (b) Clostridium
viii) (b) Acetyl Co A
ix) (c) Paneer
x) (b) 138 ºC

Section B

(Descriptive type question) 4 X 10 = 40

2. Explain the process for isolation of industrially important micro-organism.

Ans: The screening and isolation of industrially important microorganisms for the production of useful products involves screening followed by obtaining either pure or mixed cultures and further their assessment to determine which carry out the desired reaction or produce the desired product. The isolate must eventually carry out the process economically. Therefore, the selection of the culture to be used is a compromise between the productivity of the organism and the economic constraints of the process.

Criteria as being important in the choice of organism:

1. the nutritional characteristics of the organism: using a very cheap medium or a pre-determined one.
2. the optimum temperature of the organism.
3. the reaction of the organism with the equipment to be employed and the suitability of the organism to the type of process to be used.
4. the stability of the organism and its amenability to genetic manipulation.
5. the productivity of the organism, measured in its ability to convert substrate into product and to give a high yield of product per unit time.
6. the ease of product recovery from the culture.

The isolation of Industrially Important Micro-organism

- **Isolation methods utilizing selection of the desired characteristic** involve most important technique called Enrichment culture. The recognition that microorganisms in nature always exist in a mixture demanded that for the study of various physiological types, pure cultures were essential. By applying this method, result in increase of number of the organism relative to the number of the others in the original inoculum. And it starting from a mixed population and reaching to a single organism. Enrichment technique performed by 2 ways

  1. enrichment liquid culture
  2. enrichment cultures using solidified media

- **Isolation methods not utilizing selection of the desired characteristic**
  1. Screening methods

Isolation methods not utilizing of the desired characteristic

- The synthesis of some products does not give the producing organism any selective advantage which may be exploited directly in the isolation procedure. Example include Production of antibiotics and growth promoters
- Therefore, a pool of organisms has to be isolated and subsequently tested for the desired characteristic.
- The major problem is the reisolation of strains which have already been screened many times before. However the problem will be minimized by two ways

  1. Developing procedures to favor the isolation of unusual texa which are less likely to have been screened previously.

  2. Identifying selectable features correlated with the unselected industrial trait enabling an enrichment process to be developed.
- An environmental sample contains a vast variety of organism. Design of isolation media based on knowledge of only one taxon may inadvertently result in the preferential isolation of undesirable types.

3. What is del factor? Elucidate on methods of heat sterilization for achieving a desired del factor.

Ans: Culture media (for microorganism) may be sterilized by filtration, radiation, ultrasonic treatment, chemical treatment or heat. However, for practical reasons, steam is used almost universally for the sterilization of fermentation media. The destruction of micro-organisms by steam (moist heat) may be described as a first-order chemical reaction and, thus, may be represented by the following equation:

$$-dN/dt = kN$$

Where N is the number of viable organisms present,

t is the time of the sterilization treatment,

k is the reaction rate constant of the reaction, or the specific death rate.

On integration of equation the following expression is obtained:

$$N_t / N_0 = e^{-kt}$$

where No is the number of viable organisms present at the start of the sterilization treatment,

Nt is the number of viable organisms present after a treatment period, *t.*

On taking natural logarithms, equation is reduced to:

$$\ln (N_t / N_0) = -kt$$

A plot of the natural logarithm of N_t / N_0 against time yields a straight line, the slope of which equals - *k.* This kinetic description makes two predictions which appear anomalous:

(i) An infinite time is required to achieve sterile conditions (i.e. N= 0).

(ij) After a certain time there will be less than one viable cell present.

Thus, in this context, a value of *Nt* of less than one is considered in terms of the probability of an organism surviving the treatment.

As with any first-order reaction, the reaction rate increases with increase in temperature due to an increase in the reaction rate constant, which, in the case of the destruction of micro-organisms, is the specific death rate *(k).* Thus, *k* is a true constant only under constant temperature conditions. The relationship between temperature and the reaction rate constant

was demonstrated by Arrhenius and may be represented by the equation:

$$\text{din } k / dT = E / RT^2$$

where *E* is the activation energy, *R* is the gas constant, *T* is the absolute temperature.

On integration equation it gives:

$$k = A\, e^{-E/RT}$$

where *A* is the Arrhenius constant.

On taking natural logarithms, equation becomes

$$\text{In } k = \text{In } A^{-E/RT}$$

From equation it may be seen that a plot of In *k* against the reciprocal of the absolute temperature will give a straight line. Such a plot is termed an Arrhenius plot and enables the calculation of the activation energy and the prediction of the reaction rate for any temperature. By combining together equations the following expression may be derived for the heat sterilization of a pure culture at a constant temperature:

$$\text{In No/Nt} = A \cdot t \cdot e^{-E/RT}$$

Deindoerfer and Humphrey used the term In *No/* Nt as a design criterion for sterilization, which has been variously called the **Del factor**, Nabla factor and sterilization criterion represented by the term V. Thus, the Del factor is a measure of the fractional reduction in viable organism count produced by a certain heat and time regime. Therefore:

$$\nabla = \ln (N_0/N_t)$$

but $$\ln(N_0/N_t) = kt$$

and $$kt = A \cdot t \cdot e^{-(E/RT)}$$

thus $$\nabla = A \cdot t \cdot e^{-(E/RT)}.$$

The **del factor** is dependent on a temperature and time regime.

A short time and high temperature regime with rapid cooling gives advantage in terms of achieving the desired del factors

For eg to achieve a del factor of 45.7 two regime may be used i.e 130^0C with holding time of 2.44 minutes or 150^0 C with holding time of 2.7 seconds.

4. Describe the role of biotransformation processes for production of novel products.

Ans: Biotransformation: Microorganisms are extremely useful in carrying out biotransformation processes in which a compound is converted into a structurally related product by one or a small number of enzymes contained in the cells. Biotransformation is one of the important experimental methods to identify compounds, whose structure and properties resembles the parent compound. **"**Biotransformation is use of biological catalyst to convert a substrate into a product in a limited number of steps. There are two major classes of biotransformation: whole cells and enzymes. Whole cells are generally cheap, but some experience with microbiological techniques is required (except for bakers' yeast). The major advantage of biotransformations over abiotic asymmetric catalysts is that a successful process can tap the vast resources available in biotechnology.

Significance of Biotransformation:

During biotransformation, a particular drug is treated with microsomal proteins and screened for possible metabolites. This helps to identify phase-I metabolites, that may result due to oxidation,

reduction, dealkylation, epoxidation, dehalogenation, oxidative deamination, etc. Compounds upon treatment with hepatocytes or cytosol could lead to both Phase-I and Phase-II metabolites. The most common phase-II metabolites are conjugate metabolites, which are directly formed from the parent drug, or from the phase-I metabolites. Glucuronide conjugates; sulfate conjugates, methylation, acetylation, sulfation, aminoacid conjugation and glutathione conjugation are considered as common phase-II metabolites.

Biotransformation also helps in optimizing the structure of the drug. For example, formation of glutathione adducts in a specific region of the molecule suggests the possibility of toxicity in the human system. This vital piece of information helps to perform necessary modifications in the identified site, and prevents the compound from resulting in toxicity.

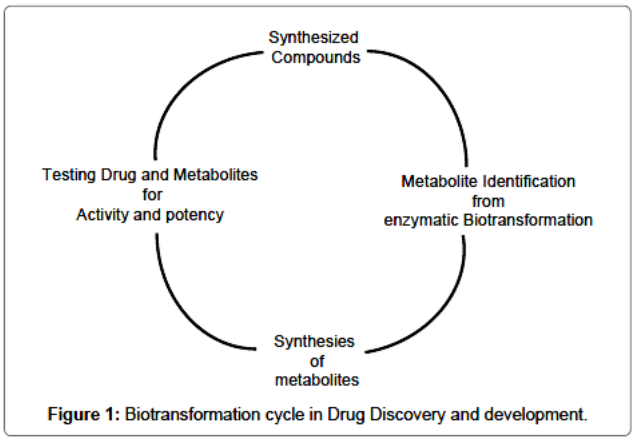
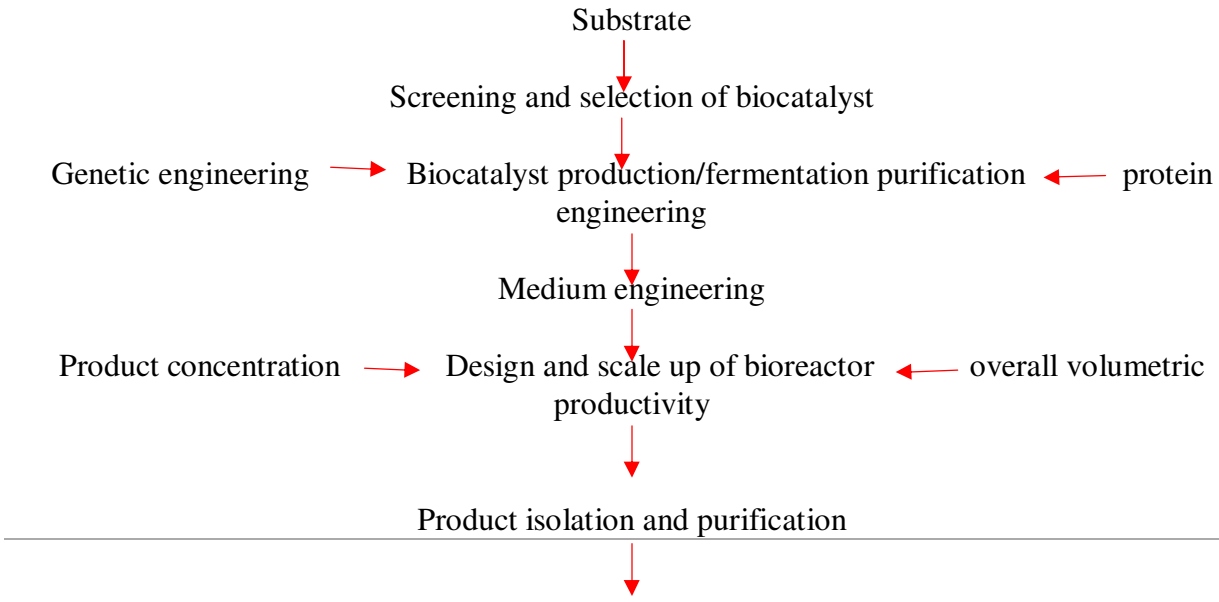


Figure 1: Biotransformation cycle in Drug Discovery and development.

Application of biotransformation:

1. Synthesis of oligosaccharides
2. Synthesis of C-C bonds
3. Synthesis of chiral intermediates
4. Redox biocatalysis (oxidative and reductive)
5. Regeneration of co-factors
6. Combinatorial biocatalyst

Biotransformation process:



5. What are the parameter that perturb steady state of a bioreactor? How a bioreactor can be sterilized?

Key factors that influence the steady state of a bioreactor are as follows:

1. Inoculum propagation procedures adopted, and the quality and quantity of inoculum used to start the fermentation.
2. Choice of medium; cheaper nutrient sources are often employed for large-scale operations due to cost constraints.
3. Industrial-scale sterilization protocols may result in greater degradation of heat-labile compounds, which affects the final quality of the medium.
4. Larger fermenters are often subject to the development of nutrient, temperature, pH and oxygen gradients, which were not experienced in smaller, well mixed, fermenters.
5. Scale-up can also alter the generation of foam, shear forces and rate of removal of carbon dioxide.

Sterilization of bioreactor:

- **Air sterilization**
  To prevent contamination of either the fermentation by air-borne microorganisms or the environment by aerosols generated within the fermenter, both air input and air exhaust ports have air filters attached. These filters are designed to trap and contain microorganisms. Filters are made of glass fibre, mineral fibres, polytetrafluoroethylene (PTFE) or polyvinyl chloride (PVC), and must be steam sterilizable and easily changed. In some circumstances, particularly where pathogenic organisms are being grown, fermenter exhaust may also undergo dry heat sterilization (incineration) as an additional safety measure.
- **Media and vessel sterilization**
  For pilot-scale and industrial aseptic fermentations the fermenter can be sterilized empty. The vessel is then filled with sterile medium, prepared in a batch or continuous medium 'cooker' that may supply several fermentations. Alternatively, the fermenter is filled with formulated medium and the two are sterilized together in one operation.
- **Continuous sterilization**
  Direct heat exchangers
  Indirect heat excahgers
  Sterilization of feeds
  Sterilization of liquid wastes

6. Describe the methods that can be used for cell disruption for further isolation of enzymes.

Ans: During production of desire products (including enzyme, biomolecules, specific compounds) some target products are intracellular, including many enzymes and recombinant proteins, several of which form inclusion bodies. Therefore, methods are required to disrupt the microorganisms and release these products. The breaching of the cell wall/envelope and cytoplasmic membrane can pose problems, particularly where cells possess strong cell walls. For example, a pressure of 650 bar is needed to disrupt yeast cells, although this may vary somewhat at different times during the growth cycle and depending upon the specific growth conditions.

General problems associated with cell disruption include the liberation of DNA, which can increase the viscosity of the suspension. This may also affect further processing, such as pumping the suspension on to the next unit process and flow through chromatography columns. A nucleic acid precipitation step or the addition of DNase can help to prevent this problem. If mechanical disruption is

used then heat is invariably generated, which denatures proteins unless appropriate cooling measures are implemented. Products released from eukaryotic cells are often subject to degradation by hydrolytic enzymes (proteases, lipases, etc.) liberated from disrupted lysosomes. This damage can be reduced by the addition of enzyme inhibitors, cooling the cell extract and rapid processing. Alternatively, attempts may be made to produce mutant strains of the producer microorganism lacking the damaging enzymes.

Cell disruption can be achieved by both mechanical and non-mechanical methods. The disruption process is often quantified by monitoring changes in absorbance, particle size, total protein concentration or the activity of a specific intracellular enzyme released into the disrupted suspension.

Physico mechanical cell disruption methods

Several mechanical methods are available for the disruption of cells.

1. Liquid shear
2. Solid shear
3. Agitation with abrasive
4. Freeze- thawing.
5. Ultrasonication

Chemical Methods

1. Detergents
2. Osmotic shock
3. Alkali Treatment
4. Enzymatic Treatment

Those based on **solid shear** involve extrusion of frozen cell preparations through a narrow orifice at high pressure. This approach has been used at the laboratory scale, but not for large-scale operations. Methods utilizing **liquid shear** are generally more effective. The French press (pressure cell) is often used in the laboratory and the high-pressure homogenizers, such as the Manton and Gaulin homogenizer (APV-type mill), are employed for pilot- and production-scale cell disruption. They may be used for bacterial and yeast cells, and fungal mycelium. In these devices the cell suspension is drawn through a check valve into a pump cylinder. At this point, it is forced under pressure (up to 1500bar) through a very narrow annulus or discharge valve, over which the pressure drops to atmospheric. Cell disruption is primarily achieved by high liquid shear in the orifice and the sudden pressure drop upon discharge causes explosion of the cells. The rate of protein release (efficiency of disruption) is independent of the cell concentration, but is a function of the pressure exerted, the number of cycles through the homogenizer and the temperature. Disruption of yeast cell preparations, for example, typically requires three passes through the pressure cell at 650 bar, whereas wild-type *Escherichia coli* generally needs 1100–1500 rials are released. As a result, the product of interest must be separated from a complex mixture of proteins, nucleic acids and cell wall fragments. Some fragments of cell debris are not readily separated, making the solution difficult to clarify. In addition, proteins may be denatured if the equipment is not sufficiently cooled and filamentous microorganisms may block the discharge valve. When used for certain categories of microorganisms, the homogenizers have to be contained to prevent the escape of aerosols.

On a small scale, manual grinding of cells with abrasives, usually alumina, glass beads, kieselguhr or silica, can be an effective means of disruption, but results may not be reproducible. In industry, **high-speed bead mills**, equipped with cooling jackets, are often used to agitate a cell suspension with small beads (0.5–0.9μm diameter) of glass, zirconium oxide or titanium carbide. Cell breakage results from shear forces, grinding between beads and collisions with beads. The efficiency of cell breakage is a function of agitation speed, concentration of beads, bead density and diameter, broth density, flow rate and temperature. Cell concentration is also a major factor (optimum 30–60% dry weight), which is an important difference from the liquid shear homogenizers described above. Maximum throughput in these systems is about 2000 L/h.

Ultrasonic disruption of cells involves **cavitation**, microscopic bubbles or cavities generated by pressure waves. It is performed by ultrasonic vibrators that produce a high-frequency sound with a wave

density of approximately 20 kilohertz/s. A transducer converts the waves into mechanical oscillations via a titanium probe immersed in the concentrated cell suspension. However, this technique also generates heat, which can denature thermolabile proteins. Rod-shaped bacteria are often
easier to break than cocci, and Gram-negative organisms are more easily disrupted than Gram-positive cells. Sonication is effective on a small scale, but is not routinely used in large-scale operations, due to problems with the transmission of power and heat dissipation. Cell disruption is a somewhat neglected area of bioprocessing, as there has been relatively little innovation and progress. Even the routinely used established mechanical methods were originally devised for other purposes.
However, some newer disruption systems are being developed to give improved large-scale disruption, often with integral containment. They include a newly designed ball mill, the CoBall Mill; the Constant Systems high-pressure disrupter, which operates at up to 2700bar; and two systems with no moving parts, the Microfluidics impingement jet system and the Glass-col
nebulizer. The Parr Instruments cell disruption bomb is designed for disrupting mammalian cells. This is a relatively gentle method that works on the principle of nitrogen decompression and does not generate heat. Nitrogen is dissolved in cells under high pressure, and sudden pressure release then causes the cells to burst.

Non-mechanical cell disruption methods

An alternative to mechanical methods of cell disruption is to cause their permeabilization. This can be accomplished by autolysis, osmotic shock, rupture with ice crystals (freezing/thawing) or heat shock. Autolysis, for example, has been used for the production of yeast extract and other yeast products. It has the advantages of lower cost and uses the microbes' own enzymes, so that no foreign substances are introduced into the product. Osmotic shock is often useful for releasing products from the periplasmic space. This may be achieved by equilibrating the cells in 20% (w/v) buffered sucrose, then rapidly harvesting and resuspending in water at 4°C.

A wide range of other techniques have been developed for small-scale microbial disruption using various chemicals and enzymes. However, some of these can lead to problems with subsequent purification steps. Organic solvents, usually acetone, butanol, chloroform or methanol, have been used to liberate enzymes and other substances from microorganisms by creating channels through the cell membrane. Simple treatment with alkali or detergents, such as sodium lauryl sulphate or Triton X-100, can also be effective. Several cell wall degrading enzymes have been successfully employed in cell disruption. For example, lysozyme, which hydrolyses b-1,4 glycosidic linkages within the peptidoglycan of bacterial cell walls, is useful for lysing Gram-positive organisms. Addition of ethylene diamine tetraacetic acid (EDTA) to chelate metal ions also improves the effectiveness of lysozyme and other treatments on Gram-negative bacteria. This is because EDTA has the ability to sequester the divalent cations that stabilize the structure of their outer membranes. Enzymic destruction of yeast cell walls can be achieved with snail gut enzymes that contain a mixture of b-glucanases. These enzyme preparations are also useful for producing living yeast spheroplasts or protoplasts.

The antibiotics penicillin and cycloserine may be used to lyse actively growing bacterial cells, often in combi-bar. During processing the temperature rises by about 2.2–2.4°C per 100 bar, i.e. by approximately 20°C over one pass at 800 bar. Consequently, precooling of the cell preparation is usually essential. The energy input necessary is approximately 0.35kW per 100bar and the throughput is up to 6000 L/h. A problem with this method of cell disruption is that all intracellular matenation with an osmotic shock. Other permeabilization techniques include the use of basic proteins such as protamine; the cationic polysaccharide chitosan is effective for yeast cells; and streptolysin permeabilizes mammalian cells.

7. Give an account of the organic acid production by fermentation.

What are Organic Acid?

This is defined as an organic compound that is an acid. The most common type of this would be the carboxylic acid group. Organic acids possess a long chain of carbons attached to a carboxyl group. This

category of acid is used widely in the food industry as both a food additive and is used also in chemical feedstocks. Fermentation processes play a major role in the production of most organic acids.

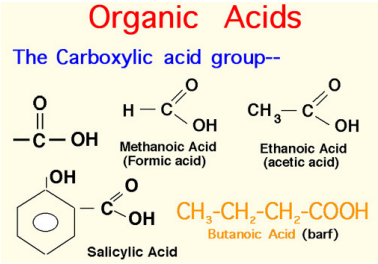


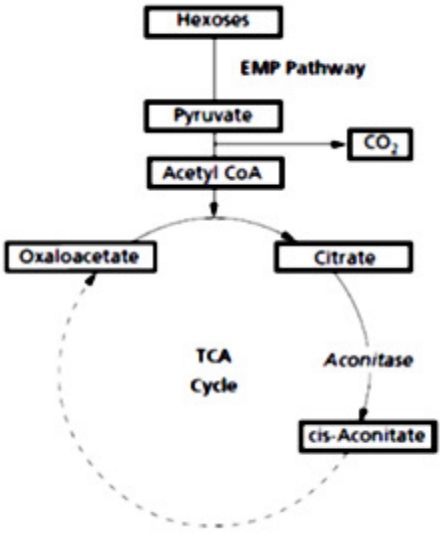
Figure 1. Diagram of the Carboxylic acid group

All acids of the tricarboxylic acid (TCA) cycle can be produced microbially in high yields, other acids can be derived indirectly from the Krebs cycle (pseudonym for the TCA cycle) such as itaconic acid, or can be derived directly from glucose (gluconic acid).

Some acids are formed as the end products from pyruvate or ethanol (lactic and acetic acid). Fungi, in particular *Aspergilli*, are well known for their potential to overproduce a variety of organic acids. These microorganisms have an intrinsic ability to accumulate these substances and it is this that provides the fungi with an ecological advantage, since they grow rather well at pH 3 to 5, while some species even tolerate pH values as low as 1.5.

Organic Acids Produced in Microbial Fermentations:

1. **Citric acid:**



Citric acid biosynthesis.

2. ***Gluconic Acid***

Gluconic acid (2,3,4,5,6-Pentahydroxycaproic acid) is used in the food and beverage, pharmaceutical, detergent and construction industries. Calcium and ferrous glutamate are used in calcium and iron deficiencies. It is essential to the tanning process of leather and is also used in metal processing. Sodium glucanate is used in cement mixes and is also used in the presence of sodium hydroxide as a sequestering agent of calcium in glass bottle washing. An enzyme, glucose oxidase, which is also produced in gluconic acid fermentation is used widely in the food industry as a flavour and colour stabilizer. More importantly in terms of health and disease, this enzyme is used in diagnostic kits to assay glucose in the urine of

diabetes patients. Similarly to citric acid fermentation, gluconic acid is produced using the organism *Aspergillus niger*. The growth medium in this fermentation is based on glucose and corn steep liquor with other nutritional requirements including ammonium salts and urea. Too much nitrogen leads to excessive growth of organism therefore producing lower acid yields. The production pH is maintained at 6.0- 7.0 until optimum growth and a set glucose oxidase level has been achieved. After this point, the pH may be allowed to drop to 3.5. The temperature is maintained throughout the process at 33°C. A great attribute of gluconic acid production is that yield exceeds 90% theoretical yield. This fermentation requires a vast amount of aeration, as the process is highly aerobic. Turbine impellers in baffled culture vessels provide this high aeration requirement. The biochemistry of gluconic acid (gluconate) production is also well understood. Unlike citric acid, where glucose is taken up by the organism and converted to citric acid, gluconic acid is produced extracellularly. In *A. niger,* the spontaneous conversion of β-DGlucose from α-D-Glucose is accelerated by the enzyme mutarose. This β-D-Glucose is converted to D-glucono-δ-lactone by glucose oxidase. This enzyme is induced by glucose presence at pH values >4 and is denaured below pH2. The conversion of D-glucono-δ- lactone to D-Gluconic acid is spontaneous at neutral pH. At lower pH, this is conversion is less effective and is facilitated by D-glucono-δ-lactonase by *A. niger*. This enzymatic process has the added advantage that no product purification steps are required after the fermentation.

3. Lactic acid

The first microbial production of an organic acid was lactic acid production in 1880. Lactic acid is produced in fermentation by gram-positive bacteria. It plays a role in the manufacturing of many food and drink products. Today, chemical methods for the production of lactic acid are the same cost as biological processes and so are very competitive. There are two kinds of recognised lactic acid bacteria, homofermentative and heterofermentative. The heterofermentative organisms produce a large number of byproducts and as such are not suitable for commercial purposes. With the homofermentative bacteria, only very little substrate is converted into by-products and cell material and a very large portion is metabolised into lactic acid making these homofermentative bacteria the much-preferred choice commercially.

The biosynthesis of lactic acid from glucose proceeds via glyceraldehyde-3-P, 1,3-di-Pglycerate and pyruvate. Theoretically, up to two moles of lactic acid are produced from 1 mole of glucose. Over 90% of this potential yield is actually attained in practice. Depending on which substrate is used in the fermentation, different microorganisms are used in lactic acid production. These are *Lactobacillus delbrueckii* and *L. leichmannii* when glucose is used as substrate, *Lactobacillus bulgaricus* with whey and *Lactobacillus pentosus* with sulfite waste liquor. All of the above organisms are facultative anaerobes and therefore, the bioreactors need not be run with complete oxygen exclusion. Lactic acid is used in a variety of food products including coffee and tea. An estimated 40,000 tonnes per year is produced worldwide. It has a high production cost and new technology is currently being investigated to try and lower the production costs and increase the market.

8. Give an account of cheese production with the help of suitable example.

Cheese production

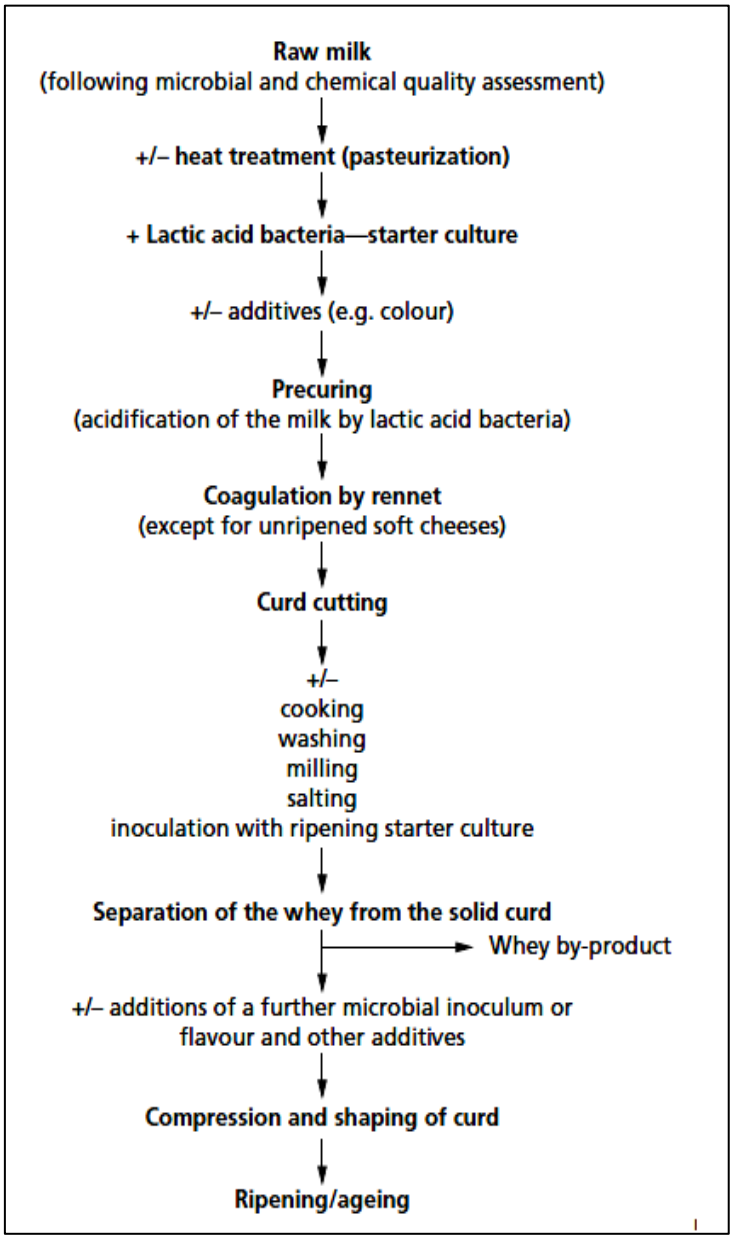
The bulk of world cheese production, which is now over 10^{10} kg/annum, is from 5\$10^{10}$ L of cow's milk. Cheese making essentially involves concentration of milk fat and protein by removing water. However, there are numerous different types of cheese with widely varying texture and flavour. Textures range from soft through to very hard, and flavours vary from mild to very strong. The vast range is due to differences in:

1 the type of milk used;

2 the diet of the milk producer, which in turn is influenced by the local soil, vegetation and climatic factors;

3 the particular strain of microorganisms used for inoculation at each phase of production, including starter and ripening stages, which contributes different organoleptic compounds and modifies the texture;
4 the processing methods employed; and
5 the environmental conditions during ripening, particularly temperature and humidity.

There is no standard method of cheese making, as limitless variations exist for all stages of the process and a vast quantity is still produced with little or no reference to the underlying science. However, for most cheeses, the process basically involves (Fig):
1 pretreatment of raw milk;
2 formation of solid curd;
3 removal of the liquid whey from the curd;
4 curd processing; and
5 ripening and ageing.



First, raw milk is checked for various chemical and microbiological quality parameters and then pasteurized. The production of coagulated milk proteins or curd is then achieved by the activities of lactic acid bacteria, such as *Lactococcus lactis*, *L. cremoris* and *Streptococcus thermophilus*. These bacteria have the ability to lower the pH through the fermentation of lactose to lactic acid, which facilitates protein coagulation. They also influence the flavour of the final product by producing specific flavorur and aroma compounds, and perform essential proteolysis and lipolysis in later maturation.

A mixed starter culture is often used, consisting of several strains of these mesophilic or thermophilic streptococci and lactobacilli, which may be prepared in heat treated milk or whey-based media. Use of defined starter cultures reduces batch-to-batch variations in both production time and levels of acid generated. An inoculum of 0.5–2.0% (v/v) is added and the fermentation is performed at around 32°C for 10–75 min. For some cheeses this may be further controlled by heat treatment at 55°C, which inhibits mesophiles and promotes the action of thermophiles. Thus, the initial selection of suitable microbial strains, the amount of starter culture used, the length of preripening and the incubation temperature are important in creating many subtle differences in the final colour, flavour and aroma.

Curd formation may be promoted, in all but soft, unripened cheeses such as cottage and cream cheeses, by the addition of specific proteolytic enzymes. Traditionally, rennin (chymosin, aspartic protease EC 3.4.23.4) is used, which is prepared in a crude form from the stomach, abomasum, of veal calves and is referred to as **rennet**. Due to a shortage of available calf chymosin and the requirement for

so-called ‘vegetarian’ cheeses, fungal proteases with similar properties to calf chymosin are now also employed. In addition, the calf chymosin gene has been introduced into several microorganisms for the commercial production of recombinant enzyme.

The milk component primarily involved in curd for mation is the protein casein, which is a mixture of α-1, α-2, β and κ caseins. k casein is important in maintaining the colloidal stability of milk proteins. Addition of rennin results in the removal of surface glycopeptide from the k casein. Consequently, the casein becomes unstable and aggregates in the presence of calcium ions to form a gel. As the gel forms it entraps fats and ultimately forms white creamy lumps, referred to as curd. Precipitated curd is soft but can be readily separated from the liquid whey by holding the mixture in cheese cloth. Semi-dried curd that remains in the cheese cloth is usually salted and other ingredients may be added, such as colouring agents, herbs, or a further microbial inoculum. It is then pressed and placed into a shaping mould or cut into blocks. For many cheeses this is followed by a period of ripening or ageing to develop the final flavour and texture.

Ripening involves the modification of proteins and fats by microbial and milk proteases and lipases that remain in the young cheese. Some countries allow acceleration of flavour development through the addition of commercial enzyme preparations. Lysozyme may also be added in the manufacture of hard-cooked Emmental, Gouda and Gruyère-type cheeses to prevent growth of the spo ilage organism, *Clostridium tyrobutyricum*, which otherwise would be troublesome in the later stages of ripening. In addition, the curd may have been inoculated with a bacterial or fungal culture before ripening. For example, *Propionibacterium freundenreichii* ssp. *shermanii* is used for the production of Swisstype cheeses, e.g. Emmental and Gruyère. As these bacteria grow they modify the flavour and generate gas bubbles that result in holes or eyes within the cheese.

Internally mould-ripened blue-veined cheeses, including Danish Blue, Gorgonzola, Roquefort and Stilton, primarily use *Penicillium roqueforti*. Traditional manufacture relies on the natural development of the mould that originates from spore populations that become established in the local cheese-making environment. Large-scale production now uses spore inocula, which are mixed into the curd before the cheeses are pressed and formed. Young cheeses are usually punctured with stainless steel rods to promote fungal growthby increasing oxygen levels within the cheese and then stored under controlled humidity at around 9°C. Cheeses are ripened for up to a year during which they develop the characteristic blue veins, and the aroma and flavour that is due to methyl ketones such as 2- heptanone.

Camembert-type and other surface-ripened cheeses use *Penicillium camemberti,* which originates naturally from the environment, or the surface of the cheese may be sprayed with a spore inoculum. The mould grows on the surface of the cheese for 1–6 months to produce the characteristic white crust or rind. At the same time, its hydrolytic enzymes are secreted into the cheese where they modify the flavour and texture.

Harit Jha