2.3 CULTURE PROCEDURES

2.3.1 MEDIA STERILIZATION

Sterilization is defined as the complete destruction or elimination of all viable organisms (in or on an object being sterilized). There are no degrees of sterilization: an object is either sterile or not. Sterilization procedures involve the use of heat, radiation, chemicals or physical removal of cells.

Media for industrial fermentations are usually sterilized. In some cases the economics of the fermentation makes it unrealistic to sterilize. The fermentations can proceed, however, these fermentations employ low pH and other contamination inhibitors (lactic acid) to hold in check the numbers of contaminating microorganisms. In other cases, sterilization is not required as the media components are poorly utilized by contaminating microorganisms. Fermentation media are sterilized by the use of: filtration, radiation, ultrasonic treatment, chemical treatment or heat (boiling or passing live steam through the medium, or by subjecting the medium to steam under pressure - autoclaving). Steam is used almost universally for the sterilization of fermentation media. The major exception is the use of filtration for the sterilization of animal cell culture.

Heat: Heat is the most important and widely used method. For sterilization, the type of heat, time of application and temperature required to ensure destruction of all microorganisms must always be considered. Endospores of bacteria are the most thermo-resistant of all cells so their destruction usually guarantees sterility.

Incineration: In this process, organisms are burned and physically destroyed. It is widely used for needles, inoculating wires, glassware, tubes etc. and objects that cannot be destroyed in the incineration process.

Boiling: Boiling is done at >100˚C for 20-30 min. It kills everything except for some endospores. To kill endospores and therefore perfectly sterilize the solution, very long or intermittent boiling is required.

Autoclaving: Autoclaving is the process of using steam under pressure in an autoclave or pressure cooker. It involves heating at 121˚C for 15-20 min under 15 psi pressure and can be used to sterilize almost anything. However heat labile substances will be denatured or destroyed. Sterilization of nutrient media is usually done using this process.

Dry Heat (Hot Air Oven): The process involves heating at 160˚C for 2 hours or at 170˚C for 1 hour. It is used for glassware, metal and objects that will not melt.

Sterilization in industry-scale fermenters (or bioreactors) is more complex. Steam is used to sterilize fermentation media. The medium can be sterilized *in situ* within the bioreactor. However, if the medium is sterilized in a separate vessel, the bioreactor needs to be sterilized before the sterile medium is added to it. Bioreactors are sterilized by passing steam through spargers. Spargers are devices that distribute gas bubbles (usually sterile air or steam) in a liquid

phase. They have particular design criteria, e.g., providing small sized bubbles (the sparger breaks the incoming air into small bubbles).

Various designs can be used such as porous materials made of glass or metal. However, the most commonly used type of sparger used in modern bioreactors is the sparge ring. A sparge ring consists of a hollow tube in which small holes have been drilled and is easier to clean than porous materials and is also less likely to block during fermentation. During sparging, steam pressure is held at 15 psi in the vessel for 20 min.

2.3.2 TEMPERATURE

As the temperature rises, the rate of chemical reactions increases – doubling for every 10°C rise in temperature. Cells should therefore grow faster as the temperature rises. However, some temperature-sensitive macromolecules (proteins, nucleic acids and lipids) will become denatured (thus non-functional) beyond a maximum limit. There is also a minimum temperature below which the lipid membrane will not be sufficiently fluid to function properly.

Microorganisms have optimal temperatures for growth. If grown at a temperature below the optimum, growth occurs slowly resulting in a reduced rate of cellular production. If the growth temperature is too high, microbial death can occur. In general, cell yield decreases with temperature. As with chemical and enzymatic reactions, cell growth varies as a function of temperature. Different groups of microorganisms have evolved to grow over different temperature ranges: **stenothermal** microorganisms can grow over a temperature range of approximately 30°C and **eurythermal** over even wider ranges. Most microorganisms will grow over a range of 25-30˚C although the actual temperature at which a particular organism grows will depend on its psychrophilic, mesophilic, moderate thermopilic or extreme thermophilic nature (Fig. 2.1).

Psychrophiles are cold loving microorganisms. Their optimum growth temperature is between -5 and 15˚C. They are usually found in the Arctic and Antarctic regions and in streams fed by glaciers. They are killed by exposure to room temperature. Their membranes contain a high proportion of unsaturated fatty acids that remain fluid at low temperatures. Membranes containing a high concentration of saturated fatty acids become non-functional at lower temperatures. The dominating character of cold-adapted enzymes is probably their enhanced turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) that compensate for the reaction rate reduction at low temperatures in order to maintain adequate metabolic fluxes. According to the current hypothesis, this optimization of the catalytic parameters can originate from the highly flexible structure of these proteins, which provides enhanced abilities to undergo conformational changes during catalysis at low temperature. Psychrophiles have enzymes that are characterized by a reduced number of surface salt bridges (in most cases, this difference arises from the replacement of the basic residue of the pair by a glutamine or an asparagine residue), fewer amino-, oxygen-, and sulfur-aromatic interactions, lower proline content and a reduced overall hydrophobicity arising from amino acid replacements occurring mainly in α-helices.

Mesophiles grow best at moderate temperatures. Their optimum growth temperature is between 25-45˚C and are found in the soil as well as on and in bodies.

Thermophiles are heat-loving with an optimum growth temperature of 45-70˚C. They are commonly found in hot springs and compost heaps. Several algal and fungal species have temperature optima up to 55˚C. Only certain prokaryotes are truly thermophilic. Thermophiles have evolved a variety of mechanisms that allow them to survive at temperatures no other organisms can thrive at. These traits include unique membrane lipid composition, thermostable membrane proteins, and higher turnover rates for various protein enzymes. One of the most important attributes to the maintenance of homeostasis within the organism is the plasma membrane surrounding the organism. Archaean thermophiles and also acidophiles, have membranes containing unique ether lipids. These tetraether lipids span the entire membrane forming a rigid monolayer that is impermeable to both ions and protons. Ether-type lipids, such as these, are much stronger than the ester-type lipids found in non-thermophilic bacteria and Eukarya. Also, the lipid composition in the membranes of the thermophiles consists of more branched and saturated fatty acids than other organisms. Having a stronger lipid complex within the membrane helps the Archaean thermophiles to withstand higher temperatures better than other organisms. Aside from having to stabilize the plasma membrane at high temperatures, thermophiles must also stabilize their proteins, DNA, RNA, and ATP. As of now, the process of heat stabilization for DNA, RNA, and ATP is unknown.

Thermophiles have developed distinct ways of heat stabilizing the proteins that are required for the maintenance of life. The relative rigidity & improved packing density of proteins from thermophiles are frequently proposed as the main structural determinants of their stability. The surface energy of the protein, along with the hydration of the non-polar groups that are exposed, are minimized. Also, hydrophobic regions are packed into a very dense core of the protein by charge-charge interactions between amino acids. There is also an increase in salt bridges and other networks, which help to stabilize the structures at higher temperatures. Finally, it has been shown that there is a distinct increase in the synthesis of chaperonin proteins after a heat shock. Chaperonins unfold and help refold proteins that are not folded properly enough to perform their required function. Increasing the number of these during high temperatures, most likely allows the cells to have a second chance at folding proteins that misfolded due to high heat.

Hyperthermophiles grow at very high temperatures with optimal growth between 70-110˚C. These organisms can be found growing near hydrothermal vents at great depths in the ocean. Most hyperthermophiles are archaeans – mainly anaerobic sulphate reducers or have other metabolism with lower requirements for thermolabile cofactors such as NADH and NADPH. When non-thermophiles are exposed to high temperatures, damage is caused to cytoplasmic membranes, ribosomes break down, enzymes are irreversibly denatured and breakage of DNA strands occurs. The actual mechanism of thermotolerance is not fully elucidated. However, the membranes of thermopiles remain intact and ribosomes work effectively at higher temperatures. Enzymes are also more heat stable. Thermophiles also have DNA with a high guanine-cytosine ratio resulting in interchain hydrogen bonds which provide greater

thermostability. These organisms also maintain higher intracellular potassium levels with the unusual counter ion, 2,3-diphosphoglycerate, which helps stabilize proteins. It is important to note that the optimum temperature for growth may be different from that of product formation.

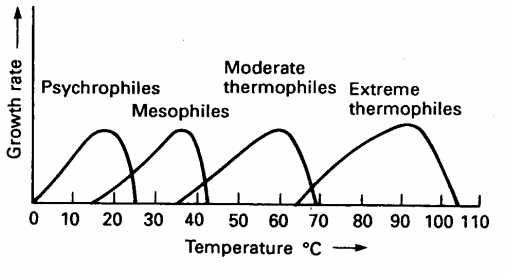

Fig. 2.1: Effect of temperature on specific growth rate of psychrophiles, mesophiles and thermophilic microorganisms (Ward, 1992).

2.3.3 pH

As with temperature, microorganisms have an optimum and a pH range in which they grow. Most microorganisms grow between pH 5 and 7. Generally fungi and yeast grow in acidic conditions (pH4-6). As the cells grow, metabolites are released into the medium, a process that can change medium pH. Therefore the pH of the medium must be monitored and be adjusted by base or acid addition to maintain a constant pH. Microorganisms can be placed into the following groups based on their optimum pH requirements:

Neutrophiles – grow best between pH 5-8. The majority of microorganisms are neutrophiles.

Acidophiles grow best below pH 5.5. Acidophiles use a variety of pH homeostatic mechanisms that involve restricting proton entry by the cytoplasmic membrane and purging of protons and their effects by the cytoplasm. To help maintain ΔpH, acidophiles have a highly impermeable cell membrane to restrict proton influx into the cytoplasm. An example of a highly impermeable cell membrane is the archaeal-specific structures composed of tetraether lipids (as opposed to the ester linkages found in bacterial and eukaryotic cell membranes). Differences in lipid head-group structures and ion permeability between the extreme acidophiles (*Ferroplasma acidarmanus* and *F. acidiphilum*) have been suggested as a reason for the different optimal growth pH of these acidophiles. The low permeability of acidophile membranes is a result of several factors including: (i) the fixed nature of the monolayer such that fracturing of these membranes does not cleave the two opposing lipid layers and opposing polar head groups; (ii) a bulky isoprenoid core; and (iii) the fact that ether linkages characteristic of these membranes are less sensitive to acid hydrolysis than ester linkages. Results for thermophilic and mesophilic acidophilic archaea indicate that there might be a stronger association between tetraether lipids and tolerance to acid gradients than previously thought. Membrane channels have a reduced pore size. Proton influx is inhibited by a chemiosmotic gradient created by a Donnan potential. A further mechanism used by acidophiles to reduce proton influx is the generation of an inside positive membrane potential ($\Delta\Psi$) [which is opposite to the inside negative $\Delta\Psi$ of neutralophiles]. The $\Delta\Psi$ is generated by a Donnan potential of positively charged molecules and inhibits the influx of protons using a chemiosmotic barrier against the proton gradient. This potential is possibly produced by a greater influx of potassium ions than the outward flux of protons. To maintain pH homeostasis, acidophiles need to be able to remove excess protons

from the cytoplasm and the ΔpH in *Bacillus acidocaldarius* and *Thermoplasma acidophilum* is created by active proton pumping. Proton uncoupling by organic acids (such as acetic or lactic acid) are harmful to acidophiles because they function as uncouplers of the respiratory chain at low pH by diffusion of the protonated form into the cell followed by dissociation of a proton. Thus, active mechanisms of organic acid degradation might be a pH homeostatic mechanism that is used by heterotrophic acidophiles.

Cytoplasmic buffering helps to maintain the intracellular pH. If protons manage to penetrate the acidophile cell membrane, a range of intracellular mechanisms help to ameliorate the ensuing biological damage. First, the buffering capacity of the cytoplasm to sequester or release protons can be used as a pH homeostasis mechanism. The presence of a large number of DNA and protein repair genes evident on several extreme acidophile genome sequences might also be related to problems associated with pH homeostasis, whereby biomolecules damaged by low pH require fast and efficient repair. The *Picrophilus torridus* genome has been shown to contain a large number of genes determining DNA repair proteins. Intracellular enzymes might be stabilized by 'iron rivets', intracellular compartmentalization of enzymes and pH gradients existing within the cytoplasm of this archaeon.

Alkalophiles grow best above pH 8.5. Alkalophiles, e.g., *Bacillus* and *Micrococcus* take up protons to maintain their internal pH at a lower value. The strategies include: (i) increased metabolic acid production through amino acid deaminases and sugar fermentation; (ii) increased ATP synthase that couples H^+ entry to ATP generation; (iii) changes in cell surface properties; and (iv) increased expression and activity of monovalent cation/proton antiporters. Among these strategies, monovalent cation/proton antiporters play an essential and dominant role in alkaline pH homeostasis in many bacteria in addition to roles in Na^+ and volume homeostasis. Similarly, Na^+/H^+ antiporters play essential roles in homeostasis of pH, Na^+ and volume in eukaryotic cells and organelles. Bacteria have multiple monovalent cation/proton antiporters (e.g., at least four in *E. coli*, five in *B. subtilis* and four in alkaliphilic *Bacillus* strains. Detailed information about individual Na^+/H^+ and $Na^+(K^+)/H^+$ antiporters is beginning to provide insights into the basis for the dominant role of specific antiporters in pH homeostasis of individual bacterial strains. Several enzymes of amino acid catabolism are also increased in abundance at alkaline pH. Deaminases provide an acid-generating mechanism that is adaptive to alkaline challenge.

2.3.4 SOLUTES AND WATER ACTIVITY

Most microorganisms contain between 70 and 80% water and a certain amount of free water is required for the performance of specific metabolic activities. Microbial cells with rigid walls placed in hypotonic (lower osmotic pressure than cell contents) solutions take up water and become turgid and those without cell walls swell and eventually burst. Water uptake and retention is problematic in dry and hypertonic conditions. Although hypertonic environments may contain considerable amounts of water, the high external levels of solutes (salts or sugars) causes water to pass out of most cells, halting growth.

The availability of water to microorganisms defined by the term water activity (A_w) is the ratio of the vapour pressure of the water in the solution surrounding the cell (P_{soln}) to the vapour

pressure of pure water (P_{water}). $P_{water} = 1$. Most bacteria cannot grow below an A_W of 0.9. Most fungi are xerotolerant and can grow at low moisture levels (≈0.7). Xerophilic fungi and osmophilic yeasts have evolved to survive at $A_w \approx 0.6$. True halophiles (salt concentrations >0.3 mmol/l) have highly modified cell walls with unusual membrane lipids. Most osmophilic, xerophilic and halophilic organisms accumulate compensating solutes which balance the osmotic strength of the external solute.

The biggest challenge is to adjust the turgor and living cells have developed two principal strategies to re-establish turgor pressure and to circumvent the detrimental consequences of water loss when exposed to increasing osmolality. On the one hand, there is the "salt-in-cytoplasm"-strategy, which means that inorganic ions, mainly K^+ and Cl^-, accumulate in the cytoplasm until the internal salt concentration is similar to the extracellular one. This strategy is found in extremely halophilic Halobacteria (Archaea) and halophilic, anaerobic Haloanaerobiales (Bacteria). On the other hand, the vast majority of prokaryotes cope with increasing osmolarity by uptake or synthesis of compatible solutes, which are defined as small, highly soluble, organic molecules which do not interfere with the central metabolism, even if they accumulate at high concentrations. This strategy is widespread and evolutionarily well conserved in all three domains of life. However, the spectrum of compatible solutes used comprises only a limited number of compounds and these can be divided into two major groups: 1) sugars and polyols; and 2) α and β amino acids and their derivatives, including methylamines. This limitation to a rather small number of compounds reflects the fundamental constraints on solutes which are compatible with macromolecular and cellular functions. Most archaeal compatible solutes resemble in structure their bacterial counterpart, with the difference that the majority of them carry a negative charge.

2.3.5 AERATION AND MIXING

Oxygen is an important substrate for aerobic organisms. Since metabolic energy production by cells is directly related to oxygen uptake rate (also called respiration rate), oxygen concentration is very strongly related to growth rate. Growth rate sharply rises to its maximum value with dissolved oxygen concentration. Optimal growth of many microorganisms usually requires large amounts of dissolved oxygen. As oxygen is sparingly soluble in water (8.4 mg/l at 25˚C) it needs to be supplied continuously - generally in the form of sterilized air to a growing culture. The air produces bubbles and the stirrer is used to break up the bubbles and mix the contents of the reactor. If airflow is inadequate or the air bubbles are too large, the rate of transfer of oxygen is low and is insufficient to meet oxygen demand. On exposure to oxygen, most microorganisms interact with it to produce highly reactive toxic products, including superoxide, hydrogen peroxide and hydroxyl radicals. These products react destructively with any organic molecules that they encounter, including lipids, proteins and nucleic acids. Microorganisms contain three groups of enzymes, viz., superoxide dismutase, catalase and peroxidise to catalyse and detoxify the free radicals. Microorganisms show a great deal of variation in their requirements for gaseous oxygen:

Obligate aerobes: are organisms that grow only in the presence of oxygen and obtain their energy through aerobic respiration.

Microaerophiles: require a low concentration of oxygen (2-20%) for growth, but higher concentrations are inhibitory. They obtain their energy through aerobic respiration.

Obligate anaerobes: are organisms that only grow in the absence of oxygen and are inhibited or killed by its presence. They obtain their energy through anaerobic respiration or fermentation.

Aerotolerant anaerobes: like obligate anaerobes, they cannot use oxygen to transform to energy however they can grow in its presence. They obtain their energy only by fermentation and are known as obligate fermenters.

Facultative anaerobes: grow with or without oxygen, but generally better with oxygen and obtain their energy through aerobic respiration if oxygen is present but use fermentation or anaerobic respiration of it is absent. Most bacteria are facultative anaerobes.