# BFSC 603: Biotechnology and Bioinformatics

## Unit 1. Introduction

## Chapter 1: Concepts, Terminologies and History

### 1.1.1. Introduction

**Biotechnology** arose from the field of zymotechnology, which began as a search for a better understanding of industrial fermentation, particularly beer.

The heyday and expansion of zymotechnology came in World War I in response to industrial needs to support the war.

The industrial potential of fermentation was outgrowing its traditional home in brewing, and "zymotechnology" soon gave way to"biotechnology."

### 1.1.2 Concepts and Terminologies

The terms biotechnology, genetic engineering and molecular biology are very much related. There are also many terminologies that have been added to the lexicon of biotechnology. A brif on these terminologies are summarized hereunder.

# 1.1.2.1. Biotechnology

### 1.1.2.1. Biotechnology

**It** is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services. It involves the use of microorganisms, such as bacteria or yeasts, or biological substances, such as enzymes, to perform specific industrial or manufacturing processes. Using bacteria that feed on hydrocarbons to clean up an oil spill is one example of biotechnology.

### 1.1.2.2. Genetic engineering

It is also called *genetic modification*, is the direct human manipulation of an organism's genome using modern DNA technology. It involves the introduction of foreign DNA or synthetic genes into the organism of interest.

# 1.1.2.3. Molecular biology

### 1.1.2.3. Molecular biology

It is the branch of biology that deals with the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins, and especially with their role in cell replication and the transmission of genetic information.

### 1.1.2.4. Genomics

**Genomics** is the study of the **genome** . The term **genome** refers to the entire **gene** tic content of an organism. Genomics is the scientific discipline of mapping, sequencing, and analyzing genomes. The entire RNA and **protein** content of an organism are referred to as **transcriptome** and **proteome**, respectively. Genomics, in the broad sense, includes transcriptomics (study of the transcriptome) and proteomics (study of the proteome), as the genetic signal can be modified during and after the **transcription** and translation processes.

### 1.1.2.5. Functional genomics

Functional genomics combine bioinformatics, DNA chip technology, animal models, and other methodologies to identify and characterize genes that cause human disease, and are therefore prime targets fir drug development.

### 1.1.2.6. Metagenomics

Metagenomics is the **cloning** of genetic material from microorganisms that cannot be grown in the laboratory into ones that can be grown so that new forms of known genes may be identified.

### 1.1.2.7. Proteomics

The proteome is defined as the expressed protein complement of a cell, tissue, or whole organism. Proteomics was first used in 1994 by Williams and Hochstrasser. The proteome, unlike the genome, varies both temporally and between tissues as the fish grows and adapts its physiology to meet the demands of a new environment. As proteins are the final determinant of phenotype—the proteome that describes the abundance, identity, posttranslational modifications, and potentially the synthesis rates of proteins—an understanding of the regulation proteome is imperative to gain a holistic view of the animal. Proteomics use mass spectroscopy (MS) techniques to identify novel functional proteins from genes that are expressed.

### 1.1.2.8. Metabolomics

Metabolomics is the scientific study of chemical processes involving metabolites. Specifically, metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind", the study of their small-

molecule metabolite profiles. The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes.

### 1.1.3. Fields of Biotechnology

Biotechnology has wide applications in all fields of science and technology. **Genetic engineering** is used in the production of drugs, human **gene therapy** , and the development of improved plants. Based on the field in which the principles of biotechnology are applied, new areas of research have emerged and have developed into a multi-dicipplinary domains of their own. A brief on such specialized fields are summarized hereunder.

#### 1.1.3.1. Agricultural biotechnology

Agricultural **biotechnology** is dated back to 10,000 BC when farmers began to select the most suitable plants and animals for breeding. Soon thereafter, Sumerians used yeast, a type of fungus, to make beer and wine in Mesopotamia. In the 1860s, **Gregor Mendel** crossed different pea plants and identified the **principles of inheritance** and marked the beginning of **conventional biotechnology**. Major advances in plant breeding followed the revelation of Mendel's discovery. Breeders brought their new understanding of **gene** tics to the traditional techniques of self-pollinating and cross-pollinating plants.

Recognising desirable traits and incorporating them into future generations is very important in plant breeding. A few of these traits can arise spontaneously through a process called **mutation**, but the natural rate of mutation is very slow and unreliable to produce all plants that breeders are looking for. In the late 1920s it was discovered that exposing plants to **x-rays and chemicals** could **increase the rate of genetic variation**, thereby increasing the pool of characteristics that breeders and farmers could choose from when looking for beneficial features for crop breeding. Examples of plants that were produced via mutation breeding include varieties of wheat, barley, rice, potatoes, soybeans and onions.

Experts in United States anticipate the world's population in 2050 to be approximately 8.7 billion persons. The world's population is growing, but its surface area is not. By increasing crop yields, through theuse of **biotechnology** the constant need to clear more land for growing food is reduced.

Countries in Asia, Africa, and elsewhere are grappling with how to continue feeding a growing population.They are also trying to benefit more from their existing resources.Biotechnology holds the key to increasing the yield of staple crops by allowing farmers to reap bigger harvests from currently cultivated land, whilepreserving the land's ability to support continued farming.

Malnutrition in underdeveloped countries is also being combated with biotechnology. The Rockefeller Foundation is sponsoring research on “golden rice”, a crop designed to improve nutrition in the developing world. Rice breeders are using biotechnology to build Vitamin A into the rice. Vitamin A deficiency is a common problem in poor countries. A second phase of theproject will increase the iron content in rice to combat anemia, which is a widespread problem among women and children in underdeveloped countries.

Similar initiatives using **gene** tic manipulation are aimed atmaking crops more productive by reducing their dependence on pesticides, fertilizers and irrigation, or by increasing their resistance to plant diseases. Increased crop yield, greater flexibility in growing environments, less use of chemical pesticides and improved nutritional content make agricultural biotechnology, quite literally, the future of the world’s foodsupply.

The plant biotechnology has following applications:

1. Plant Cell and **Tissue Culture** .

2. Production of pesticide, herbicide and salt tolerant plants. For example, an “insect protection” gene (Bt) has been inserted into several crops - corn, cotton, and potatoes - to give farmers new tools for integrated pest management. Bt corn is resistant to European corn borer. This inherent resistance thus reduces a farmers pesticide use for controlling European corn borer, and in turn requires less chemicals and potentially provides higher yielding Agricultural Biotechnology.

### 1.1.3.2. Animal biotechnology

The animal biotechnology has the following application.

1. The development of vaccines to protect animals from disease,

2. The production superior calves through superovulation and embryotranfer technology,

3. The production of several calves from one embryo (embryo splitting/ **cloning** ),

4. Increase of animal growth rate,

5. Rapid disease detection by molecular immunological techniques.

6. Monoclonal **antibody** production

7. Recombinant vaccine production, and

8. **Transgenic** technology in animal production.

In summary, modern biotechnology offers opportunities to improve product quality, nutritional content, and economic benefits. The genetic makeup of plants and animals can be modified by either insertion of new useful genes or removal of unwanted ones. Biotechnology is changing the way plants and animals are grown, boosting their value to growers, processors, and consumers.

### 1.1.3.3. Industrial Biotechnology

Industrial **biotechnology** applies the techniques of modern molecular biology to improve the efficiency and reduce the environmental impacts of industrial processes like textile, paper and pulp, and chemical manufacturing. For example, industrial biotechnology companies develop biocatalysts, such as **enzymes**, to synthesize chemicals. Enzymes are **protein**s produced by all organisms. Using biotechnology, the desired enzyme can be manufactured in commercial quantities.

Biotechnology also produces biotech-derived cotton that is warmer, stronger, has improved dye uptake and retention, enhanced absorbency, and wrinkle- and shrink-resistance.

Some agricultural crops, such as corn, can be used in place of petroleum to produce chemicals. The crop's sugar can be fermented to acid, which can be then used as an intermediate to produce other chemical feedstocks for various products. It has been projected that 30% of the world's chemical and fuel needs could be supplied by such renewable resources in the first half of the next century.

### 1.1.3.4. Environmental Biotechnology

Environmental **biotechnology** is used in waste treatment and pollution prevention. Environmental biotechnology can more efficiently clean up many wastes than conventional methods and greatly reduce our dependence on methods for land-based disposal.

Every organism ingests nutrients to live and produces by-products as a result. Different organisms need different types of nutrients. Some bacteria thrive on the chemical components of waste products. Environmental engineers use bioremediation, the broadest application of environmental biotechnology, in two basic ways. They introduce nutrients to stimulate the activity of bacteria already present in the soil at a waste site, or add new bacteria to the soil. The bacteria digest the waste at the site and turn it into harmless byproducts. After the bacteria consume the waste materials, they die off or return to their normal population levels in the environment.

Bioremediation, is an area of increasing interest. Through application of biotechnical methods, enzyme **bioreactors** are being developed that will pretreat some industrial waste and food waste components and allow their removal through the sewage system rather than through solid waste disposal mechanisms. Waste

can also be converted to biofuel to run **gene**rators. Microbes can be induced to produce **enzymes** needed to convert plant and vegetable materials into building blocks for biodegradable plastics.

In some cases, the byproducts of the pollution-fighting microorganisms are themselves useful. For example, methane can be derived from a form of bacteria that degrades sulfur liquor, a waste product of paper manufacturing. This methane can then be used as a fuel or in other industrial processes.

### 1.1.3.5. Fisheries Biotechnology

**Biotechnology** is also used in the fisheries field for increasing fish production through various techniques. Fisheries biotechnology can be broadly classified into aquaculture biotechnology, marine biotechnology, algal biotechnology and processing biotechnology.

Since 1980s, there has been a burst of biotechnology activity in research and development related to various **fish species**, in particular those used in aquaculture production. Biotechnology has played a major role in the areas of induction and control of

maturation and spawning, sex control (andro **gene** sis and gynogenesis), sex inversion in protandrous species like sea bass and protogynous species like the grouper, production of triploid, tetraploid and **transgenic** fishes. Traits that are being tested in fish species such as **carp, trout, salmon and channel catfish** include growth rates that are three to eleven times faster with more **efficient feed utilisation, increased tolerance to cold water and improved disease resistance**. Accelerated growth rates mean that fish reach marketable size sooner, thereby reducing overhead costs for fish farmers. In addition, researchers use the human interferon gene to improve disease resistance in carp, which could reduce the amount of **antibiotic** s needed to keep fish healthy and reduce the costs incurred from losses due to disease.

The first (and to date only) genetically engineered fish to be sold commercially is the fluorescent Glofish®, a zebra fish modified to glow red, which came onto the US market in 2004.

Other areas include disease diagnosis (molecular and immunodiagnostic kits), **hybridoma** technology, and management (probiotics, vaccines, immunostimulants), cell and **tissue culture** , conservation of germplasm (cryopreservation of fish gametes), extraction of bioactive substances from marine organisms including marine bacteria, marine algae, marine invertebrates and fishes.

### 1.1.3.6. Other Applications

Biotechnical methods are now used to produce many **protein** s for pharmaceutical and other specialized purposes. A harmless **strain** of *Escherichia coli* bacteria, given a copyof the **gene** for human insulin, can make insulin. As these genetically modified (GM) bacterial cells age, they produce human insulin, which can be purified andused to treat diabetes in humans. Products of modern **biotechnology** include artificial blood vessels from collagen tubes coated with a layer of theanticoagulant heparin.

**Gene therapy** – altering DNA within cells in an organism totreat or cure a disease – is one of the most promising areas of biotechnologyresearch. New genetic therapies are being developed to treat diseases such ascystic fibrosis, AIDS and cancer.

DNA fingerprinting has become one of the most powerful andwidely known applications of biotechnology today. DNA from samples of hair, bodily fluids or skin at a crime scene are compared with those obtained fromthe suspects.

1.1.4. Historical events related to biotechnology

The Hungarian **Karl Ereky coined the word "biotechnology"** in 1919 to describe a technology based on converting raw materials into a more useful product. For Ereky, the term "biotechnologie" indicated the process by which raw materials could be biologically upgraded into socially useful products.

- In1920, Leads city council, U.K. established the Institute of Biotechnology.
- During1970s, Biotechnology emerged as a new discipline.
- In 1978, European Federation of Biotechnology was established.

Biotechnology is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and service (The Organisation for Economic Co-operation and Development, OECD, 1981). The "Scientific and Engineering Principles" refer to microbiology, **gene**tics,

- biochemistry,etc. and "biological agents" mean microorganisms, **enzymes**, plant and animal cells.
- **In 1982, Government of India set up, the National Biotechnology Board and in 1986, it be came a separate department, Department of Biotechnology in the Ministry of Science and Technology.**
- United Nations proposed for the establishment of International Centre for **Genetic Engineering** and Biotechnology (ICGEB) in 1988. It has 2 centres, New Delhi (India) and Trietse.
- In the 1940s, penicillin was discovered in England and it was produced industrially in the United States using a deep fermentation process. The enormous profits and the public expectations penicillin gave rise to a radical

shift in the standing of the pharmaceutical industry. Doctors used the phrase "miracle drug".

- A number of discoveries made during the 1960s and 1970s shed light on how distinct fragments of DNA could be isolated .

- The work of Swiss molecular biologist **Werner Arber** focused on specialized enzymes that digest, or “restrict,” the DNA of **viruses** infecting bacteria. These enzymes were subsequently called as “ **restriction enzyme s” that** could also act like molecular scissors to cut DNA.
- In 1970 American molecular biologist **Hamilton Smith** and colleagues determined that restriction enzymes could cleave DNA molecules at precise and predictable locations. Hamilton concluded that the enzymes were able to recognize specific **nucleotide sequence** s. Scientists quickly realized that restriction enzymes could be used in the laboratory to manipulate DNA.
- In 1973 American biochemist **Herb Boyer** used restriction enzymes to produce a DNA molecule with genetic material from two different sources. This **splicing** technique is now known as **recombinant DNA** .
- Boyer inserted foreign genes into **plasmid** s and observed that the plasmids could replicate to make many copies of the inserted genes. In subsequent experiments, Boyer, American biochemist Stanley Cohen, and other researchers demonstrated that inserting a recombinant DNA molecule into a host bacteria cell would lead to extremely rapid replication and the production of many identical copies of there combinant DNA.
- This process, known as **cloning** , gave scientists the power to make many copies of desired DNA for molecular study.
- The speed and efficiency of DNA cloning were vastly improved in the 1980s with the invention of **polymerase chain reaction** (PCR). Developed by American biochemist **Kary Mullis** , PCR enables scientists to produce large amounts of **DNA sequence** s ina test tube. In a matter of hours, the process can produce millions of **clone** d DNA molecules.
- In the late 1970s and early 1980s, British biochemist **Frederick Sanger** and his associates developed **DNA sequencing techniques** . Sanger’s methods, which used special compounds called dideoxy nucleotides, rapidly yielded the exact nucleotide sequence of a desired sample. With the use of automated equipment, the new techniques transformed genetic sequencing into a speedy, routine laboratory procedure.
- **1.1.4.1. Other significant events**
- 6000B.C- Bread making (involving yeast fermentation)
- 1857AD- Pasteur proves that yeasts are living cells that cause alcohol fermentation
- 1928– Alexander Fleming discovers penicillin from *Penicillium notatum*
- 1953-DNA structure and function elucidated
- 1970-Smith *et al*. report restriction **endonuclease** from *Haemophilus influenzae* that recognizes specific DNA target **sequence** s

- 1972- **Walter Fiers** and his team at the Laboratory of Molecular Biology of the **University of Ghent** ( **Ghent** , **Belgium** ) were the first to determine the sequence of a **gene** : the gene for **bacteriophage MS2** coat **protein** .
- 1973- Tong *et al*. injected mRNA and rRNA from mature eggs of crucian carp and common carp into newly fertilized crucian carp eggs, in order to induce character variation in goldfish.
- 1973- Tong and Niu, transplantednuclei between gold fish (*Carassiusauratus*) and *Rhodeus sinensis* forthe purpose of studying the developmental variations between the integratednuclei and the pure heterologous nuclei, and the effects of **cytoplasm** on thenucleus.
- 1974- Parker defined probiotics are “organisms and substances which contributeto intestinal microbial balance”.
- 1976 - **Walter Fiers** andhis team determine the complete **nucleotide** - sequence of **bacteriophage** MS2-RNA
- 1975-Kohler and Milstein report monoclonal antibodies
- 1977- DNA is **sequenced** forthe first time by **Fred Sanger** , **Walter Gilbert** , and **Allan Maxam** workingindependently. Sanger's lab sequence theentire **genome** of **bacteriophage Φ-X174** .
- 1979- **Paulien Hogeweg** coined the term *bioinformatics* for the study of informatic processesin biotic systems.
- 1980 - Gordon *etal*. revolutionized the procedure for producing **transgenic** animals based onthe **microinjection** of **clone** d DNA into the pronucleus of fertilized eggs at theone-cell stage.
- 1982 - Induced the first viable tetraploid fish, rainbow trout
- 1982-Palmiter *et al.,* produced firsttransgenic mouse; rat **gene transfer** red to mouse
- 1983 - **Kary B. Mullis** discoversthe **polymerase chainreaction** enabling theeasy amplification of DNA
- 1984-Transgenic pig, rabbit, and sheep by microinjection of foreign DNA into eggnuclei
- 1984- Maclean and Talwar reported microinjection of cloned DNA intorainbow trout (*Oncorhynchus mykiss*)eggs.
- 1985-First transgenic fish was produced, Zhu produced transgenic goldfish
- 1986 - Fletcher *et al*.,showed that AFP injection to seawater-acclimatized rainbow trout lowered thefreezing point of the whole fish in proportion to the circulating anti-freezeprotein concentration.
- 1986- Chen *et al*.transplanted cell nuclei from a grass carp blastula cell line intounfertilized, enucleated eggs of crucian carp, thus creating the first "test-tube fish".
- 1989-The **human** gene that encodes the **CFTR** proteinwas sequenced by **Francis Collins** and **Lap-Chee Tsui** . Defects in this gene cause **cystic fibrosis**
- Human **Genome** Project started

- 1990- Shujian *et al.* transplanted cell nuclei of themutant cell line (AHZC-88), which was resistant to the grass carphemorrhagic virus, into unfertilized grass carp eggs using electric fusion, and raisedthree of the fish to the fry stage.
- 1995-The genome of *Haemophilus influenzae* is the first genome of a free living organism to be sequenced.
- 1996- ***Saccharomyces cerevisiae*** is the first **eukaryote** genomesequence to be released.
- 1998-The first genome sequence for a multicellular eukaryote, ***Caenorhabditis elegans*** , is released.
- 1999- Zhiyuan Gong *et al.*, at the **National University of Singapore** produced transgenic Zebrafish by inserting a gene called **green fluorescent protein** (GFP), originally extracted from a **jellyfish** , that naturally produced brightgreen **bioluminescence** .
- 2001-First draft sequences of the human genome are released simultaneously by the **Human Genome Project** and **Celera Genomics** .
- 2003- Successful completion of Human Genome Project with 99% of the genomesequenced to a 99.99% **accuracy** .
- 2003- Gong *et al.*, developed transgenic zebrafish (*Danio rerio*) for ornamental and bioreactor system by strong expression of fluorescent proteins in the skeletalmuscle.
- **Chapter 2: Nucleic Acids - Structure, Chemistry & Genetic Code**

### 1.2.1. DNA as genetic material

- The structure of DNA encodes all the information needed by every cell to function and thrive.
- DNA carries hereditary information in a form that can be copied and passed intact from **gene** ration to generation.
- A gene is a segment of DNA.
- The biochemical instructions found within most genes, known as the **genetic code** , specify the chemical structure of a particular **protein** .
- The DNA structure of a gene determines the arrangement of **amino acids** in a protein, ultimately determining the type and function of the protein manufactured.

The studies that have revealed the chemistry of genes began in Germany in 1869 when Friedrich Miescher isolated nuclei from pus cells (white blood cells) in waste surgical bandages. He found that these nuclei contained a novel phosphorus bearing substance that he named nuclein. Nuclein is mostly chromatin, a complex of deoxyribonucleic acid (DNA) and chromosomal protein (Chromatin = DNA +Protein).

By the end of the nineteenth century both DNA and ribonucleic acid (RNA) had been separated from the protein.

By the beginning of 1930s, P. Levene, W. Jacobs, and others had demonstrated that RNA is composed of a sugar (ribose) plus four nitrogenous bases, and that DNA contains a different sugar (deoxyribose) plus four bases. They discovered that each base is coupled with a sugar-phosphate to form a **nucleotide** .

1.2.1.1 Evidence that genes are made of DNA (or sometimes RNA)

Transformation in Bacteria

Frederick Griffith laid the foundation for the identification of DNA as the gene tic material in 1928 with his experiments on transformation in the bacterium *Pnuemococcus*, now known as *Streptococcus pneumoniae*.

DNA: The transforming material

Oswald Avery, Colin Mac Leod, and Maclyn Mc Carty showed the transforming substance to be DNA in 1944 in virulent cells of *Streptococcus pneumoniae*.

In 1952, A.D. Hershey and Martha Chase performed experiment in T2 bacteriophage . The phage is composed of protein and DNA only. The experiment showed that the genes of phage are made of DNA.

### 1.2.1.2 The chemical nature of Nucleotides

By the mid 1940s, biochemists know the fundamental chemical structures of DNA and RNA. When they broke DNA into its component parts, they found these constituents to be

nitrogenous bases, phosphoric acid, and the sugar deoxyribose. Similarly, RNA yielded bases and phosphoric acid, plus a different sugar ribose.

- The four bases found in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T). But in RNA Uracil (U) replaces thymine.
- Adenine and Guanine are purines and are two ringed structures.
- Others are single ringed and are called pyrimidines.
- These structures constitute the alphabet of **gene** tics.
- Ribose contains a hydroxyl (OH) group in the 2 - position. Deoxyribose lacks the oxygen and simply has a hydrogen.
- The bases and sugars in RNA and DNA are joined together into units called Nucleosides.
- The subunits of DNA and RNA are nucleotides, which are nucleosides with a phosphate group attached through a phosphodiester bond.
- 1.2.2. DNA Structure
- Linus Pauling elucidated the - helix for DNA structure, an important feature of protein structure. Indeed, the a- helix, held together by hydrogen bonds,

laid the intellectual ground work for the double helix model of DNA proposed by Watson and Crick. Maurice Wilkins and Rosalind Franklin used X-ray diffraction to analyse the three-dimensional structure of DNA at Kings College in London. Watson and Crick performed no experiments themselves. They used other group's data to build a DNA model.

- Erwin Chargaff studies (1950) of the base composition of DNAs from various sources revealed the following.
- · The content of purines always equaled the content of pyrimidines.
- · The amounts of adenine and thymine were always equal, as were the amounts of guanine and cytosine.
- · These findings, known as Chargaff's rules, provided a valuable confirmation of Watson and Crick's model.
- · The most crucial piece of the puzzle came from an X-ray diffraction picture of DNA taken by Franklin in 1952. Franklin's X-ray work strongly suggested that DNA was a helix.

### 1.2.2.1.The Double helix

**Polynucleotides**

DNA molecules form chains of building blocks called nucleotides. Each nucleotide consists of a sugar molecule called deoxyribose that bonds to a phosphate molecule and to a nitrogen-containing compound, known as a base. DNA uses four bases in its structure: adenine (A), cytosine (C), guanine (G), and thymine (T).

The order of the bases in a DNA molecule—the **genetic code** —determines the amino acid **sequence** of a **protein** .

In the cells of most organisms, two long strands of DNA join in a single molecule that resembles a spiraling ladder, commonly called a double helix.

- Alternating phosphate and sugar molecules form each side of this ladder.
- Bases from one DNA strand join with bases from another strand to form the rungs of the ladder, holding the double helix together.
- The pairing of bases in the DNA double helix is highly specific—adenine always joins with thymine, and guanine always links to cytosine.
- These base combinations play a fundamental role in DNA's function by aiding in the replication and storage of gene tic information. Complementary base pairing enables to predict the sequence of bases on one strand of a DNA molecule if the order on the corresponding or complementary DNA strand is known.
- Watson and Crick found that the best model that satisfied all the X-ray data was a double helix with the sugar phosphate chain on the outside and the bases on the inside.

- The two chains run in an anti parallel fashion with one chain having a 5'→3' orientation and the other having a 3'→ 5' orientation.
- The width of the helix was found to be 2 nm. The purine and pyrimidine bases were stacked 0.34 nm apart in a ladder.
- The helix made one full turn every 3.4 nm and, therefore, there should be 10 layers of bases stacked in one turn.
- Since the width of the helix is 2 nm it can accommodate only 2 strands.
- Each step would contribute a pair of bases, with each base attached to one of the sugar-phosphate backbone.
- In a given DNA, adenine is equal to thymine and guanine to cytosine.
- The two strands of DNA are held together by hydrogen bonds. There are two hydrogen bonds for A = T pairing and three bonds for C º G pairing. C º G pairing is stronger than A = T pairing.
- Helical structure is right handed.
- The fifth (5- prime, of 5') carbon of the pentose ring is connected to the third (3 - prime, of 3 ') carbon of the next pentose ring via a phosphate group, and the nitrogenous bases stick out from this sugar-phosphate back bone.
- By convention, DNA sequence s are read from 5'→3' with respect to the polarity of the strand.

Watson and Crick model suggested a copying mechanism for DNA. Since one strand is the complement of the other, the two strands can be separated, and each can serve as the template for building a new strand.

Watson and Crick were aware of this potential and they wrote in the Journal Nature, "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material".

1.2.2.2. Genes made of RNA

Most genetic systems studied to date contain genes made of DNA. But some viruses , including several phages, plant and animal viruses (e.g., HIV, the AIDS virus), have RNA genes. Sometimes viral RNA genes are double-stranded but usually they are single-stranded.

A group of viruses, referred to as retrovirus es, has RNA as the genetic material. These tumour viruses can integrate with the host genome DNA, only after the RNA makes a DNA copy. Thus, these viruses carry the gene for reverse transcriptase catalyses the conversion of RNA to DNA.

The central dogma says that the flow of information is unidirectional i.e. DNA → RNA → Protein .

With the discovery of the enzyme reverse transcriptase, it is now clear that RNA can also go back to DNA and the central dogma is now represented as: RNA → DNA → Protein.

1.2.2.3. Variety of DNA structures

The structure for DNA proposed by Watson and Crick represents B form of DNA. B form is present in most DNA in the cell.

A form differs from the B form in several aspects. The plane of a base pair is no longer perpendicular to the helical axis, but tilts 20 degrees away from horizontal. Also, the A helix packs in 11 base pairs per helical turn instead of 10 found in the B form, and turn occurs in 31 angstroms instead of 34.

The distance between base pairs, is only 2.8 nm instead of 3.4 nm, as in B-DNA.

Both the A and B form DNA structures are right handed; the helix turns clockwise.

Alexander Rich and his colleagues discovered in 1979 DNA can exist in an extended left-handed helical form. Because of the zigzag look of this DNA's backbone when viewed from the side, it is often called Z DNA. There is evidence that living cells contain small proportion of Z-DNA. The distance between base pair is 4.5 nm and number of bases per turn is 12.

RNA-DNA hybrid strand assumes the A form.

Normal DNA has 2 grooves (major and minor). Z- DNA has single groove.

1.2.3. Properties of DNA

1. DNA denaturation or DNA melting

The temperature at which the DNA strands are half denatured is called the melting temperature, or Tm. When a DNA solution is heated, the bonds that hold the 2 strands together become weaker and finally break. This is known as **DNA denaturation.** The amount of strand separation or melting is measured by the absorbance of the DNA solution at 260 nm. Nucleic acids absorb light at this wave length. When cooled, the two strands will reunite. The GC content of a DNA has a significant effect on its Tm. The higher a DNA's GC content, the higher its Tm. C º G pairing form 3 hydrogen bonds, whereas A = T pairs have only 2. In addition to heating, DMSO and formamide also disrupt the hydrogen bonding between DNA strands and promote denaturation. Lowering the salt concentrations of the DNA solution also aids denaturation.

2. Annealing or Renaturation

Once the two strands of DNA separate, they can reunite under the proper conditions. This is called annealing or renaturation. Factors that contribute to renaturation are:

1. Temperature - Best temperature for renaturation of a DNA is about 25$^{o}$C below its Tm.

2. DNA concentration - The higher the concentration, the faster the annealing.

3. Renaturation time - If longer time allowed for annealing, the more will occur.

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### 1.2.5. Activities of genes

A gene is a unit of information which is held as a code in a discrete segment of DNA. This code specifies the amino acid **sequence** of a **protein** . The sequence information for a single gene was not continuous along the DNA, but was interspersed with pieces of non- coding sequence. The coding parts of a gene sequence are **exon** s, and the non- coding parts are **intron** s. Before a gene can be expressed, the DNA that encodes has to be transcribed into RNA. A gene participates in 3 major activities.

1. A gene can be replicated-genetic information can be passed from generation to generation unchanged.

2. The sequences of bases in the RNA depends directly on the sequences of bases in the gene. Most of these RNAs, in turn, serve as templates for making protein molecules. Thus, most genes are essentially blueprints for making proteins. The production of protein from a DNA blueprint is called gene expression.

3. A gene can accept occasional changes, or mutations.

### 1.2.6. Mitochondrial DNA

DNA is also present inside a cell as extra-chromosomal **gene** s in mitochondria. Several mitochondria are present in each cell. In plants, the photosynthetic organelles, chloroplasts also contain DNA.

Unlike the chromosomal DNA, there is no meiosis and replication appears to be a simple copying process. Extra chromosomal genes present in mitochondria are normally circular molecules of around 16 kb in length. Because there are large numbers of mitochondria in an egg, but very few in a spermatozoan, it is hardly surprising to find that mt DNA present in a sexually reproduced offspring is usually inherited entirely from its mother. This maternal only inheritance of mt DNA is the normal situation in almost all animals (but *Mytilus* sp. is an exceptional one).

In contrast to the nuclear **genome** , the mitochondrial genes of animals are very efficient and have no **intron** s. In addition there is virtually no ‘ **junk DNA** ’ or repetitive **sequence** s in mitochondrial genome although the control region does often vary in length due to tandem repeats.

Mitochondria **protein** coding genes code for **enzymes** that are involved in electron transport system. They include seven subunits of NADH dehydrogenase, cytochrome b, cytochrome c oxidase and ATP synthetase.

The **mitochondrial genome** of fish contains 13 genes coding for proteins, two genes coding for ribosomal RNA, 22 genes coding for transfer RNA molecules and one non-coding section of DNA which acts as the initiation site for mt DNA replication and RNA **transcription** . This is called the control region.

The rate of mutation in animal mt DNA is higher than in the nuclear DNA (about 5 to 10 times higher). This means that the rate of evolution is greater in mtDNA than in nuclear DNA.

Analysis of mitochondrial DNA for determination of population relationships is particularly attractive for three reasons.

i) it is relatively small (For e.g., in rainbow trout -$16.5X10^3$ bp and catfish – 17 kb) and less complex than nuclear DNA.

ii) mt DNA exhibits a more rapid evolution than nuclear DNA and thus, allows for detection of relatively recent sequence divergence.

iii) the inheritance of mt DNA is apparently strictly maternal, thereby avoiding the complication of sexual recombination of genetic material.

**Restriction enzyme** analysis of mt DNA from several populations of rainbow trout result in their differentiation. Analysis of mt DNA by restriction **endonuclease** s has been used to distinguish three species of catfishes from the Arabian Gulf (*Arius bilineatus, A. thalassinus and A. teniispinis*).

### 1.2.7. Genetic code

Most **gene** s encode **protein** s and only a small part of the total DNA coding regions of genes act as a template for the protein. Proteins are made up of **amino acids** . It is the **sequence** of amino acids which give the protein its specific properties. DNA template is first transcribed into mRNA. The mRNA template is then translated into a chain of amino acids.

There are 20 different amino acids which are used to build up proteins. Which amino acid is signaled by which particular codes? This remained one of the great mysteries until the early 1960s.

The most outstanding work in breaking of the genetic code was done by Marshall Nirenberg and his associates in the early 1960s. They devised an elegant technique, called the triplet binding test, and discovered the first word of code dictionary.

A system was developed for synthesizing proteins ***in vitro*** ; the system included a cell extract containing ribosomes, tRNAs and other cellular components. Into this, Nirenberg added artificially synthesized mRNA molecules of known **nucleotide** sequences. When synthetic mRNAs consisting entirely of a single type of

nucleotide were added, **polypeptide** s composed of only a single type of amino acid were formed. Thus, phenylalanine was formed when polyuridylic acid (poly U) was added.

Marshall Nirenberg, Severo Ochoa, Hargobind Khorana, Francis Crick and many others contributed significantly to decipher the genetic code.

- **They figured out that the order in which amino acids are arranged in proteins.**
- **On the basis of a variety of experiments, it was found out that a particular sequence of 3 bases (triplet) would code for a particular amino acid and this triplet is referred to as codon .**
- **For example, if the mRNA has in its sequence a triplet code AUG, the corresponding amino acid in the protein would be methionine.**
- **Similarly, the sequence UUU would code for phenylalanine. Thus, the codons for all the 20 naturally occurring amino acids in proteins were figured out.**
- **Thus, in a mRNA molecule, the sequence of bases read in blocks of three at a time starting from a particular position in a non-overlapping fashion would automatically decide the sequence of amino acids in the poly peptide derived from that mRNA.**
- **Thus, 4 bases when arranged in the form of triplet code can generate $4^3$ or 64 codons .**
- **Of these, 3 codons serve as STOP (Non-Sense) codons (UAG, UAA, UGA) which simply tell the translation machinery to terminate. Not recognized by tRNAs.**
- **One codon AUG serve as initiating codon. Thus, many amino acids have more than one codon and codons specifying the same amino acid are said to be degenerate and differ in only the third base.**

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### 1.2.7.1. Properties of the Genetic code

1. The code is highly de gene rate, meaning that most of the amino acids are coded for more than one amino acids. Leucine, serine and arginine have 6 different codon s. Proline, threonine and alanine, have four. Isoleucine has three. Methionine and tryptophan have only one codon.

1. The code is not overlapping. There is no punctuation or spacing between different codons. The starting signal for protein synthesis is the codon AUG (for methionine).

2. The code appears to be highly universal that is, it is the same for various different kind of organisms. Coding regions can be transferred from one organism to another and the correct protein produced. However, a few exceptions to this are known. For example, in yeast mitochondria, UGA codes for tryptophan instead of stop. In Paramecium, UAA and UAG code for glutamine instead of stop codon.
3. Point Mutation will cause change in the amino acid sequence .

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Normal gene frame | BIG | FAT | CAT | ATE | RAT |
| Delete base (F) | 1BIG | ATC | ATA | TER | AT- |
| Add base (X) | 1BIG | FAT | CXA | TAT | ERA + |

The universality provides strong evidence that life on earth started only once. When the first living forms appeared some 3 billion years ago, the genetic code was established and it has not changed since then through out the evolution of living organisms. Once the initial code was established, there were strong selective pressures to maintain it invariant because the change in a single codon would change amino acids in a great many proteins at the same time and these multiple mutations would in all likelihood be lethal.

The selective pressure has been less strict in mitochondrial DNA. Mitochondria code only for few proteins and have their own protein synthetic machinery. The overall code has been maintained.

## Chapter 3: Organization of genome in prokaryotes and eukaryotes

### 1.3.1. Introduction

If an organism is to survive the processes that enable information to be copied from gene s and then used to synthesize protein s must be regulated. Different cells within an organism share the same set of chromosomes. In each cell some genes are active while others are not. For example, in humans only red blood cells manufacture the protein hemoglobin and only pancreas cells make the digestive enzyme known as trypsin, even though both types of cells contain the genes to produce both hemoglobin and trypsin. Each cell produces different proteins according to its needs so that it does not waste energy by producing proteins that will not be used.

A variety of mechanisms regulate gene activity in cells. One method involves turning on or off gene transcription , sometimes by blocking the action of RNA polymerase , an enzyme that initiates transcription.

Gene regulation may also involve mechanisms that slow or speed the rate of transcription, using specialized regulatory proteins that bind to DNA. Depending on an organism's particular needs, one regulatory protein may spur transcription for a particular protein, and later, another regulatory protein may slow or halt transcription.

### 1.3.2. Gene structure

Transcription proceeds from left to right, regardless of the orientation of the gene in the chromosome. This means that the promoter lies to the left of the coded region. Taking the gene organization first:

Transcription starts at the transcription initiation site, and stops when it encounters the polyA attachment site.

Transcription produces mRNA as a copy of the DNA, from the initiation site to the polyA attachment site.

A set of enzymes then attaches a series of a hundred or more A's to the mRNA called the polyA tail. This tail appears to protect the mRNA from degradation by enzymes.

So mRNA is simply a single strand of bases, copied from the genomic DNA, from the initiation site and ending with a polyA tail.

The start codon , for translation, is always AUG, which encodes methionine and at the end is a stop codon UGA. These codons define the coding region. The region of about 30 bases between the transcription initiation and start codon is called the upstream untranslated region (UTR). The region between the stop codon and the polyA attachment site is called the downstream, and in some genes contain sequence s which control mRNA stability.

### 1.3.3. Genes in development

Gene regulation helps individual cells within an organism function in a specialized way. Other regulatory mechanisms coordinate the genes that determine how cells develop. All of the specialized cells in an organism, including those of the skin, muscle, bone, liver, and brain, derive from identical copies of a single fertilized egg cell. Each of these cells has

the exact same DNA as the original cell, even though they have vastly different appearances and functions. Genes dictate how these cells specialize.

Early in an organism's embryonic development the overall body plan forms. Individual cells commit to a particular layer and region of the embryo, often migrating from one location to another to do so. As the organism grows, cells become part of a particular body organ or tissue, such as skin or muscle. Ultimately, most cells become highly specialized—not only to develop into a neuron rather than a muscle cell, for example, but to become a sensory neuron instead of a motor neuron. This process of specialization is called differentiation. At each stage of the differentiation process, specific genes known as developmental control genes actively turn on and switch off the genes that differentiate cells.

One class of developmental control genes, known as homeotic genes, directs the formation of particular body parts. Activating one set of homeotic genes instructs part of an embryo to develop into a leg, for example, while another set initiates the formation of the head. If a homeotic gene becomes altered or damaged, an organism's body development can be dramatically disrupted. A change in a single gene in some insects, for instance, can cause a leg to grow where an antenna belongs. Homeotic genes work by regulating the activity of other genes. Homeotic genes code for the production of a regulatory protein that can bind to DNA and thus affect the transcription of one or more genes. This enables homeotic genes to initiate or halt the development and specialization of characteristics in an organism.

Nearly identical homeotic genes have been identified in varied organisms, such as insects, worms, mice, birds, and humans, where they serve similar embryonic development functions. Scientists theorize that homeotic genes first appeared in a single ancestor common to all these organisms. Sometime in evolutionary history, these organisms diverged from their common ancestor, but the homeotic genes continued to be passed down through generations virtually unchanged during the evolution of these new organisms.

The information present in the gene is not always used. Many genes remain silent and are expressed only when the gene product is needed. However, there are certain genes whose products are constantly needed for cellular activity. These are known as 'house–keeping genes'.

### 1.3.4. Gene expression in prokaryotes

An average bacterium, contains one thousands the DNA content of a typical eukaryotic cell. The bacterial chromosome contains a single circular DNA molecule associated with a few **protein**s and is not enclosed within a limiting membrane unlike that in the eukaryotic cell. Bacteria can divide very rapidly. The doubling time is also referred to as generation time and in some bacteria, this can be as low as 20 minutes from a single origin of replication and can proceed bidirectionally.

The bacterium *Escherichia coli* has about 2,500 genes. **The expression of these genes** is usually controlled to achieve maximum cellular economy. This means that genes will be turned on or off as per the requirement. A set of genes will be switched on when there is necessity to handle and metabolise a new substrate. When these genes are turned on, **enzymes** are produced, which metabolise the new substrate. The phenomenon is known as induction and the small molecules eliciting this induction are referred to as inducers.

Similarly, when a metabolite needed by the bacterium is provided in excess from outside, the bacterium stops making it and thus conserves its reserves. This is achieved by the added metabolite turning off a set of genes involved in producing that metabolite in the bacterial cell. This phenomenon is known as feed back repression.

As against the processes of induction and repression as already indicated a set of genes are constantly expressed to take care of house keeping functions such as glycolysis. These genes which are constantly expressed are referred to as constitutive.

In 1961, Francois Jacob and Jacques Monod, at the Pasteur Institute in Paris, proposed that metabolic path ways are regulated as a unit. For example, when the sugar lactose is added to the cultures of *E. coli*, it induces three enzymes necessary to break down the lactose into glucose and galactose.

### 1.3.4.1. Bacterial operons

Lac operon

**Lac operon** consists of 3 **gene** s, lac Z, Y and A coding for β-galactosidase, permease and transacetylase catalyzing a catabolic pathway.

- The genes for these three **enzymes** occur adjacent to each other and thus are linked. These are referred to as **structural gene** s, since they have the information to code for the amino acid **sequence** and thus directly decide the structure and function of the individual **protein** s of the pathway.
- These 3 genes are regulated as a unit by a single switch operator O. This entire unit is referred to as an operon.
- RNA **polymerase** binds to the promoters region P and initiates **transcription** . However, under normal conditions transcription cannot proceed, since a repressor protein coded by the *i* gene binds to the operator and blocks RNA polymerase movement.
- In the presence of the inducer lactose, the repressor protein structure is modified such that the repressor cannot bind to the operator any more.
- This leads to the transcription of the operon and induction of β-galactosidase and the other two enzymes.
- β-galactosidase cleaves lactose into glucose and galactose and once this happens induction will cease. The genes are expressed or not expressed

depending on whether the operator switch is on or off. When the switch is on, the three genes are transcribed by RNA polymerase into a single stretch of mRNA covering all the three genes.

- Each gene segment is referred to as a **cistron** and the long messenger RNA covering all the cistrons is known as polycistronic.
- **1.3.5. Gene Expression in Eukaryotes**
- Gene regulation in eukaryotes is more complex than in bacteria and other prokaryotes. Higher eukaryotes have several thousand genes. Eukaryotes are multicellular organisms which can also undergo differentiation. Thus, the cells in the undifferentiated stage not only grow and divide, but are also destined to become part of specialized tissues such as the liver, spleen or heart in an animal and the leaf, root, stem or flower in an angiosperm. Thus regulation of **gene expression in the eukaryotic cell** is very complex. Most multicellular organisms contain different types of cells that serve specialized functions. The cells of an animal's heart, blood, skin, liver, and muscles all contain the same genes. But in order to carry out their specific functions within the body, each cell must produce different **protein** s and respond to changing environmental stimuli, such as
- glucose levels in the blood or body temperature. Such specialization is possible only with sophisticated gene regulation.
- The information on the eukaryotic gene for assembling a protein is not continuous, but split. However, when messenger RNA is formed from such genes, the unwanted RNA regions are removed and the regions coding for **amino acids** are joined together. This process is referred as **splicing** . Thus, bases in the messenger RNA and amino acids in proteins are collinear even in eukaryotic cells, although the genes are split. The regions of a gene, which become part of a mRNA and code for different regions of the protein, are referred to as **exon** s. The regions which do not form part of RNA processing before mRNA formation are referred to as **intron** s. In eukaryotes, genes involved in coding for the **enzymes** of a particular metabolic pathway need not to be linked. Sometimes they are present even on different chromosomes. However, such genes are regulated together just as in bacterial **operon** s. The basic processes of induction and repression is constantly regulated by the changing environment in the cell. Thus during growth and development, small molecules such as **hormone** s, vitamins, metal ions, chemicals and invading pathogens can induce or repress certain genes and this would result in the production or absence of certain proteins. This ultimately leads to the operation or non-operation of metabolic pathways leading to altered cell function. This is the underlying molecular basis of growth, development, differentiation and disease brought about by the influence of the environment on gene expression.
- Eukaryotes use a variety of mechanisms to ensure that each cell uses the exact proteins it needs at any given moment. In one method, eukaryotic cells use **DNA sequence** s called enhancers to stimulate the **transcription** of

genes located far away from the point on the chromosome where transcription occurs. If a specific protein binds to an enhancer site on the DNA, it causes the DNA to fold so that the enhancer site is brought closer to the site where transcription occurs. This action can activate or speed up transcription in the genes surrounding the enhancer site, thereby affecting the type and quantity of proteins the cell will produce. Enhancers often exert their effects on large groups of related genes, such as the genes that produce the set of proteins that form a muscle cell.

- Gene regulation can also take place after transcription has occurred by interfering with the steps that modify mRNA before it leaves the nucleus to take part in translation. This process typically involves removing exons (segments that code for specific proteins) and introns. These sections of the mRNA can be modified in more than one way, enabling a cell to synthesize different proteins depending on its needs.

## Chapter 4: DNA replication, transcription and translation.

1.4.1. Introduction

Prior to cell division, the DNA material in the original cell must be duplicated so that after cell division, each new cell contains the full amount of DNA material. The process of DNA duplication is usually called replication. In this process each strand of the original double-stranded DNA molecule serves as template for the reproduction of the complementary strand. Through DNA replication, two identical DNA molecules have been produced from a single double-stranded DNA molecule. The replication is termed semi conservative since each new cell contains one strand of original DNA and one newly synthesized strand of DNA.

In a **cell** , DNA replication begins at specific locations in the **genome** , called " **origins** ". Unwinding of DNA at the origin, and synthesis of new strands, forms a **replication fork** .

At a specific point, the double helix of DNA is caused to unwind possibly in response to an initial synthesis of a short RNA strand using the enzyme helicase. **Protein** s are available to hold the unwound DNA strands in position. Each strand of DNA then serves as a template to guide the synthesis of its complementary strand of DNA.

- **DNA polymerases** are a family of **enzymes** that carry out all forms of DNA replication. DNA **polymerase** III is used to join the appropriate **nucleotide** units together. **A DNA polymerase can only extend an existing DNA strand paired with a template strand; it cannot begin the synthesis of a new strand.**

- To begin synthesis of a new strand, a short fragment of DNA or **RNA** , called a **primer** , must be created and paired with the template strand before DNA polymerase can synthesize new DNA.
- Once a **primer** pairs with DNA to be replicated, DNA polymerase synthesizes a new strand of DNA by extending the 3' end of an existing nucleotide chain, adding new **nucleotides** matched to the template strand one at a time via the creation of **phosphodiester bonds** .
- The energy for this process of DNA polymerization comes from two of the three total phosphates attached to each unincorporated **base** . (Free bases with their attached phosphate groups are called **nucleoside triphosphates** ))
- When a nucleotide is being added to a growing DNA strand, two of the phosphates are removed and the energy produced creates a **phosphodiester bond** that attaches the remaining phosphate to the growing chain.
- DNA polymerases are **gene** rally extremely accurate, making less than one error for every $10^7$nucleotides added.
- DNA polymerases also have proofreading ability; they can remove nucleotides from the end of a strand in order to correct mismatched bases.
- **1.4.2. Origins of a replication**
- For a cell to divide, it must first replicate its DNA. This process is initiated at particular points within the DNA, known as "origins", which are targeted by **protein** s that separate the two strands and initiate DNA synthesis. Origins contain **DNA sequence** s
- recognized by replication initiator proteins (e.g.dnaA in *E coli* and the Origin Recognotion Complex in yeast).
- These initiator proteins recruit other proteins to separate the DNA strands at the origin, forming a bubble.
- Origins tend to be "AT-rich" (rich in adenine and thymine bases) to assist this process, because A-T base pairs have two hydrogen bonds (rather than the three formed in a C-G pair)—strands rich in these **nucleotide** s are **gene** rally easier to separate due to the positive relationship between the number of hydrogen bonds and the difficulty of breaking these bonds.
- Once strands are separated, RNA **primer** s are created on the template strands.
- More specifically, the leading strand receives one RNA primer per active origin of replication while the lagging strand receives several; these several fragments of RNA primers found on the lagging strand of DNA are called Okazaki fragments, named after their discoverer.
- DNA **polymerase** extends the leading strand in one continuous motion and the lagging strand in a discontinuous motion (due to the Okazaki fragments).
- **RNase** removes the RNA fragments used to initiate replication by DNA Polymerase, and another DNA Polymerase enters to fill the gaps.
- When this is complete, a single nick on the leading strand and several nicks on the lagging strand can be found. Ligase works to fill these nicks in, thus completing the newly replicated DNA molecule.

- As DNA synthesis continues, the original DNA strands continue to unwind on each side of the bubble, forming two **replication forks** . In bacteria, which have a single origin of replication on their circular chromosome, this process eventually creates a " **theta structure** " (resembling the Greek letter theta: θ). In contrast, eukaryotes have longer linear chromosomes and initiate replication at multiple origins within these.

### 1.4.3. The replication fork

- The replication fork is a structure that forms within the nucleus during DNA replication.
- It is created by helicases, which break the hydrogen bonds holding the two DNA strands together.
- The resulting structure has two branching "prongs", each one made up of a single strand of DNA.
- These two strands serve as the template for the leading and lagging strands which will be created as DNA **polymerase** matches complementary **nucleotide** s to the templates.
- The templates may be properly referred to as the leading strand template and the lagging strand template.
- **1.4.4. Leading strand and Lagging strand**
- The **leading strand** is the template strand of the DNA double helix so that the replication fork moves along it in the 3' to 5' direction. This allows the new strand
- synthesized complementary to it to be synthesized 5' to 3' in the same direction as the movement of the replication fork. On the leading strand, a **polymerase** "reads" the DNA and adds **nucleotides** to it continuously. This polymerase is **DNA polymerase III** (DNA Pol III) in **prokaryotes** and presumably **Pol ε** in **eukaryotes** .
- The **lagging strand** is the strand of the template DNA double helix that is oriented so that the replication fork moves along it in a 5' to 3' manner. Because of its orientation, opposite to the working orientation of DNA polymerase III, which moves on a template in a 3' to 5' manner, replication of the lagging strand is more complicated than that of the leading strand. On the lagging strand, **primase** "reads" the DNA and adds **RNA** to it in short, separated segments. In eukaryotes, primase is intrinsic to **Pol α** . DNA polymerase III or **Pol δ** lengthens the primed segments, forming **Okazaki fragments** .
- **Primer removal** in eukaryotes is also performed by Pol δ. In prokaryotes, **DNA polymerase I** "reads" the fragments, removes the RNA using its **flap endonuclease** domain (RNA **primer** s are removed by 5'-3' **exonuclease** activity of polymerase I , and replaces the RNA **nucleotide** s with DNA nucleotides (this is necessary because RNA and DNA use slightly different kinds of nucleotides).
- **1.4.5. Dynamics at the replication fork**

- As helicase unwinds DNA at the replication fork, the DNA ahead is forced to rotate. This process results in a build-up of twists in the DNA ahead. This build-up would form a resistance that would eventually halt the progress of the replication fork.
- **DNA topoisomerases** are **enzymes** that solve these physical problems in the coiling of DNA. Topoisomerase I cuts a single backbone on the DNA, enabling the strands to swivel around each other to remove the build-up of twists. Topoisomerase II cuts both backbones, enabling one double-stranded DNA to pass through another, thereby removing knots and entanglements that can form within and between DNA molecules.
- **1.4.6. Termination of replication**
- Because bacteria have circular chromosomes, termination of replication occurs when the two replication forks meet each other on the opposite end of the parental chromosome.
- *E coli* regulate this process through the use of termination **sequence**s which, when bound by the Tus **protein**, enable only one direction of replication fork to pass through. As a result, the replication forks are con**strain**ed to always meet within the termination region of the chromosome.
- Eukaryotes initiate DNA replication at multiple points in the chromosome, so replication forks meet and terminate at many points in the chromosome; these are not known to be regulated in any particular manner.
- Because eukaryotes have linear chromosomes, DNA replication often fails to synthesize to the very end of the chromosomes (**telomeres**), resulting in telomere shortening. This is a normal process in **somatic cells** — cells are only able to divide a certain number of times before the DNA loss prevents further division (this is known as the **Hayflick limit**).
- **1.4.7. Types of replication**
- Three models of **DNA replication** were proposed:
- 1. Semi conservative replication would produce two copies that each contained one of the original strands and one new strand. Semi conservative replication describes the method by which **DNA** is replicated in all known cells.
- 2. Conservative replication would leave the two original template **DNA** strands together in a double helix and would produce a copy composed of two new strands containing all of the new DNA base pairs.
- 3. Dispersive replication would produce two copies of the **DNA** , both containing distinct regions of DNA composed of either both original strands or both new strands.
- The **deciphering** of the structure of DNA by **Watson** and **Crick** in 1953 suggested that each strand of the **double helix** would serve as a template for synthesis of a new strand. However, there was no way of knowing how the newly synthesized strands might combine with the template strands to form two double helical **DNA** molecules.

- The semiconservative model seemed most reasonable since it would allow each daughter strand to remain associated with its template strand. The semiconservative model was confirmed by the **Meselson-Stahl experiment** .

### 1.4.7.1. Meselson-Stahl experiment

The semi-conservative theory can be confirmed by making use of the fact that DNA is made up of nitrogen bases. **Nitrogen** has an isotope N15 (N14 is the most common isotope) called heavy nitrogen. The experiment that confirms the predictions of the semi-conservative theory makes use of this isotope.

1. Bacterial (*E. coli*) DNA is placed in a media containing heavy nitrogen (N15), which binds to the DNA, making it identifiable.
2. This DNA is then placed in a media with the presence of N14 and left to replicate only once. The new bases will contain nitrogen 14 while the originals will contain N15
3. The DNA is placed in test tubes containing caesium chloride (heavy compound) and centrifuged at 40,000 rpm.
4. The cesium chloride molecules sink to the bottom of the test tubes creating a density gradient. The DNA molecules will position at their corresponding level of density (taking into account that N15 is more dense than N14)
5. These test tubes are observed under uv-rays. DNA appears as a fine layer in the test tubes at different heights according to their density

According to the semi-conservative theory, after one replication of DNA, we should obtain 2 hybrid (part N14 part N15) molecules from each original strand of DNA. This would appear as a single line in the test tube. This result would be the same for the dispersive theory. On the other hand, according to the conservative theory, we should obtain one original DNA strand and a completely new one i.e. two fine lines in the test tube placed separately one from the other. Up to this point, either the semi-conservative or the dispersive theories could be truthful, as experimental evidence confirmed that only one line appeared after one replication. In order to conclude between those two, DNA had to be left to replicate again, still in a media containing N14.

In the dispersive theory, after 2 divisions we should obtain a single line, but further up in the test tube, as the DNA molecules become less dense as N14 becomes more abundant in the molecule. According to the semi-conservative theory, 2 hybrid molecules and 2 fully N14 molecules should be produced, so two fine lines at different heights in the test tubes should be observed.

Experimental evidence confirmed that two lines were observed providing evidence for the semi-conservative replication theory.

### 1.4.8. Transcription

Transcription is the synthesis of mRNA from a DNA template. It is like DNA replication in that a DNA strand is used to synthesize a strand of mRNA. Only one strand of DNA is copied. A single **gene** may be transcribed thousands of times. After transcription, the DNA strands rejoin.Three steps are involved in transcription: initiation, elongation and termination.

### 1.4.8.1.Initiation

RNA **polymerase** recognizes a specific **base sequence** in the DNA called a promoter and binds to it. The promoter identifies the start of a gene, which strand is to be copied, and the direction that it is to be copied. RNA polymerase unwinds the DNA, and the base pairs are disrupted, producing a "bubble" of single-stranded DNA. Like DNA replication, transcription always occurs in a 5' to 3' direction. That is, the new ribo **nucleotide** is added to the 3'end of the growing chain. Unlike replication , however, only one of the strands acts as template on which the RNA strand is built. As RNA polymerase binds promoters in a defined orientation, the same strand is always transcribed from a given promoter.

The choice of promoter determines which stretch of DNA is transcribed and is the main step at which regulation is imposed. That is, the decision of whether or not to initiate transcription of a given gene is chiefly how a cell regulates which **protein** s it will make at any given time.

### 1.4.8.2. Elongation

RNA polymerase assembles bases that are complimentary to the DNA strand being copied. RNA contains uracil instead of thymine. Once the RNA polymerase has synthesized a short stretch of RNA (approximately ten bases), it shifts into the elongation phase. During elongation, the enzyme performs an impressive range of tasks in addition to the catalysis of RNA synthesis. It unwinds the DNA in front and re-anneals it behind, it dissociates the growing RNA chain from the template as it moves along, and it performs proofreading functions.

### 1.4.8.3. Termination

Once the polymerase has transcribed the length of the gene, it must stop and release the RNA product. This step is called termination. A termination code in the DNA indicates where transcription will stop. The mRNA produced is called an mRNA transcript.

### 1.4.9. Processing the mRNA Transcript

In eukaryotic cells, the newly-formed mRNA transcript (also called heterogenous nuclear RNA or hnRNA) must be further modified before it can be used. The

eukaryotic gene s consist of blocks of coding sequence s separated from each other by blocks of non-

coding sequences. The coding sequences are called **exon** s and the intervening sequences are called **intron** s. As a consequence of this alternating pattern of exons and introns, genes bearing non-coding interruptions are often said to be “split”. Exons are too short whereas introns are too long. A cap is added to the 5’ end and a poly-A tail (150 to 200 Adenines) is added to the 3’end of the molecule. The newly-formed mRNA has regions that do not contain a genetic message.

Like the uninterrupted genes of prokaryotes, the split genes of eukaryotes are transcribed into a single RNA copy of the entire gene. Thus, the primary transcript for a typical eukaryotic gene contains introns as well as exons. The primary transcripts of split genes must have their introns removed before they can be translated into **protein** . Introns are removed for the pre-mRNA by a process called RNA **splicing** . This process converts the pre-mRNA into mature messenger RNA and must occur with great precision to avoid the loss, or addition, of even a single **nucleotide** at the sites at which the exons are joined.

DNA is located in an organelle called the **nucleus** . **Transcription** and mRNA processing occur in the nucleus. The nucleus is surrounded by a double membrane. After the mature mRNA transcript is produced, it moves out of the nucleus and into the **cytoplasm** through pores in the nuclear membrane.

### 1.4.10. Translation

Translation is the process where ribosomes synthesize **protein** s using the mature mRNA transcript produced during **transcription** . There is a specific tRNA for each of the 20 different **amino acids** . A tRNA molecule transports an amino acid to the ribosome. The 3-letter **anti codon** on the tRNA molecule matches the 3-letter code (called a **codon** ) in the mRNA. The tRNA with the anticodon "UAC" bonds with methionine. It always transports methionine. Transfer RNA molecules with different anticodons transport other amino acids.

## Unit 2: Genetic Engineering

- **Chapter 1 : Recombinant DNA Technology**
- **2.1.1. Introduction**
- The **recombinant DNA technology** is also referred to as **gene cloning** or molecular cloning. The discovery of **restriction enzyme** s and many other **enzymes** which can be useful as tools in gene manipulation have brought ***in vitro*** recombining of DNA to a reality and dependence on *in vivo* recombinational events came to an end.

- Gene cloning may be aimed at getting more copies of a particular gene in desired host cell. In a way it is **gene amplification** achieved through gene cloning.
- But it is also possible to have expression of gene in desired host cells after cloning. This will result into formation of product of that gene into new host cells. This can be useful for production of **protein** s into cells which are convenient to cultivate. Cloning can be done in bacterial host (e.g. *E .coli*) or in eukaryotes (e.g. yeast).
- **Basically cloning involves 4 steps.**
- 1. The **vector** DNA is cleaved with one or more restriction enzymes.
- 2. The DNA to be **clone** d, the target or insert is joined to the vector, generating a recombinant molecule.
- 3. The recombinant DNA molecule is introduced into the host bacterial cell.
- 4. Transformed colony is selected and amplified.
- **2.1.2. Enzymes commonly used in recombinant DNA technology**
- **2.1.1. Restriction endonuclease s (RE)**
- Restriction endonucleases are bacterial enzymes that are hydrolases and cleave phosphodiester bonds of double stranded DNA at specific palindromic sites within the chain to produce $5^1$ $PO_4$ and $3^1$ OH ends.
- They are called as "restriction endonucleases" mainly due to their natural function in restricting the growth of the virus that attack bacteria. The enzymes do this by binding to the viral DNA and cleaving it at highly specific rites within or adjacent to a particular **sequence** know as recognition sequence.
- The site specificity is important since it enables the bacteria to defend its own DNA against attack by the **restriction enzyme** s by methylating the corresponding sites of its own DNA. Thus each types of bacterium produces a few restriction endonucleases, but its own DNA is not cleaved by its own restriction enzyme as it is **methylated** and thus protected from cleavage by its own enzymes. The existence of restriction enzymes were observed by Werner Arber (1968).
- About hundreds of different restriction enzymes have been purified, and many of them are commercially available. These restriction enzymes are utilized to cut any extremely long length of DNA into a series of appropriate sized fragments, from which a fragment containing the desired **gene** is **probe** d.
- **There are three types of restriction endonucleases : Type I, II and III.**
- Type I and III recognize specific nonpalindromic sequences in the DNA chain, but cleave the chain at different sites away from the recognition site, thus producing DNA fragments of different length and ends.
- Type II restriction endonucleases, however, recognize specific palindromic sequences that range generally from 4–8 **nucleotide** s and cut the chain within the site, thus producing specific DNA fragments with known ends. For this reason, they are very useful in **genetic engineering** .

- **Palindrome** s in DNA are sequences that read the same sequence of bases from either end (e.g. MALAYALAM)
- The restriction enzyme ECO RI, recognizes the GAATTC sequence and cuts the two strands at the sites shown producing staggered or sticky or cohesive $5^1$ and $3^1$ ends. Alu I cuts the AGCT sequence producing blunt or flush ends.
- At present more than 1200 restriction enzymes with different specificities for different palindromic sequences have been purified from various types of bacteria.
- Certain restriction endonucleases purified from different bacteria recognize the same palindromic sequence, but may or may not cut at the same site and are named **isoschizomers**,
- e.g. Dpn I and Sau 3A cut at GATC and G$^m$ATC.
- Hpa II cuts CCGG, but cannot cut C$^m$CGG.
- Msp I cuts both CCGG and C$^m$CGG. Such *isoschizomers* are used for finding out if a DNA is methylated at a specific site.
- The restriction enzymes are named by taking the first letter of the generic name and the first two letters of the species, e.g. the Eco RI. *E. coli* R – **strain** , I is the first enzyme. Specific site – GAATTC – Eco RII – Second enzyme, specific site – CCAGC -.
- The frequency of occurrence of a palindrome in a DNA strand depends on the length of the palindrome. Site frequency = ¼ n where, n = length of the restriction site sequence. For example, an AGCT palindrome occurs at intervals of 256 bp, whereas GAATTC occurs at intervals of 4096 bp.
- Restriction enzymes have helped in the isolation of specific genes from various species including that of man and transferring it to another species, and thus cross the species barrier, though in nature, two different species do not cross-breed. They have helped in the development of recombinant DNA and genetic engineering technology which are of immense benefit for mankind.

### 2.1.2.2. DNA polymerase and reverse transcriptase

DNA polymerases synthesize complementary **nucleotide sequence** on a template nucleotide strand.

- **DNA polymerase I**, isolated from *E. coli*, synthesizes a complementary strand on a template DNA in $5^1 \rightarrow 3^1$ direction.
- It also possess low level of **exonuclease** activity in both $5^1 \rightarrow 3^1$ and $3^1 \rightarrow 5^1$ directions.
- This enzyme is used in labeling of DNA to prepare **probe** .

i) **Klewnow enzyme** is the large fragment of the DNA Polymerase I of *E. coli.*

- It possess $5^1 \rightarrow 3^1$polymerase activity and $3^1 \rightarrow 5^1$ **exon** uclease activity but lacks the exonuclease activity in $5^1 \rightarrow 3^1$ direction.

ii) **Tag DNA polymerase** is isolated from a bacterium *Thermus aquaticus,* living in hot springs and active even at 94ºC.

- This enzyme is highly thermostable for which it is used for DNA amplification during **polymerase chain Reaction (PCR)** . It does not have $3^1 \rightarrow 5^1$ exonuclease activity and hence cannot carry out proof reading.

**Reverse Transcription of mRNA**

Recently, a superior method of selecting desired **gene** s has been discovered, which is called reverse **transcription** of mRNA.

- The desired **protein** is first refined and purified, and next it is administered to a rabbit to stimulate the synthesis of an **antibody** against it.
- Antibody formation is a natural defense mechanism. When pathogenic bacteria or other extraneous substances invade our bodies, we recognize them as foreign and produce specific proteins which can bind to them, leading to inactivation or destruction of the invader.
- These binding proteins are called **antibodies**. Antibody specificity for antigens is extremely high.

Following this step, the antibodies produced in the rabbit are mixed with homogenized cells in which the desired protein is being synthesized. The antibodies specifically bind to the proteins while in the process of being synthesized.

- The anti-body protein-m-RNA ribosome complex sediments and m-RNA is extracted from this sediment.
- Next, the m-RNA is mixed with the enzyme "**reverse transcriptase**". (This enzyme was found in some **viruses** having RNA as genetic information instead of DNA. The virus utilizes reverse transcriptase to catalyze the reverse process of synthesizing a complementary DNA chain on an RNA template).
- The genes specifying the desired protein can be obtained by using reverse transcriptase to make a complementary single-stranded DNA molecule synthesized on the m-RNA template.
- Then this single stranded DNA is converted into a double-stranded complementary DNA molecule by using the enzyme **DNA polymerase**.

**2.1.3. Vectors**

Vectors are the carrier DNAs into which 'foreign' DNAs or **gene** s of interest are spliced to make a **recombinant DNA**.

- Vectors along with this 'foreign' DNAs (i.e. recombinant DNA) are then introduced into appropriate host cell and are maintained for study or expression.
- Organisms with chimeric property can be produced with the help of **cloning** vectors.
- There are two types of vectors, **cloning vectors and expression vectors.**

**i) Cloning vectors** are used for obtaining millions of copies of **clone** d DNA segment. The cloned genes in these vectors are not expected to express themselves at **transcription** or translational level. Cloning vectors are used for creating **genomic library** or preparing the **probe** s or **genetic engineering** experiments or other basic studies. Most cloning vectors were originally derived from naturally occurring extrachromosomal elements such as **bacteriophage** s and **plasmid** s.

**ii) Expression vectors** allow the expression of cloned gene, to give the product ( **protein** ). This can be achieved through the use of promoters and expression cassettes and **regulatory gene** s ( **sequence** s). Expression vectors are used for **transformation** to generate **transgenic** plant, animal or microbe where cloned gene expresses to give the product. Commercial production of product of cloned gene may also be achieved by high level expression using the expression vectors.

2.1.3.1 Plasmids

The occurrence of plasmids in *E. coli* came to light in the early 1950's through the pioneering work of Joshua Lederberg in the USA and William Hayes in England.

- Plasmids are gene tic elements that are stably inherited without being a part of the chromosome (s) of their host cells.
- They are found in bacteria and fungi of many kinds but not in higher eukaryotes and are not essential to the survival of the host cell.
- They may be composed of DNA or RNA and may be linear or circular.
- Double-stranded DNA plasmids appear to exist as predominantly covalently closed circular molecules in bacterial cells.
- Both circular and linear plasmids are found in yeast and other fungi. Linear yeast plasmids composed of either RNA or DNA, can encode protein toxins that inhibit the growth of sensitive yeasts.

Plasmids code for molecules that ensure their replication and stable inheritance during cell replication, and they also encode many products of considerable medical, agricultural, and environmental importance. For example, they code for toxins that greatly increase the virulence of pathogenic bacteria. They can also confer resistance to antibiotic s, and they enable bacteria belonging to the genus *Rhizobium* to fix atmospheric $N_2$.

Plasmids are widely used in molecular biology because they provide the basis for many vector s that are used to clone and express genes. The smallest bacterial plasmids are about 1.5 kb and the largest are greater than 1500 kb. The vast majority are circular. However, several very large linear DNA plasmids, up to 500 kb long, have been found in species of *Streptomyces* and *Nocardia*. Smaller plasmids are much desirable for gene cloning experiments. Larger plasmids are less in number whereas smaller ones are more in number.

The number of molecules of a plasmid found in a single bacterial cell is termed as copy number. It ranges from 1 to more than 50 per cell but this number is specific for a given plasmid residing in bacterial cell.

Plasmids with larger copy number are more useful for gene cloning experiments.

Plasmid PBR 322 is derived from transposon $Tn^3$, plasmid pMBI, and plasmid pSC 101. pMBI- replicon,$Tn^3$ - ampicillin transposon, pSC 101 – tetracycline resistance region.

# 2.1.3.2 Shuttle vectors and Bacteriophages

### Shuttle vectors

- Specialized vectors have been made that can replicate in more than one organism.
- This allows the same gene to be expressed in different hosts. Shuttle vectors must have separate origins of replication and separate selection mechanisms for each host organism.
- In order for a shuttle vector to grow in both yeast and *E. coli*, it must have several essential elements;
- two origins of replication, one for *E. coli* and one for yeast;
- a yeast centromere sequence so that it is partitioned into the daughter cells during yeast replication;
- selectable markers for both yeast and *E. coli*; and
- a multiple cloning site for inserting the gene of interest.

- Phage has a linear DNA molecule so a single break creates two fragments.
- Foreign DNA can be inserted between them and two fragments can be joined.
- Such phages when undergo lytic cycle in host will produce more chimeric DNA.
- Wild type lambda phage could accommodate only 2.5 kb of foreign DNA.

- Phage vectors are restructured by removing nonessential genes and making vector DNA smaller so that larger insert can be accommodated in phage head during packing.
- Lambda phage such prepared has one Eco RI site and accommodates 20-25kb of foreign DNA.
- They are used for preparing genomic library of eukaryotes.

### 2.1.3.3 Cosmids

Cosmids are the novel cloning vectors which possess properties of both plasmid and phage.

- Cosmids were first developed in 1978 by Barbara Hohn and John Collins.
- Cosmids contain a *cos* site of phage (which is essential for packaging of nucleic acid into protein coat) plus essential features of plasmid (such as plasmid origin of replication, a gene for drug resistance) and several unique restriction sites for insertion of DNA to be clone d.
- Cosmids can be perpetuated in bacteria in plasmid form, but can be purified by packaging *in-vitro* into phages.
- Advantage of using cosmid vector is that larger DNA can be cloned than what is possible with phage of plasmid.

For cloning foreign DNA into cosmid vector, cosmid DNA is first linearised by cutting it with appropriate RE. Foreign DNA which is to be cloned is also treated with the same RE. Subsequently, cosmid DNA and foreign DNA fragments are mixed in presence of $T_4$ DNA ligase .

### 2.1.3.4 Yeast cloning vectors

· Yeast cloning vectors are the carrier DNA molecules into yeasts.

· Yeasts are eukaryotes.

· Yeast artificial chromosome (YACS) are most sophisticated yeast vectors.

· They have centromeric and telomeric region of a chromosome. These regions are needed to allow chromosome to be replicated in yeast cells.

· Due to origin of replication that is present, replication of DNA occurs. These elements are placed in single DNA fragment which can be used as vector to clone foreign DNA into yeasts.

· The advantages of YAC include very large piece of DNA can be cloned. Only single copy of YAC is present per cell.

### 2.1.3.5 Shuttle vectors

Specialized vectors have been made that can replicate in more than one organism.

- This allows the same **gene** to be expressed in different hosts. Shuttle vectors must have separate origins of replication and separate selection mechanisms for each host organism.
- In order for a shuttle vector to grow in both yeast and *E. coli*, it must have several essential elements;
- two origins of replication, one for *E. coli* and one for yeast;
- a yeast centromere **sequence** so that it is partitioned into the daughter cells during yeast replication;
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- Wild type lambda phage could accommodate only 2.5 kb of foreign DNA.
- Phage vectors are restructured by removing nonessential genes and making vector DNA smaller so that larger insert can be accommodated in phage head during packing.
- Lambda phage such prepared has one Eco RI site and accommodates 20-25kb of foreign DNA.
- They are used for preparing **genomic library** of eukaryotes.

## 2.1.4. Cloning a Gene

DNA cloning or molecular cloning is the technique of producing identical copies of DNA in a larger amount with the help of host. The recombinant DNA must be introduced into a cell, within which it may replicate freely. In addition, it is required that the introduced gene also be expressed within this cell.

## 5.4.1. Cloning in Prokaryotes

After ligation of target into **vector** , the recombinant DNA is multiplied in a suitable prokaryotic host. Various bacterial host commonly employed are :

1) Gram positive bacteria *E. coli*: host for **plasmid** .

2) Gram negative bacteria, *Bacillus subtilis*: host for plasmid, phages and **cosmid** vectors.

- **Chimeric DNA** is inserted into bacterial cells to create transformed bacterial cells.
- Colonies of transformed cells are selected and used for multiplication in suspension cultures.
- The rDNA is retrieved and isolated from the host cell when required.

***E. coli:***

- Most commonly employed gram positive bacterial host which is termed as **workhorse** of **genetic engineering** .
- After suitable modifications, several commercially valuable **protein** s have been expressed from transformed *E. coli* cells.

***Bacillus subtilis:***

- Utilized in a large number of fermentation industries.
- Absolutely non-pathogenic.
    - Most species secrete proteins which allow the desired protein to be collected **exogenous** ly.
- ***Streptomyces* species:**
    - Gram negative bacteria producing more than 60 percent of known **antibiotic** s.
    - Genetic engineering may help in improving their drug producing ability and synthesizing novel antibiotics.

## 2.1.4.1. Cloning foreign DNA into the circular DNA of a plasmid

- The cell's total **genome** is digested using a restriction **endonuclease** that produces **sticky end** s to get the **`foreign DNA'**.
- The circular plasmid is also cut open with the same endonuclease.
- The thousands of DNA fragments of the total genome and of open plasmids are annealed and ligated to get recombinant plasmids in which at least one or two carry the desired **gene** .
- The highly heterogeneous mixture of recombinant and parental plasmids so produced is introduced into the ***competent cells* of *E. coli*** by the process called **transformation** .
- The cells are made competent by treating with high concentration of $ca^{++}$ which increases the permeability of the cell wall.
- The cells were then placed on a selective medium containing an **antibiotic** to which the plasmid carries the resistance gene.
- The transformed cells grow to give colonies of cells on the plate, each colony being formed by a single transformed cell.

## 2.1.4.2. Cloning in Eukaryotes

*E. coli* offers limitation to its use in cloning eukaryotic genes as **splicing** of mRNA cannot take place. Therefore, eukaryotic cells are used to **clone** and express eukaryotic genes. Among eukaryotes, DNA cloning has been done mostly in yeast cells.Yeast is becoming an important organism for the commercial production of medically important proteins such as viral vaccines. It is of great value as an organism for basic research, for producing heterologous proteins and as a vehicle for cloning large segments of DNA.

## 2.1.5. Preparation of a DNA Library

A DNA library is a storehouse of **gene** tic information maintained in bacteria instead of books. These bacteria are **clones** created by recombinant DNA, and the foreign DNA they hold is the library's store of information. DNA libraries are helpful to scientists who require a plentiful supply of particular DNA segments to do their work. These repositories of genetic information are stored in small tubes, which can easily be shipped to other researchers for study.

Each library has a unifying theme. For example, a library may contain the entire chromosomal DNA, or **genome** , of a given organism, or it may consist of genes that are active within certain types of cells, such as heart cells. To create a library of the human genome, DNA from all the human chromosomes would be cut into many pieces. These pieces would be randomly inserted into **vector** s, such as **plasmid** s, which would then be placed into a population of bacteria.Taken together, the entire population of bacteria would contain all the DNA of the human chromosomes.

DNA library is a collection of **clone** d DNA fragments. There are two types of DNA library:

- The **genomic library** contains DNA fragments representing the entire genome of an organism. The **genomic library** phage vectors, instead of plasmid vectors.λis normally made by
- The **cDNA library** contains only complementary DNA molecules synthesized from mRNA molecules in a cell.

The advantage of **cDNA** library is that it contains only the coding region of a genome. To prepare a cDNA library, the first step is to isolate the total mRNA from the cell type of interest. Because eukaryotic mRNAs consist of a **poly-A tail** , they can easily be separated. Then the enzyme reverse transcriptase is used to synthesize a DNA strand complementary to each mRNA molecule. After the single-stranded DNA molecules are converted into double-stranded DNA molecules by DNA **polymerase** , they are inserted into vectors and cloned.

## 2.1.5.1 Colony hybridization

It is the preferred choice for screening the colonies to identify and isolate the colony which contains the desired gene.

- In this technique the cells are first plated on selective plates.
- A replica of the colonies is made on nitrocellulose filter disc which is placed on the surface of a second plate.
- The colonies are allowed to grow on the master plate and the nitrocellulose disc.
- The disc is then removed and placed in alkali to lyse the bacteria *in situ* and to denature their DNA.
- The single-stranded DNA binds to the nitrocellulose filter in the position originally occupied by the bacterial colony.
- The filter is than baked at $80^0$C, following which it is incubated with a solution containing the radiolabelled c DNA probe under conditions which favour nucleic acid hybridization.
- The unhybridized material is removed by extensive washing, thus allowing the identification of colonies containing sequence s complementary to the probe by autoradiography .
- Colonies which give a positive autoradiograph signal can then be picked from the master plate and cultured in order to provide sufficient cells carrying the desired gene.

## 2.1.5.2. Probes

A probe is a piece of DNA or RNA used to detect specific nucleic acid **sequence** s by **hybridization** (binding of two nucleic acid chains by base pairing). They are radioactively labeled so that the hybridized nucleic acid can be identified by **autoradiography** . The size

of probes ranges from a few **nucleotide** s to hundreds of kilobases. Long probes are usually made by **cloning** .

Originally they may be double-stranded, but the working probes must be single-stranded. Short probes ( **oligonucleotide** probes) can be made by chemical synthesis. They are single-stranded.

Suppose we have cloned a specific gene in yeast and want to find its homologous gene in human, then we may use the specific yeast gene as a probe to detect its homologous gene from the human genomic library.

On the other hand, if we know the conserved sequence in the specific gene between yeast and human, we may use oligonucleotide probes containing only the conserved sequence. Typically, an oligonucleotide about 20 nucleotides long is sufficient to screen a library.

In some cases, we have known the partial sequence of a **protein** and want to detect its gene in the library. Then we may synthesize oligonucleotide probes based on the known **peptide** sequence. Since an amino acid may be encoded by several DNA triplets, many different oligonucleotide probes are often needed.

### 2.1.5.3. Screening

Once a particular DNA fragment is identified, it can be isolated and amplified to determine its sequence. If we know the partial sequence of a gene and want to determine its entire sequence, the probe should contain the known sequence so that the detected DNA fragment may contain the gene of interest.

## 2.1.6. cDNA cloning

cDNA cloning plays a major role in current molecular biology. **Construct** ion of a c **DNA library** is a highly sophisticated technology that involves a series of enzymatic reactions. The quality and integrity of a cDNA library greatly influence the success or failure in the isolation of the cDNAs of interest.

- If eukaryotic **gene** is to be **clone** d and expressed in prokaryotic cell, then directly cutting the source DNA into suitable fragments alone will not be sufficient.
- The difference in the gene organization of eukaryotes and prokaryotes is important.
- **Intron s** the segments of **noncoding sequence s** are present in eukaryotic gene. Theses introns are transcribed into mRNA. Such precursor mRNA in eukaryotic cell undergoes post- **transcription** al modification and removal of introns occurs to give rise to processed mRNA.
- **Processed mRNA** then gives rise to **protein** product. In fact there are also post-translational changes occurring in eukaryotic cell. Thus, to get expression in the form of protein product introns have to be removed.
- Bacteria or yeasts do not have necessary **splicing** mechanism for removal of introns. Hence eukaryotic gene if directly cloned in bacteria or yeast will give rise to precursor mRNA but not the protein product at end.
- This difficulty can be overcome by cDNA route.

The general principles begin with a mRNA that is transcribed into the first-strand DNA, called a **complementary DNA** or **cDNA**, which is based on **nucleotide bases complementary to the mRNA template**.

- This step is catalysed by AMV **reverse transcriptase** using oligo(dT) **primer** s.
- The second-strand DNA is copied from the first- strand cDNA using **DNA polymerase I**, thus producing a double-stranded cDNA molecule.
- Subsequently, the double-stranded cDNA is ligated to an adapter and then to an appropriate **vector** via **T4 DNA ligase** .
- The recombinant vector-cDNA molecules are then packaged **in vitro** and cloned in a specific host, generating a **cDNA library**.
- Specific cDNA **clones** can be "fished" out by screening the library with a specific **probe** .

The messenger RNA is isolated from an appropriate tissues. For example, to obtain the cDNA of **growth hormone** (GH) gene, pituitary should be used for mRNA preparation, since it is the place where GH is synthesized.

- Taking advantage of the poly–A tail at the $3^1$ end of most mRNAs, mRNA is isolated from total cellular RNA by selective binding to and elution from oligo-dT cellulose or poly–U sepharose column.
- The oligo (dT) is complementary to poly (A), so it binds to the poly (A) at the $3^1$ -end of the mRNA and primes DNA synthesis, using the mRNA as the template.
- By using reverse transcriptase enzyme, cDNA can be synthesized from mRNA.

After the mRNA has been copied, ssDNA (the "first strand") is formed and the mRNA is removed with alkali or **ribonuclease H (Rnase H**). This enzyme degrades the RNA part of RNA/DNA hybrid - remove the RNA from first strand of cDNA.

Next second DNA strand is made using the first as template. We need primer oligo (dc) tail at $3^1$ end of the first strand is build, using the enzyme **terminal transferase** and one of the deoxyribonucleoside triphosphates (dCTP). The enzyme adds dCs, one at a time, to the $3^1$ end of the first strand. To this tail, a short oligo (dG) is hybridized, which primes SS syntheses.

DNA polymerase called **klenow fragment** is used. The klenow fragment contain the DNA polymerase activity and the $3^1 \rightarrow 5^1$ **exonuclease** activity, but it lacks the $5^1 \rightarrow 3^1$ **exon** uclease activity normally associated with DNA polymerase I.

Once a double stranded DNA is produced, it is ligated to a vector. **Sticky end** s are made since cDNA lack sticky ends. To solve this oligo (dc) is added on to cDNA using terminal transferase and dCTP. In the same way oligo (dG) is attached to the ends of vector and allowed the oligo (dC)s to anneal to the oligo (dG)s. This brings the vector and cDNA together in a rDNA that can be used directly for **transformation** . **Plasmid** vectors can be used.

Gene libraries of chum salmon, rainbow trout, common carp, grass carp and tilapia were already constructed and available.

There are three steps that are critical for success or failure in the construction of a cDNA library.

- First is the purity and integrity of the mRNAs used for the synthesis of the first-strand cDNAs. Any degradation or absence of specific mRNAs will result in partial-length cDNAs or complete loss of the specific cDNAs, especially for some rare mRNAs.
- The second important step is to obtain full-length cDNAs. If this procedure is not performed well, even if one has a very good mRNA source, the cDNA library is not so good. In that case, one may "fish" out only partial-length cDNAs or no positive clones at all. Once double-strand cDNAs are obtained, they are much more stable as compared with mRNAs.
- A third essential step in cDNA cloning is the ligation of cDNAs with adaptors to vectors. If the ligation fails or is of low efficiency, *in vitro* packaging of recombinant λDNAs cannot be carried out effectively. In order to construct an excellent cDNA library, elimination of RNase contamination must be carried out whenever possible.

Two major strategies are described for the construction and screening of cDNA library.

- One is subtracted cDNA library in which cDNAs are derived from mRNAs expressed in a specific cell or tissue type but not in another type. The cell/tissue type-specific cDNA clones are greatly enriched in the library, which allows one to readily isolate specific cDNAs copied from rare mRNAs.
- The other is the complete expression cDNA library that includes all cDNA clones from all mRNAs in a specific cell/tissue type.
- **2.6.1.Construct ion and screening of a complete expression of cDNA library**
- A complete cDNA library theoretically contains all cDNA clones corresponding to all mRNAs expressed in a cell or tissue type.
- An expression cDNA library refers to one in which all cDNAs are cloned in the sense orientation so that all the cDNAs in the library can be induced to express their mRNAs and proteins.
- As a result, this cDNA library can be screened with specific antibodies against the expressed protein of interest or with a specific nucleic acid probe. In theory, this type of cDNA library preserves as much of the original cDNAs as possible, which can allow one to 'fish" out any possible cDNA clones by screening the cDNA library as long as specific probe or specific antibodies are available.
- Jiang *et al.* (1989) showed that **antifreeze protein** (AFP) existed in the serum of winter flounder, *Psuedopleuronectes americanus* and they prepared and purified the AFP mRNA. A gene probe was synthesized according to the AFP gene sequence of *Pseudopleuronectes americanus*, and subsequently hybridized with the mRNA from *P. americanus*.
- A single-stranded cDNA was enzymatically synthesized with reverse transcriptase. Double-stranded cDNA synthesize was carried out with polymerase and SI nuclease treatment. The cDNA was then placed in an *E. coli* JM83 cell using pUC 19 as the vector, thus enabling the cloning of *P. americanus* cDNA.
- The construction of gene libraries for common carp and grass carp were first reported by Zhu et al. (1990). GH of common carp and grass carp were screened and hybridized with the GH gene probe of Salmo salar and gene cloning was also carried out in fishes.

# Chapter 2 : Transgenic fish production

## 2.2.1 Introduction

An organism that has a foreign or modified **gene transfer** red to its **genome** using the ***in vitro*** **gene** tic techniques is called a genetically modified organism (GMO) or a **transgenic** organism.

- Gordon *et al*. (1980) produced transgenic animals by **microinjection** of **clone** d DNA into the pronucleus of fertilized eggs at the one-cell stage.
- Palmiter *et al.* (1982) introduced **growth hormone** gene into mice and produced giant mouse of 44 gms whereas, normal grows upto only 29 g.
- Attempts to produce transgenic fish began in the mid-1980s. Maclean and Talwar (1984) reported microinjection of cloned DNA into rainbow trout (*Oncorhynchus mykiss*) eggs.

Zhu *et al*. (1985) microinjected fertilized eggs of goldfish with metallothionein promoter fused with the human growth **hormone** gene.

Transgenic technology has been successfully used to develop fast-growing super-fish stocks for

- human consumption,
- to produce pharmaceuticals,
- to test water contamination in both developed and developing countries.
- Several laboratories now have GM fish with increased growth performance caused by extra copies of GH genes. So far, fast growing fish by transferring growth hormone gene have been developed for several aquacultural species.
- Several species including loach, common carp, crucian carp, Atlantic salmon, channel catfish, tilapia, medaka and northern pike containing either human, bovine, or salmonid growth hormone genes grew 10-80% faster than non-transgenic fish in aquaculture conditions.
- Some of the experiments demonstrated that growth can be enhanced through transgenesis from 10% up to an incredible 30-fold.

## 2.2.2. Advantages of fish as transgenics

- Fish produce large quantities of eggs; external fertilization make it relatively simple to insert novel DNA.
- Research on transgenic fish is currently under development for at least 35 species of fish worldwide, as well as for a variety of mollusks, crustaceans, plants, and marine microorganisms, for various purposes.
- Transfer of cloned DNA was reported in a number of fish species; e.g.,
- common carp (*Cyprinus carpio*),
- catfish (*Clarias gariepinus*, *Ictalurus puncatatus*, *Heteropneustus fossilis*),
- salmon (*Salmo salar*), rainbow trout,
- tilapia (*Oreochromis niloticus*),
- goldfish (*Carassius auratus*), loach (*Misgurnus fossilis*), Medaka (*Oryzias latipes*), Zebrafish (*Brachydanio rerio*), northern pike (*Esox lucius*),

- rosy barb (*Barbus conchonius*), sword tail (*Xiphophorus*) and gilthead seabream (*Sparus auratus*).

## 2.2.3. Selection of species Genes

For the aquacultural importance, Indian major carps, Common carp, Channel catfish, Chinese carps, Salmon, Trout and Tilapia are the best species for the transgenic project.

Improvement of growth rate, imparting disease and environmental stress resistance are some important traits for transgenesis.

## 2.2.3.1. Growth hormone gene

Growth is a complex biological process involving genetic, hormonal, nutritional and environmental factors.

- '**Growth hormone '** (GH) is produced by the anterior lobe of the pituitary. It increases growth by stimulating appetite and improving the food conversion efficiency.
- GH is a **protein** hormone having a molecular weight of about 22 kilodaltons.
- Injection of recombinant bovine or chicken GH also caused significant increase in the growth rate in Coho salmon, Rainbow trout and some other fishes.
- However, it is difficult to practice this technique in cultured species of fishes because GH may get digested in the gut, if given through feed.
- Transgenic fish carrying GH gene will produce growth hormone **endogenous** ly by passing the necessity of **exogenous** hormone treatment.
- GH gene has been **clone** d in some fishes either from the **genomic library** or from the c **DNA library** .
- Zhu *et al*., in 1985 first reported the production of transgenic gold fish by **microinjection** of human growth hormone gene (hGH) which was linked with mouse metallothionein gene promoter (mMT).
- Growth hormone gene was subsequently transferred into several species of fishes including Loach, Common carp, Crucian carp, Atlantic salmon, Channel catfish, Medaka and Zebrafish.
- In late 1980s the gene **construct** s used for transgenic fish production were primarily from the non-piscine sources. Human, bovine or salmonid growth hormone gene fused with some viral gene promoter or mouse metallothionein gene promoter were used in these studies.
- Higher growth rate at the range of 10 to 80% was achieved by using these gene constructs.
- The studies conducted at Auburn University in transgenic common carp showed 20 to 40% increase in the growth rate. Similar studies conducted in Scotland have resulted in a test animal almost 11 times heavier than the normal one. Instead of normal size of 7 lbs, the genetically altered pacific salmon attained a weight of 80 lbs.
- In few cases, no difference was observed between the transgenic and their non-transgenic siblings.
- The transgenic Atlantic salmon produced by Devlin *et al*., (1994) carrying 'all-fish gene' showed dramatic improvement in growth.
- In India, transgenic fish research has been initiated in carps (National Institute of Immunology, New Delhi), tilapia, zebrafish, catfish, carp (Madurai Kamaraj

University, Madurai) and catfish (Centre for Cellular and Molecular Biology, Hyderabad).

### 2.2.3.2. **Antifreeze protein** genes

Production of cold resistant fish variety is useful for establishing aquaculture industry in the temperate region, where water gets frozen during winter. The gene responsible for imparting cold resistance was cloned from winter flounder (*Pseudopleuronectes americanus*), which lives in the polar sea. This species avoids freezing of its blood even at –7°C temperatures by producing a set of **anti-freeze proteins (AFP**).

- AFP are produced in the liver and exported to the blood stream.

- When produced at high concentration (10-20 mg/ml), AFP inhibits the growth of ice crystal formation in the blood, which helps to protect fish from freezing.
- Following the discovery of AFP and the isolation of their genes, efforts have been made for developing cold resistant variety.
- Fletcher *et al*. (1986) showed that AFP injection to seawater-acclimatized rainbow trout lowered the freezing point of the whole fish in proportion to the circulating anti-freeze protein concentration.
- This experiment revealed the feasibility of providing freeze protection to animals by transgenesis (Fletcher *et al*., 1988).

### 2.2.3.3. Disease resistance

Fish has poorly developed **antibody** dependent immunity. Efforts to produce disease resistance in fish stocks by transgenesis have begun recently. The potential of Rainbow trout lysozyme gene as a bacterial inhibitor was assessed in Atlantic salmon.

Lysozyme is a nonspecific antibacterial enzyme present in the blood, mucus, kidney, and lymphomyeloid tissues in fish (Hew *et al*., 1995). Rainbow trout contain elevated levels of lysozyme (10- to 20-fold higher than in Atlantic salmon) and a rainbow trout lysozyme **cDNA** construct with an ocean pout AFP promoter has been created.

Rainbow trout were recently reported to have 2 distinct types of lysozymes, with only type II having significant bactericidal activity (Mitra *et al.*, 2003). The gene for type II lysozyme was amplified and **sequence** d for future use in transgenic immune system enhancement of farmed fish.

The potential of Rainbow trout lysozyme gene as a bacterial inhibitor was assessed in Atlantic salmon. There is enormous promise in the application of transgenesis for enhancing fish health.

### 2.2.4. Reporter genes

Reporter gene s are ideal for expression assays.

Reporter gene is defined as a gene whose products detects or marks the cells, tissues, organisms that express the gene from those that do not.

Reporter genes isolated from prokaryotes, *E. coli*, are used in fishes,e.g.,

- lac Z gene,
- Cat (Chloramphenicol Acetyl Transferase gene),
- luciferase,
- green fluorescent protein gene,
- winter flounder anti-freeze protein,
- chicken, crystalline and
- carp a -globin.

## 2.2.5. Methods of gene transfer

The two most commonly used techniques are microinjection and electroporation.

## 2.2.5.1. Microinjection

Microinjection is the most common method of **gene transfer** . It involves the use of an injection pipette, the dimensions of which depend on the target species: e.g., pipettes of inner diameter of 3-5 µm are used for tilapia. Soon after fertilization the **gene** is microinjected into the **cytoplasm** since the egg nucleus is not visible in the fishes. The site of injection varies from species to species. In fish eggs, the nucleus or pronucleus cannot be seen using conventional light microscopy, mainly because of the opaqueness of the chorion and /or the cytoplasm. Hence, most **transgenic** fish studies have opted for cytoplasmic injection of DNA, following fertilization.

The target is the thin layer of ooplasm under the chorion or developing blastodisc. The injection pipette must penetrate the chorion (which is often thick and opaque, except in some species such as catfish and medaka, which have transparent and thin chorions) and the membrane of the fertilized egg.

Several methods of pretreatment have been reported, including a two-step method which involves

- piercing the chorion with a broken pipette before microinjection into its ooplasm, dechorionation and prevention of chorion hardening,
- microinjection through the micropyle and
- microinjection before hardening of the chorion.

The possibility of damaging eggs during microinjection is high and this technique requires a great deal of skill.

- The survival rates of different species of transgenic fish produced by this method have been reported to range from 5 to 90%.
- Linearised DNA rather than circular DNA is injected for the greater probability of the former to get integrated into the host's **genome** .
- Higher amount of DNA is used for cytoplasmic gene transfer than when it is injected to the pronucleus.
- Some species has softer chorion such as catfish, Zebra fish.
- Small volume of the solution 1-2 nl of DNA containing $>10^7$ copies should be injected.

- The rate of survival and integration of the transgene after microinjection varies widely in different species of fishes and in different batches of the same species.
- Although microinjection is time consuming, laborious, species-specific and technically demanding, it remains the most widely used method for gene transfer in fish.

## 2.2.5.2. Electroporation

Electroporation is another method of **gene transfer** . It utilizes a series of short electrical pulses to make the membrane porous and permeable to DNA incorporation.

- Embryos and sperms can be electroporated.
- It is less labour intensive and does not require special expertise for **gene** transfer as needed in the case of **microinjection** .
- It is easier to do this in spermatozoa than in embryos, which possess tough chorion.
- The gene transfer efficiency and integration rate do not differ much between electroporation and microinjection methods.
- In zebra fish 0.1 milli second pulses of 125/cm for batches of 200 eggs.
- This technique has been tested on medaka, zebrafish, common carp, catfish and loach.
- When compared with different methods of gene transfer (microinjection, sperm-mediated, chromosome mediated and through electroporation), despite lower survival, electroporation technique ensures a higher transfer efficiency.

## 2.2.5.3. Other gene transfer techniques include

- electroporation
- electrofusion,
- high velocity microprojectiles,
- blastula chamber injection,
- direct gene transfer into fish muscles *in vivo*, embryonic cells,
- sperm binding, and
- chromosome mediated gene transfer.

Electroporation, electrofusion, high velocity microprojectiles, sperm binding, and chromosome mediated gene transfers are examples of mass gene transfer techniques.

## 2.2.6.4. PCR amplification

It is based on repeated cycles of denaturation, annealing of **oligonucleotide primer** s complementary to the gene, and primer extension by Taq **polymerase** . The amplified fragment can then be recognized as a discrete fragment on a gel or on a southern blot.

Expression assays are aimed at detecting the presence of **reporter gene** products such as CAT and ß- gal in host cells.

## 2.2.6. Detection of transgenes

Most studies on **transgenic** fish have used hybridized slot, **southern blot** and northern blot techniques to detect transgenes.

### 2.2.6.1. Southern blot hybridization

It is the most widely used method.

- In this method, fragments of DNA generated by restriction digestion, are subjected to agarose gel **electrophoresis** .
- The separated fragments are then transferred to a nitrocellulose or nylon membrane by a blotting technique.
- The DNA of interest can be detected by hybridizing the membrane to a radioactive **probe** , which bears the same homology as the DNA.

### 2.2.6.2. Northern blot

It is based on the same principle as southern blot, but RNA is used instead of DNA.

- It measures accumulation of RNA transcripts and it is extremely useful in studies of gene expression.
- The difference between slot and southern blotting is that **slot blotting** does not require the genomic DNA to be cut with **restriction enzyme** s prior to transfer to a nylon membrane or nitrocellulose filter.
- Analysis of degraded DNAs and of multiple samples are possible with slot blotting.
- However, slot blot hybridization is not as informative as southern blot hybridization because it does not indicate integrations or rearrangements involving the transgene.

### 2.2.6.3. Western blotting

It is used for identifying and characterizing specific gene products.

- It involves the transfer of **protein** s from acrylamide gels to nitrocellulose membrane by electrophoresis.
- The membrane is then probed with an **antibody** to detect the protein of interest.

***In situ*** **hybridization** is the hybridization of nucleic acids within cytological preparations. This method shows the localization of transgenes.

### 2.2.7. Glofish

The **GloFish** is a patented brand of **gene**tically modified (GM) fluorescent **zebrafish** (*Danio rerio*) with bright red, green, and orange **fluorescent** color.

Zhiyuan Gong *et al.* (1999) at the **National University of Singapore** were working with a gene called **green fluorescent protein** (GFP), originally extracted from a **jellyfish** , that naturally produced bright green **bioluminescence** .

- They inserted the gene into a zebrafish embryo, allowing it to integrate into the zebrafish's **genome**, which caused the fish to be brightly fluorescent under both natural white light and ultraviolet light.
- Their goal was to develop a fish that could detect **pollution** by selectively fluorescing in the presence of **environmental toxins** . The development of the always fluorescing fish was the first step in this process. Shortly thereafter, his team developed a line of **red fluorescent zebra fish** by adding a gene from a **sea coral**, and **yellow fluorescent zebra fish**, by adding a variant of the jellyfish gene.
- Later, a team of **Taiwanese** researchers at the National University of Taiwan, headed by Huai-Jen Tsai, succeeded in creating a **medaka** (rice fish) with a fluorescent green color. Taiwan became the first to authorize sales of a genetically modified organism as a pet.
- In addition to the red fluorescent zebrafish, trademarked as "**Starfire Red**", Yorktown Technologies released a green fluorescent zebrafish and an orange fluorescent zebrafish in mid-2006.
- The new lines of fish are trademarked as "**Electric Green**" and "**Sunburst Orange**", and incorporate genes from sea coral.
- Despite the speculation of aquarium enthusiasts that the eggs are pressure treated to make them infertile, it has been found some GloFish are indeed fertile and will reproduce in a captive environment.
- The original zebrafish (*Danio rerio*) from which the GloFish was developed is a native of rivers in **India** and **Bangladesh**.
- It measures 3 cm long and has gold and dark blue stripes.
- Although not originally developed for the ornamental fish trade, it is the first genetically modified animal to become publicly available as a pet.

### 2.2.8. Food safety of transgenic (GM) fish

GM food safety depends on the

- nature of the **gene** ,
- the transgene product it encodes and
- the resulting phenotype. In addition, it is important to ensure that the insertion of a new gene has not affected an **endogenous** gene or had other pleiotropic effects.
- Ethics and animal protection concerns allows the development of healthy and safe fish only.
- Transgenic fish have received extra copies of GH genes, resulting in only moderately raised levels of circulating GH.

- GH is a **protein hormone** which is degraded along with all other food protein. Meat from fish modified with GH is regarded as completely safe for human consumption.

A National Research Council study maintains there is a low to moderate food safety risk from GM fish. Since transgene can introduce new protein into a food product, there are concerns that this technique could introduce an allergen, known or previously unknown, into the food supply.

Berkowitz and Krypsin-Sorensen (1994) discussed food safety issues posed by transgenic fish.

- If the animal's health is not negatively affected by transgenes or transgene product, it can be inferred that GM fish do not represents health hazards for human consumption.
- Concerns have been voiced of the possible risks of consumption of transgenes, their resulting protein, potential production of toxins by aquatic transgenic organisms, changes in the nutritional composition of foods, activation of viral **sequence** s and allergenicity of transgenic products.
- These risks have been analyzed, and while the majority of **genetic modification** to foodstuffs will be safe the greater potential for risk and harm is allergenicity.
- In the case of fast-growing GH fish, symptoms similar to acromegaly can be observed in some of the animals with higher growth levels, although the general impression at present is that the majority of transgenics are healthy.

About 98 percent of the dietary DNA from fish including GMOs is degraded by digestive **enzymes** relatively quickly but use of **viruses** as **vector** s, might increase the risk factor significantly as these are organisms which are adapted to integrating into host **genome** s.

### 2.2.9. Environmental impact of transgenic fish

The possible impacts from the escape of GM organisms from aquaculture facilities are of great concern to some scientists and environmental groups. Critics and scientists predict that

- GM fish could breed with wild populations of the same species and potentially spread undesirable gene s.
- Transgenic fish that have been modified so as to enable them to withstand wider ranges of salinity or temperature, could be more difficult or impossible to eradicate, similar to an invasive species.
- Escaped transgenic fish could harm wild fish through increased competition or predation.

- Critics fear that GM fish might disrupt the ecology by competing with native fish for scarce resources. The con sequence s of such competition would depend on many factors, including the size of the wild population, the number and specific genetic strain of the escaped fish, and local environmental conditions.

Other potential safeguards also exist.

Only sterile GM fish be approved for culture in ocean pens. Fertilized fish eggs that are subjected to a heat or pressure shock retain an extra set of chromosomes. The resulting *triploid* fish do not produce normal eggs or sperm, and females do not exhibit maturation of the ovary or reproductive behaviors. Thus, all-female lines of triploid fish are the best current method to ensure non-breeding populations of GM fish.

### 2.2.10. Conclusion

Gong *et al*. (2003) developed **transgenic** zebrafish (*Danio rerio*) for ornamental and bioreactor system by strong expression of fluorescent **protein** s in the skeletal muscle. The fish muscle has a capacity producing up to 27 mg of foreign protein per gram of wet tissue.

- Commercialization of transgenic fish has began in some countries such as Chile, China, Cuba and New Zealand.
- Legal consumption of transgenic fish in the US will likely to occur soon.
- However, in Europe and Japan, conservative approaches to the development of transgenic fish will prevail politically for many years.
- Because of these concerns, transgenic fish will likely be utilized commercially to a greater extent in developed countries.
- However marketing of these transgenic food fish remains a controversial issue due to ecological and food safety concerns.
- The future success and application of transgenic fish will depend upon by successful demonstration of a lack or potential lack of environmental risk, food safety, appropriate government regulation and labeling, public education and development of **gene** tic sterilization for transgenic fish.
- Appropriate, well executed public education may be necessary to gain broad consumer acceptance of transgenic fish from an environmental standpoint and perhaps in relationship to how “organic” a transgenic fish may be.

## Unit 3: Cell Culture

### Chapter 1: Cell culture and Cell lines

### 3.1.1. Introduction

**Cell culture** refers to cultures derived from dispersed cells taken from the original tissue. These cultures have lost their histological properties and often some of the biochemical properties associated with it. A large number and variety of continuous fish cell cultures have been developed during the past four decades since the first such cell culture was reported.

Basically the fish cell culture differs only slightly from the much more widely used techniques of mammalian cell and **tissue culture** . The major differences being,

- first in temperature requirements and tolerances and
- second, in osmolarity of salines and media.

For freshwater fishes, the mammalian type solutions are entirely satisfactory but for marine fishes, satisfactory results are obtained with increased osmolarity.

The important factor responsible for the development of fish culture is its application in fish virology. A virus is an obligate intracellular parasite and as such, can replicate only within a living cell.

### 3.1.2. Stages in cell culture

There are two types of cell growth.

- Adherent cultures
- Suspension cultures

#### 3.1.2.1. Adherent cultures

- These cells depend on an **anchorage for proliferation**.
- They are subject to **contact inhibition** which means, they grow as an adherent monolayer and stop dividing when they reach such a density that they touch each other.
- Most cells except mature hemopoietic cells grow in this way.
- They need protease treatment to break the bond between cells and substratum.

#### 3.1.2.2. Suspension cultures

- Cells cultured from blood, spleen or bone narrow adhere poorly to the culture dish. Because in *in vivo* they are kept under suspension.

### 3.1.3. Types of cell culture

- Freshly isolated cells from the parent body undergoing ***in vitro*** cultivation is known as **primary cultures** until they are passaged or sub-cultivated.

- They are usually heterogenous, and have a low growth fraction, but are more representative of the cell types in the tissue from which they were derived and in the expression of the tissue specific properties.
- After several subcultures a **cell line** will either die out (finite cell line) or "transform" to become a **continuous cell line**.

### 3.1.3.1. Primary cell culture

The methodologies and growth media for the preparation and maintenance of fish cell cultures **gene** rally do not differ from those used for the culture of cells from homeotherm vertebrates.

- The selection of fish species and appropriate tissues for the initiation of primary cell cultures is usually dictated by the cell type or function to be studied and/or the ultimate use of the cell culture.
- The main sources are embryonic cells and reproductive cells or gonadal tissue, due to their rapid multiplication.
- Other sources are gill tissue, connective tissues, skeletal, cardiac, epithelial cells, neural cells, heart, kidney, liver and spleen and endocrine cells.
- In many ways, the initiation of cell cultures from fish is actually easier than from hoemotherm vertebrates. Unlike mammalian cells, which must be kept near $37^{o}C$, most fish cells easily tolerate or even prefer wide range of temperatures $< 37^{o}C$. Therefore, tissue samples can be collected at field sites, placed in growth media, and transported on ice or even at ambient temperatures to the laboratory for preparation.

Cell suspensions for monolayer cultures are usually prepared by standard methods of

- enzymatic dissociation, usually Trypsin – EDTA.
- Fish tissues are usually dissociated at very low temperatures or at temperatures approximately those of the species natural environment.
- In some cases when enzymatic dissociation has failed to produce actively dividing cells or when relatively small volumes of tissue are available and the number of viable cells can be expected to be relatively small, success in initiating monolayer cultures has been achieved starting with **explants cultures**.
- Naturally, for certain types of study, tissue explant and organ culture are the methods of choice.
- Migration of dividing cells out of the explants frequently results in foci of small cell monolayers surrounding the explant, which can usually be sub cultured following enzymatic duration.

Primary fish cell cultures usually consists of a variety of cell types including both **epithelial-like** and **fibroblast-like** cells as well a variety nondividing cells.

Following several subcultures, however, one cell type usually becomes predominant.

The ratio at which subcultures can be made from primary cultures varies considerably. Few passages are made at relatively low ratios such as 1:2 or 1:3.

### 3.1.3.2. Continuous cell cultures

"New" fish cell cultures can be sub cultured for varying periods of time before reaching senescence.

Fish cell lines that can be sub-cultured several times eventually develop into continuous cell lines.

## 3.1.4. Commonly used media for fish cell culture

- Medium 199, Eagle's Minimum Essential Medium (MEM) and Eagle's basal medium (BME).
- Other synthetic media suitable for fish cell culture are CMRL 1066, Leibovitz L-15, McLoy's 5a, NCTC 109, and Puck's medium.
- Among all these media MEM is suggested best for fish cell and **tissue culture** .
- In some cases with certain marine fish cell lines such as the grunt fin line, GF, it may be necessary to increase the NaCl concentrations of standard media.

## 3.1.5. Requirements of cell culture

### 3.1.5.1. Serum additives

- Human cord serum is found to be excellent for fish cell culture. But as the cost is higher, calf serum replaces human cord serum.
- Fetal bovine serum can also be used.
- The usual level of serum is 10-15%.

### 3.1.5.2. Other additives

Other additives used in the culture media are

- products of human ascitic fluid,
- bovine aminoic fluid,
- chick embryo extract,
- lactalbumin hydrolysate,
- serum ultrafiltrate, peptone,
- yeast extract, and
- whole egg ultrafiltrate.

### 3.1.5.3. pH

Practically the pH range is not so critical.

- Most of the cells can grow well at a pH range of 7.2 – 7.8 (7.4 optimum).
- Although bicarbonate buffered media are usually employed, organic buffers such as HEPES can also be used if desired.
- Also, fish cells require $CO_2$ either from bicarbonate in sealed vessels or by propagation in a $CO_2$ incubator.
- Fish cell cultures **gene** rally do not require periodic changes (feeding) of growth medium between sub-cultures.

### 3.1.5.4. Antibiotic s

- For routine purposes, media containing 100 IU of penicillin, 100 m g of streptomycin, and 25 IU of mystatin per milliliter and chlortetracycline at 50 m g in lieu have been used.

### 3.1.5.5. Growth temperature

Fish cell cultures generally retain viability and / or proliferate over a wide range of incubation temperatures. The optimal growth temperature and the temperature range over which a particular culture will grow usually reflect the fish species and its natural environment.

- Temperatures of $15^{o}$–$20^{o}$C are usually optimal for cells from “cold water” species such as salmon and trout; however, cells from these species can frequently be maintained and even will proliferate at temperatures ranging from $2^{o}$ to $27^{o}$C.
- Intermediate or “cool-water” species have a somewhat higher limit and an optimum between $20^{o}$ and $28^{o}$C.
- Most “warm-water” fish cell cultures do not tolerate relatively low incubation temperatures, but may grow even at $37^{o}$C. Generally, the optimum temperature for these cells is between $25^{o}$ and $35^{o}$C.

The ability to grow over an extremely broad temperature range makes fish cell cultures uniquely useful for a variety of purposes, particularly studying temperature effects on metabolism, virus replication, and other cell process.

### 3.1.5.6. Culture vessels

Virtually all fish cell lines are anchorage–dependent and must be maintained as monolayer cultures on some solid substrate like standard culture vessels such as flasks, dishes micro carrier beads, etc.

Microcarrier beads yields two to three times greater per unit volume of medium than standard monolayer cultures. The efficiency of micro carriers in growth vessel and medium requirements provide significant advantages for the large-scale production of fish cell cultures, **viruses** and cellular products.

### 3.1.6. Preparation of fish for explants

#### 3.1.6.1.Tissues

- **External tissues** : Fin, skin, barbels, cornea and caudal and trunk portions should be washed in cold chlorinated tap water and rinsed in sterile BSS.
- **Antibiotic s** such as polymyxin-B (2000 IU/ml), streptomycin (100-1500 m g/ml) and penicillin can be used via bath treatment for gill purification, followed by treatment with BSS.

- Immersion of eggs for 1 sec in 95% ethanol and then transferring to sterile water should be employed for aseptic removal of embryos.
- *Gambusia* in gravid condition is immersed momentarily in methiolate and washed twice in 70% alcohol. The fish is dried with sterile cotton and aseptically the embryos are removed.

**Internal tissues**

- Sterile embryos may be obtained by surface sterilization of either eggs or gravid females.
- Unless an animal is infected and with the exception of the digestive tract, internal tissues of fishes are sterile and their aseptic removal is simple.
- Prior to opening the fish, the area of incision or when feasible the entire fish is topically disinfected or sterilized.
- It is advantageous to remove scales from heavily scales fish. Isopropanol (70%),
- Ethanol (70%) and 500 ppm available chlorine solution are used to disinfect the external surfaces.

#### 3.1.6.2.Seeding density for primary monolayer culture

- The cells to be cultured are harvested by centrifugation. It is **gene** rally agreed that 200 g for 10 min is both adequate and safe.
- Cells from many fishes readily tolerate centrifugation at $20^{o}C$ or even higher but frictional heating coupled with high ambient temperature may injure cells from cold water fishes.
- The cell density varies from 1 to $3x10^{5}$ cells/ml.

#### 3.1.6.3. Seeding density for cell lines

The seeding density for subcultures of cell lines will vary with the cell, the medium and the particular need. The usual density various from $10^4$ to $10^5$ cells/ml but $10^3$–$10^4$ cells/ml can be adequate under good conditions.

### 3.1.6.4. Choice of explant in the order of decreasing importance

Embryo, gonad, swim bladder, fin, mesentery, cornea, gill, heart and skin.

### 3.1.7. Flow chart for primary cell culture from fin fish

Fish
Swab with 70% alcohol or betadine to sterilize the external surfaces
↓
Remove caudal fin, gills and scales aseptically
↓
Cut the tissues in to rate fine pieces aseptically
↓
Wash the tissues with phosphate bufferd saline (PBS) 2-3 times
↓
Place the washed tissue in a sterile China dish or tissue culture flask and add 1-5 ml of Leibowitz's L-15 medium, until the tissue is just submerged.
↓
Incubate at 28$^o$C – 29$^o$C for 24-72 hrs. The cells from the explant migrate into the surrounding medium and form a confluent monolayer
↓
A confluent monolayer may be formed in 3-4 days

### 3.1.8. Flow chart for primary cell culture from shrimp

Shrimp (8-15 cm)
↓
Anesthetize (cold water 4$^o$C/40 min or dip in 10% hypochloride)
↓
Rinse in 7% tincture of iodine to sterilize the external surfaces of shrimp and wash with Leibowitz's solution to remove tincture of iodine.
↓
Dissect under dissection microscope aseptically to get isolated tissues of the required parts.
↓
The sterile tissues are collected in a pistri dish
↓
Trypsinize each tissue separately with 0.1% Trypsin at 37$^o$C for 20 min.
↓
Trypsinization yields isolated /single cells from the tissue mass by enzymatic action
↓
Wash the trypsinized cells with Leibowitz's medium to completely remove the trypsin. Repeat the process 2-3 times

Collect the cells by centrifuging at 800-1000 g for 5-10 min
↓
Suspend the washed cell pellet in 5-10 ml medium in a 25cm2 tissue culture flask (medium – Eagles minimum essential medium or Grace insect medium or Leileowitz's L-15 medium)
↓
Incubate at 25$^{o}$C for 24-48 hrs
↓
Watch under a microscope for the formation of a monolayer

**Secondary culture**

- The cell culture is called a primary culture until it is subcultured for the first time, after which it becomes a secondary culture.
- The subsequent cell cultures are known as *cell lines*.
- Since the primary cell culture is heterogenous, we go for selection or cloning of cells for obtaining particular cells.

### 3.1.9. Cell cloning

- This is the process of producing **gene** tically homogenous cells. This can be done by
- i) Dilution cloning
- ii) Selective media

### 3.1.9.1. Flow chart for dilution cloning

- **Flow chart for dilution cloning**
- Monolayer of cells
- ↓
- Remove the medium and add trypsin
- (After few minutes the cells are in suspension)
- ↓
- Add medium to the cells
- ↓
- Count cells
- ↓
- Dilute cells to 10-100 cells/ml
- ↓
- Seed cells in a multi-well dish
- ↓
- Let the cells settle down again
- ↓
- Grow up clones for characterization
- ↓
- Select clone

- Here chemicals or monoclonal antibodies are used to kill other cells other than the desired cells.

**Cell separation**

- This is an alternative way to cell cloning. Here cells are separated by means of their size, density, charge, surface area or specific affinities. For this flow cytometry, flow cytofluorimetry, fluorescence activated cell sorter (FACS) are used.

### 3.1.10. Storage

- For short term preservation (4.6 months) the storage temperature is 4-6$^{o}$C. For long term storage liquid nitrogen is used.

#### 3.1.10.1. Long-term storage

- Fish cell cultures can be stored frozen in liquid nitrogen or in ultra cell freezers using standard methodologies for freezing and thawing.
- Salmonid cell lines can be kept at 4$^{o}$– 6$^{o}$C for a period of 4–6 months.
- Cell lines from warm-water species generally cannot be stored at low temperature as that of cold-water species.
- Cell lines kept at sub-optimal temperatures for extended periods of time can easily be recovered by adding fresh growth medium and incubating at optimal temperature for 24–72 hrs before sub-culturing.

Freezing is done at three stages, first at 0$^{o}$C for 30 min, then at -20$^{o}$C for 60 min and thirdly at -70$^{o}$ C for 6 months and finally at -196$^{o}$C for one or two years in liquid nitrogen.

In order to protect from damages of cells during storage, DMSO 7.5% and glycerine 10% are used along with medium. Freezing of cells is done mainly for three reasons.

i) During cell line the cells may change their enzyme activity, chromosome number, etc. Therefore it is essential to freeze these cells at a particular stage of cell line and then rejuvenated.

ii) There may be contamination in cell line. To prevent this cells are frozen at periodic intervals.

iii) In an established cell line the cells can be cultured to a maximum of 50 times. In some other cell line, cells are likely to die at any time. Such cell lines can be sub-cultured only for 30 times. Freezing of these cells may extend the period of cell line.

### 3.1.11. Application of fish cell cultures

Fish cell cultures have found more widespread applications as ***in vitro*** models for studying cyto **gene** tics, cellular physiology, host-pathogen relationships, viral and environmental carcinogenesis and toxicology.

### 3.1.11. 1. Isolation and identification of fish viruses

The first cell line (RTG–2) was developed from trout and used to facilitate the isolation of infectious pancreatic necrosis virus (1PNV) by Wolf and Quimby (1962). There has been a rapid increase in the number of continuous cell cultures from carp, loach, tilapia, perch, milkfish, grouper, snakehead, seabream, and eels. These new cell lines are being used to isolate previously undetected and unknown viruses and for comparative studies of these viruses.

2. Fish cell cultures are very useful in *in vitro* models for studying the replication and genetics of viruses, the effects of antiviral drugs, and the production of experimental vaccines.

3. Fish cells have been utilised for determining karyotypes and other aspects of cytogenetics such as chromosomal polymorphism and speciation, chromosomal abnormalities and evolution.

4. Organ cultures of pituitary glands derived from tilapia, and monolayer pituitary cell cultures from tilapia, rainbow trout have been used to study the production of the **growth hormone prolactin**. Also, pituitary organ cultures from rainbow trout and cell culture from trout, carp and gold fish have been employed as *in vitro* systems for studying the mechanism of **production and regulation of gonadotropin**.

5. Cultured kidney tissue has been useful in comparing testosterone–dependent changes *in vivo* and *in vitro* in the structure of the renal glomeruli of teleost fishes.

6. Gonadal cell and organ cultures have contributed to studies on the effects of testosterone on spermatogenesis, endocrine activities of isolated folicular cells, and function of selected **enzymes** in the steroid negative–feedback regulation of gonadotropic **hormone** release.

7. Increasing use is being made of fish cell cultures in the field of toxicology, both as *in vitro* systems for studying the metabolism of various toxicants and as sensitive indicator models for testing the **cytotoxic** ity of aquatic pollutants.

8. Both primary cultures and established cell lines are also sensitive and can be used in assay systems for screening aquatic pollutants for cytotoxicity.

9. Fish cell cultures have been utilized for more detailed investigations of the processes leading to the proliferation and differentiation of tumours and tumour cells. Fish cell cultures are also used for testing and evaluating the effects of carcinogens such as the use of primary cultures of fish hepatocytes for investigating carcinogenic effects of dimethylnitrosamine, aflatoxin B1, benzo(a)pyrene, and N-methyl-N'-nitro-N-nitrosoguanidine.

10. Cell and organ cultures have facilitated studies of the immune response in fish. Cell cultures were also used to gain a better understanding of how fish macrophages and lymphocytes differentiate and function in the immune response. *In vitro* systems have been used to study the effects of various substances such as **antibiotic** s on the modulation of cells of the immune system as well as the function and comparative phylogenetics of various lymphokines such as interleukin 1. *In vitro* systems have been particularly useful in studying both antigen–specific and nonspecific cell-mediated immunity.

*In vitro* techniques to detect **antibody** –producing cells (plaque–forming cells, PFC and antigen–binding cells (rosette–forming cells, RFC) can be used to monitor the immune response in fish immunized with vaccines for bacterial pathogens.

### 3.1.11.2. Marine invertebrate tissue culture

The countries like Japan, China, United States, Canada and India initiated marine invertebrate tissue culture. Among these countries, Japan is the pioneer country carrying out research in pearl oyster for the purpose of producing *in-vitro* pearl through tissue culture. Culture of mantle tissue of pearl producing molluscs has been undertaken in recent years. The latest breakthrough obtained in the culture of mantle tissue of *P. fucata* and the abalone *Haliotis varia* is a milestone in tissue culture research. It created the possibilities of not only the production of pearls in large numbers but also different coloured pearls.

In an organ culture, the mantle tissue of a pearl oyster kept in nutrient rich medium resulted in the formation of nacreous layer with organic matrix and a pearl sac within 3 months after organization of cultures. The basic technology developed through tissue culture method can totally eliminates the dependence on natural environment for pearl production. It provides scope for manipulation of the technique to produce pearls of the desired quality.

By organizing explants cultures of pearl producing mollusc, the epithelial cells capable of producing aragonite crystals may be collected and stored in cell bank. The cells can be used at any time for the production of quality pearls in ***in vitro*** . The cells in suspension would form the pearl sac that would secrete nacre to form a pearl. Isolation and the type of epithelial cells that would secrete the aragonite crystals, which form the top quality pearls, can be done.

## Chapter 2: Hybridoma Technology

### 3.2.1. Introduction

Our knowledge of the immune system of fish and fish diseases is extremely limited when compared to our knowledge of large animals. At present, fish farming (aquaculture) is becoming an increasingly important food production industry, and may play a significant role as a food source in the future. For this reason, application of the latest biotechnological advances, including MAbs, to the aquaculture industry, is extremely important. MAbs are being adopted for purposes of immunoassay and immunotherapy.

**Hybridoma** technology is a technology of forming hybrid cell lines (called **hybridomas** ) by fusing a specific **antibody -producing B cell with a myeloma** (B cell cancer) cell that is selected for its ability to grow in **tissue culture** . The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal antibodies (in contrast to polyclonal antibodies).

Hybridoma technology for the production of monoclonal antibodies (MABs) has contributed significantly to aquaculture. Monoclonal antibodies are being employed in disease, pathogen classification, epidemiological analysis and development of vaccines.

The idea of a " magic bullet " was first proposed by Paul Ehrlich who at the beginning of the 20th century postulated that if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. In the 1970s the B-cell cancer multiple myeloma was known, and it was understood that these cancerous B-cells all produce a single type of antibody. This was used to study the structure of antibodies, but it was not yet possible to produce identical antibodies specific to a given antigen .

Production of monoclonal antibodies involving human–mouse hybrid cells was described by Jerrold Schwaber in 1973. The invention was conceived by Prof. Pieczenik, with Prof. John Sedat, as a witness and reduced to practice by Cotton and Milstein, and then by Kohler and Milstein.

Georges Köhler , César Milstein , and Niels Kaj Jerne in 1975; who shared the Nobel Prize in Physiology or Medicine in 1984 for the discovery. The key idea was to use a line of myeloma cells that had lost their ability to secrete antibodies, come up with a technique to fuse these cells with healthy antibody-producing B-cells, and be able to select for the successfully fused cells.

### 3.2.2. Production of monoclonal antibodies

Monoclonal **antibody** production is initiated by the immunisation of BALB/c mice with immunogens. e.g., **protein** , carbohydrate, nucleic acid or combinations of these. They can also be produced from impure antigen by selecting single cell **clone** after the fusion.

- Antibodies are produced by differentiated B-cells (plasma cells) and because each parent B-cell has the capability of producing antibodies of a particular specificity, the antibodies secreted by a B-lymphocyte clone are identical and therefore, is a source of homologous antibodies.
- Plasma cells are, however, short-lived and cannot be grown in culture.
- Therefore, fusion of these cells with immortal myeloma cells produces **hybridoma** cells with the ability to grow in culture and to secrete antibody with a defined specificity.
- Chemical selection, screening of the antibodies produced and **cloning** of the hybridoma cells lead to the ultimate production MAbs.
- Myeloma cell lines used in fusions have been selected because they do not produce antibody molecules, although some of the commercially available cell lines do produce immunoglobulin heavy or light chain molecules. For this reason P3x63. Ag8-653 (653) and Sp2/0-Ag14 (Sp2/0) are the most frequently used cell lines in hybridoma technology.
- Hybridoma cells can be prepared by fusing myeloma cells and antibody – producing cells which have been isolated from different mouse species, but the success rate of fusion is greatly increased if both cell types come from the same **strain** of mouse (e.g., BALB/c).
- Originally, Kohler and Milstein used Sendai virus as the fusion agent, but polyethylene glycol (PEG) is now routinely being used to fuse the cells.
- Even in efficient fusions, only approximately 1% of the initial cell numbers result in fusion.
- This leaves a large number of unfused cells, both spleen and myeloma cells still present in the culture.
- The spleen cells from the mouse die within 3 days of culture and therefore, do not pose a problem.
- However, the myeloma cells quickly adapt to the culture conditions and will outgrow the hybridoma cells resulting from the fusion.
- Removal of the myeloma cells is therefore, essential and is achieved by chemical selection. Commercially available myeloma cells are defective in one of the **enzymes** of the salvage pathway of purine **nucleotide** biosynthesis. Cell lines 653 and SP2 have mutations of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) **gene** . Addition of aminopterin to the culture medium blocks the *de novo* nucleotide synthesis pathway and forces the cell to use the salvage pathway in which HGPRT uses **exogenous** hypoxanthine and thymidine. Myeloma cells defective in HGPRT are unable to use this pathway and therefore, die in culture.
- The only cells able to grow in HAT (hypoxanthine, aminopterin, thymidine) culture medium are the hybridoma cells, which are unable to synthesize

DNA via *de novo* nucleotide synthetase pathway and rely on the salvage pathway for DNA synthesis (a characteristic provided by the spleen cell part of the hybridoma).

- Positive **clones** producing specific antibodies are usually identified by ELISA and are selected, expanded and cloned using a limiting dilution technique. Positive hybridomas are normally cloned three times before they are considered MAb producing cells.
- The resulting MAbs are extremely specific and are therefore, very useful diagnostic tools.
- In addition, hybridoma cell lines have the advantage of providing an unlimited supply of the antibody in the cell supernatant, which allows standardisation of the MAb reagents.

### 3.2.3. Application of Monoclonal Antibodies in Fish Farming

Though the technology for MAb production has been in existence for more than 25 years, yet this application to fish farming is still in its infancy.

Today, monoclonal antibodies to several viral and bacterial pathogens of fish and shellfish are available in the market (Table 1). It has been possible to develop rapid, simple, cheap, specific and sensitive MAb based immunodiagnostic kits for several microbial pathogens.

MAb based diagnostic kits such as ELISA and immunodot have even been simplified to the field level for use by farmers. Furthermore, detection of minute serological difference among bacterial and viral variants of fish and shellfish is possible by MAb based epitope analysis. This has helped immensely in serological and epidemiological studies.

- Monoclonal antibodies were produced against enterotoxin of *Vibrio cholerae,* a brackishwater and estuarine bacterium which causes cholera.
- MAbs based ELISAs have been used for studies of *Vibrio anguillarum* **strain** s and for rapid diagnosis of clinical cases of Enteric Red mouth (*Yersinia ruckeri*) and furunculosis *(Aeromonas salmonicidae*) in fish farms.
- MAbs are also used to study piscine parasities. MAbs have been developed against *Bonamia ostreae*, *Ceratomyxa shastia*, *Cryptobia salmonsitica*, *Perkinsus maximus* are pathogenic protozoan of shell fish.
- MAbs have also been employed for analysis of lymphocyte receptors and characterization of lymphocyte population in carp, for immunopurification of salmon prolactin and for development of sandwich ELISA system for both salmon prolactin and somatotropin.
- MAbs to *A. hydrophila*, EUS fungus *Aphanomyces invadans* and white spot virus of shrimp have been produced and being used in diagnosis in India.
- Application of a MAb against virus:- Infectious hematopoietic necrosis (IHN), caused by IHN virus (IHNV), is a severe and acute epizootic among

salmonid fish. This disease is now widespread. MAbs against IHNV HV - 7601, were produced.

- Detection of Infectious Pancreatic Necrosis virus ( IPNV ) by ELISA. ELISA could be used for the identification of different serotype of IPNV.
- **3.2.4. Specificity and commercial availability of monoclonal antibodies for use in aquaculture**

| **Specificity** | **Availability** |
|---|---|
| *Aeromonas salmonicida* | Diag Xotics Inc*, 27 Cannon Road, Wilton CT 06897 USA |
| *Renibacterium salmoninarum* | Aquatic Diagnostics Ltd., Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK Diag Xotics Inc*, 27 Cannon Road, Wilton CT 06897 USA |
| Infectious Pancreatic necrosis virus (IPNV) | Diag Xotics Inc*, 27 Cannon Road, Wilton CT 06897 USA Test-Line Ltd Clinical Diagnostics, Krizikova 70, 61200 Brno, Czech Republic* |
| White spot virus (WSV) | Diag Xotics Inc*, 27 Cannon Road, Wilton CT 06897 USA |
| Taura syndrome virus (TSV) | Diag Xotics Inc*, 27 Cannon Road, Wilton CT 06897 USA |
| Spring viraemia of carp virus (SVCV) | Test-Line Ltd Clinical Diagnostics, Krizikova 70, 612 00 Brno, Czech Republic* |
| *Viral haemorrhagic* Septicaemia virus (VHSV) | Test-Line Ltd Clinical Diagnostics, Krizikova 70, 612 00 Brno, Czech Republic* |
| Snakehead (*Channa striata*) IgM | Aquatic Diagnostics Ltd. Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK |
| Catfish IgM (*Clarias sp.*) | Aquatic Diagnostics Ltd. Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK |

- *MAbs included as part of a kit

## Unit 4: Molecular Techniques

## Chapter 1: PCR: Principles and applications in Fisheries

### 4.1.1. Introduction

**Polymerase** chain reaction (PCR) helps in **gene** rating numerous copies of DNA from a small initial sample.

The polymerase chain reaction (PCR) process was discovered in 1983 by Kary Mullis who was awarded the Nobel Prize for chemistry in 1993.

- Polymerase chain reaction is basically a technique that allows the selective amplification of any fragment of DNA, provided the **DNA sequence** s flanking the fragment are known.
- The system works so well because amplification of a target DNA **sequence** is exponential.
- Each heating and cooling cycle results in the doubling of the amount of template, hence after 20 cycles the yield of PCR product is approximately one million copies of the single target DNA molecule.
- The original procedure used the DNA polymerase I Klenow fragment from *E. coli.* This had the drawback that between each cycle the DNA had to be denatured (94°C) and new enzyme added.
- To circumvent this problem thermostable DNA polymerase isolated from the bacteria *Thermus* aquaticus YT1, which grows in the hot springs of Yellowstone National park is used.
- The enzyme works optimally at 72°C and can also withstand heating to 94°C for short periods of time.
- This means that during 20 cycles of PCR the enzyme does not have to be replenished.

PCR amplification requires

- two **oligonucleotide primer** s, selective primer and reverse primer,
- four dNTPs (deoxy **nucleotide** triphosphates),
- magnesium ions in molar excesses of the dNTPs and
- a thermostable DNA polymerase to perform DNA synthesis. The quantities of oligonucleotide primers, dNTPs, and Mg++ may vary for each specific application. The conditions need to be optimized for different DNA fragments and oligonucleotide primers.

### 4.1.2. Steps involved in PCR

#### 4.1.2.1. DNA denaturation

- Denaturation occurs when the reaction is heated to 92-96 ° C. The time required to denature the DNA depends on its complexity, the geometry of the PCR tube, the thermal cycler, and the volume of the reaction. For **DNA sequence** s with high G+C content larger denaturation time is required. Template DNA strands are entirely separated. **Gene** rally, 94-96 ° C for 2-3 minutes is sufficient.

#### 4.1.2.2. Annealing

- The **oligonucleotide primer** s hybridize to their complementary single - stranded target **sequence** s. The temperature of this step varies from 37 to 65 ° C, depending on the homology of the primers for the target sequence as well as the base composition of the oligo **nucleotide** s. Primers are present at a significantly greater concentration than the target DNA, and are shorter in length. As a rule, lowering the annealing temperature from the calculated Tm will increase the likelihood of non-specific amplification. As the temperature is increased through Tm, specificity will increase and yield will decrease.

#### 4.1.2.3. Extension

- Last step is the extension of the oligonucleotide primer by a thermostable **polymerase** . Temperature of 72 ° C is used for extension. The time required to copy the template fully depends on the length of the PCR product. The extension rates of thermostable polymerases are between 2 and 4 kbp min. Significant breakthrough in PCR is that it can amplify segments of up to 45 kb efficiently.

### 4.1.3. Reaction components

#### 4.1.3.1. **Primer s**

- Primers should be at least 18-20 (17-30) **nucleotide** s in length and should have a G/C content between 40 to 60% (otherwise low melting temperature is needed).
- The specificity of the PCR depends upon the primers.
- The aim of good primer design is to maximize both the specificity and efficiency of the amplification reaction.

#### 4.1.3.2. Buffer

pH range 8.3-8.8.

#### 4.1.3.3. Mg++ concentration

- Mg++ concentration can severely affect the efficiency of PCR as a con **sequence** of its complexing with dNTPs and the Mg++ requirement of the enzyme.
- An excess of Mg++ results in increased non specific priming whereas too low Mg++ levels reduce product yield.
- Optimum Mg++ concentration should be attained empirically by titrating in 0.5 mM increment between 0.5 mM and 5 mM. Mg++ is essential for enzyme activity.

### 4.1.3.4. Template

The ideal template for a PCR is free from contaminants such as nucleases.

### 4.1.3.5. Polymerase

DNA polymerase, an enzyme, can lengthen a short strand of DNA, called an **oligonucleotide** primer, if the strand is bound to a longer "template" strand of DNA. The polymerase does this by adding the appropriate complementary nucleotide to the three prime end of the bound primer. Optimum enzyme concentration is 0.005 – 0.025 units/ m l. Higher concentration may cause an increase in non-specific product **gene** ration. The DNA polymerase has a $5^1 \rightarrow 3^1$ polymerase activity but lacks $3^1 \rightarrow 5^1$ **exonuclease** activity. The enzyme has a half life of up to 40 min at 95 ° C but is destroyed within a few minutes at 100 ° C.

### 4.1.3.6. Thermal cycling

When optimizing PCR it is important to ensure complete thermal equilibrium of the reaction mix. Reaction volume (including oil or wax layer) and tube wall thickness are critical variables to consider when setting up cycling profiles. Reactions are carried out in 0.5 ml or 0.2 ml reaction tubes.

### 4.1.3.7. Final volume of the reaction

PCR requires rapid changes of temperature, which are accomplished by the thermal cycler. As a general rule, reactions are usually between 20 and 100 m l. Large – volume samples will be inefficiently heated and cooled, while small – volume reaction render insufficient product for manipulation and analysis.

#### 4.1.4. Different versions of PCR

The basic protocol of PCR has been improved to develop several versions.

4.1.4.1. **Two step PCR** - the assay is done in two steps. In the second step 1-5% of the product developed in the first step is used for amplification using same set of **primer** s for further increasing the sensitivity.

4.1.4.2. **Nested PCR** - the assay is carried out in two steps. In the second step **internal primers** are used to amplify to increase the specificity and sensitivity.

4.1.4.3. **RT PCR** where RNA is converted to **cDNA** for amplification.

4.1.4.4. ***In-situ* PCR**- DNA in tissue is detected *in situ*.

4.1.4.5. Single tube PCR with several set of primers directed to different regions of pathogen or primers for two pathogens are used.

4.1.4.6. Real time PCR

**Real-time polymerase chain reaction** or *quantitative real time PCR* (Q-PCR/qPCR) is used to amplify and simultaneously quantify a targeted **DNA** molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The procedure follows the general principle of PCR and its key feature is that the amplified DNA is detected as the reaction progresses in ***real time***. Frequently, real-time PCR is combined with **reverse transcription** to quantify **messenger RNA** and **Non-coding RNA** in cells or tissues.

**4.1.4.7. Hot-start PCR**

In this technique, initial denaturation is performed in the absence of polymerase or primers. The temperature of the reaction mix is then maintained at 70–90 ° C until all the components are combined.

**4.1.5. Applications of PCR**

• Amplification of small amounts of DNA for further analysis by DNA fingerprinting.

• The analysis of ancient DNA from fossils.

• Mapping the human (and other species) genome.

• The isolation of a particular gene of interest from a tissue sample.

• Generation of probes: large amount of probes can be synthesized by this technique.

• Analysis of mutations: Deletions and insertions in a gene can be detected by differences in size of amplified product.

• Diagnosis of monogenic diseases (single gene disorders)

• Detection of microorganisms: Especially of organisms and viruses that are difficult to culture or take long time to culture or dangerous to culture.

• The PCR has even made it possible to analyze DNA from microscope slides of tissue preserved years before.

• Detection of microbial genes responsible for some aspect of pathogenesis or antibiotic resistance.

• Crucial forensic evidence may often be present in very small quantities, e.g. one human hair, body fluid stain (blood, saliva, semen). PCR can generate sufficient DNA from a single cell.

· The sensitivity of PCR allows the detection of pathogens that would be difficult to identify with conventional techniques.

· PCR is widely used for screening shrimp seed and brood for serious viral pathogens such as WSSV, YHV, IHHNV and TSV.

· PCR is ideal for studies in epidemiology, genotyping, health certification, quarantine and for screening for development of SPF stocks.

### 4.1.6. Limitations of PCR

PCR is an extremely sensitive technique but is prone to contamination from extraneous DNA, leading to false positive results. Another potential problem is due to cross-contamination between samples. It is for this reason that sample preparation, running PCR and post-amplification detection must be carried out in separate rooms.

Concentration of Mg is very crucial as low $Mg^{2+}$ leads to low yields (or no yield) and high Mg2+ leads to accumulation of nonspecific products. Non-specific binding of primers and primer-primer dimmer formation are other possible reasons for unexpected results. Reagents and equipments are costly, hence can’t be afforded by small laboratories.

## Chapter 2: Molecular and Immunological Techniques applied in fisheries

### 4.2.1.Molecular techniques

A wide range of techniques is now available for the study of molecular **gene** tics in fisheries. Molecular genetic approaches began to be used in fisheries in the 1950s.

These initial studies were of blood group variants, primarily in tunas, salmonids and cod.

The techniques such as **electrophoresis** , DNA fingerprinting, Dot and slot blotting of DNA, Gene sequencing, DNA chip or DNA microarray, Nucleus transplantation and **Cloning** were described.

### 4.2.1.1. Electrophoresis

There are two types of electrophoresis, **Protein** or allozyme electrophoresis and DNA electrophoresis.

Protein or allozyme electrophoresis provides an indirect assessment of nuclear DNA (nDNA) variability. Population structure can be analysed by these techniques. The use of allozyme electrophoresis for describing population structure is probably at its most advanced stage in the commercial anadromous salmonid fishes. Hatchery stocks of Atlantic Salmon, have been reported as having up to 20-30% less heterozygosity than natural populations

PCR and recombinant DNA techniques create large amounts of DNA segments. To study the structure of these segments, researchers use a process known as gel electrophoresis.

- Used to identify **gene** s in any organism that have previously been identified in other organisms, such as fruit flies.
- It can also be used to compare the DNA found from blood or hair samples at a crime scene with the DNA of a suspect in the crime.

### 4.2.1.2. DNA Fingerprinting

- DNA Fingerprinting technique can be used as a powerful **marker** system in identification in fisheries.
- i) Used to verify the identity of cultured cell lines and various lines of clonal fishes, including those obtained by gyno **gene** sis and androgenesis.
- ii) Useful as tools in demographic analysis of fish population. Their parents can be identified. i.e., identification of individuals and pedigree.
- iii) The fragments detected by DNA fingerprinting can also be used in **gene linkage analysis**. If a commercially important gene tightly linked to a fingerprinting marker, the transmission of the gene can be determined by inspection of the marker. This will be of great value in genetic improvement of fish.
- iv) Fish pathogens can be identified. .
- v) Individual specific pattern have been observed in rainbow trout (*O. mykiss*), Atlantic salmon, chum salmon (*O. keta*), coho salmon (*O. kisutch*) with M13 phage or **probe** s.

- vi) Useful in annexing paternal genetic contribution in gynogenetic fish.
- vii) Assessment of inbreeding rates,
- viii) to study the action of specific genes,
- ix) as genetic markers to identify individuals and family groups and the **labelling** of broodstocks to secure ownership property.

### 4.2.2. Dot and slot blotting of DNA

- Dot or slot blotting analysis was first developed by Kafatos *et al*. (1979). Dot and slot blotting are simple techniques for immobilizing bulk unfractionated DNA on a nitrocellulose or nylon membrane. Hybridization analysis can then be carried out to determine the relative abundance of target **sequence** s in the blotted DNA preparations. Dot and slot blots differ only in the geometry of the blot, a series of spots giving a hybridization pattern that is amenable to analysis of by densitometric scanning.
- A large number of samples can be applied at once, enabling many different DNAs to be sequenced in a single hybridization experiment. The technique has found many application over the years.
- For instance, in **genome** analysis, information on the **gene** tic significance of a **DNA sequence** can often be obtained by using the sequence as a hybridization **probe** to dot blots of DNA prepared from related species. The rationale is that most genes have homologues in related organisms. For e.g. a coding sequence from the human genome will probably hybridize to related sequences in dot blots prepared from DNA of various mammals.

### 4.2.3 Gene chip or DNA microarray

A DNA microarray is a multiplex technology used in molecular biology . Microarray technology evolved from southern blot ting.

- Fragmented DNA is attached to a substrate and then probe d with a known gene or fragment.
- It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotide s , called features, each containing picomoles ($10^{-12}$ moles) of a specific DNA sequence , known as probes (or reporters ).

### 4.2.3.1. Principle of microarrays

- · Hybridization between two DNA strands, the property of complementary nucleic acid **sequence** s to specifically pair with each other by forming hydrogen bonds between complementary **nucleotide** base pairs .
- · A high number of complementary base pairs in a nucleotide sequence means tighter non- covalent bonding between the two strands.
- · After washing off of non-specific bonding sequences, only strongly paired strands will remain hybridized.

- · So fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the strength of the hybridization determined by the number of paired bases, the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot.
- · Microarrays use relative quantization in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

### 4.2.4. Types of microarray

- Many types of array exist and the broadest distinction is whether they are spatially arranged on a surface or on coded beads:
- The traditional solid-phase array is a collection of orderly microscopic "spots", called features, each with a specific probe attached to a solid surface, such as **glass** , **plastic** or **silicon biochip** (commonly known as a ***genome*** *chip*, *DNA chip* or *gene array*). Thousands of them can be placed in known locations on a single DNA microarray.
- The alternative bead array is a collection of microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes, which do not interfere with the fluorescent dyes used on the target sequence.

### 4.2.5. Uses

- · to measure changes in expression levels,
- · to detect single nucleotide polymorphisms (SNPs), or
- · to **genotype** or resequence mutant genomes.
- · to detect DNA (as in **comparative genomic hybridization** ), or detect
- · RNA (most commonly as **cDNA** after **reverse transcription** ) that may or may not be translated into **protein** s. The process of measuring gene expression via **cDNA** is called **expression analysis** or **expression profiling** .

### 4.2.6. Gene therapy

- A recent development in **gene** tic technology known as **gene therapy** focuses on curing inherited disorders. Researchers have replaced defective genes with normal **alleles** , inactivated a mutated gene, or inserted a normal form of a gene into a chromosome.
- The earliest success in human gene therapy involved the treatment of infants who cannot produce adenosine deaminase (ADA), an enzyme important to normal function of the immune system. Scientists have successfully inserted the normal allele for the gene that codes for the enzyme into cells in ADA-deficient children. Preliminary evidence indicates that this gene therapy

leads to better immune function in recipients. Researchers are also exploring gene therapy's potential to help treat people with many other conditions, including certain cancers, hemophilia, heart disease, and cystic fibrosis.

- Although the United States Food and Drug Administration (FDA) has approved more than 400 clinical trials in gene therapy, this method of treating disease remains far from an unqualified medical success. Treatments usually produce some improvement in the underlying condition, but not enough to consider the therapy suitable for large-scale use. The death of a patient involved in a gene therapy experiment in 1999 caused the National Institutes of Health (NIH), a federal agency that monitors gene therapy studies, to reevaluate the safety and effectiveness of gene therapy clinical trials.

### 4.2.7. Nucleus transplantation

- Studies on nucleus transplantation in fishes were initiated in the early 1960's in China.
- Tong *et al*. (1963) first demonstrated the technique, to study the interrelationship between the cell nucleus and **cytoplasm** . The nucleus of a crucian carp egg was removed with a glass microneedle after removing the egg capsule with forceps, and put into Holtfreter's solution in an ice bath. Then nuclei from the middle or late blastula stage of the common carp were transplanted into the enucleated, unfertilized crucian carp eggs.
- In 1973, Tong and Niu, transplanted nuclei between gold fish (*Carassius auratus*) and *Rhodeus sinensis* for the purpose of studying the developmental variations between the integrated nuclei and the pure heterologous nuclei, and the effects of cytoplasm on the nucleus. They concluded that character expression (or **gene** tic expression) was not completely controlled by the nucleus, or by the cytoplasm. In fact, it resulted from interactions between both nucleus and cytoplasm.
- Nuclear-cytoplasmic hybrid fishes have been obtained from the combination of nucleus and cytoplasm between two intergeneric species of freshwater teleost using the technique of **electric fusion**, i.e. the combination of the nucleus of carp (*Cyprinus carpio* red variety) and the cytoplasm of crucian carp (*Carassius auratus* red variety).
- Morphological characteristics of those hybrid fish that have been examined sofar are similar to those of donor nucleus parental species. Some of the hybrid fish grow to normal adults. The $F_3$, $F_4$, $F_5$ descendents have been spread for farm culture. **Protein** content of nucleo-cytoplasmic hybrid is 3.78% higher. Fat content 5.88% lower and the growth rate 15-23 percent faster than those of its parents.
- Chen *et al.* (1986) transplanted cell nuclei from a grass carp blastula cell line into unfertilized, enucleated eggs of crucian carp, thus creating the first " test-tube fish". They also obtained two fish by transplanting crucian carp kidney cell nuclei into enucleated crucian carp eggs, and another three fish

by transplanting gold fish kidney cell nuclei into enucleated crucian carp eggs as well. These fish grow to reach sexual maturity.

- Mao Shujian *et al.* (1990) transplanted cell nuclei of the mutant cell line (AHZC- 88), which was resistant to the grass carp hemorrhagic virus, into unfertilized grass carp eggs using electric fusion, and raised three of the fish to the fry stage.
- These examples demonstrate that fish somatic cells have developmental **totipotency** . It shows that many different types of cells have the ability to develop to the fry stage, and some have continued to sexual maturity. Thus, there is a possibility of selecting disease resistant or cold resistant cell lines for donors and developing a good **strain** through nuclear transplantation. More basic work in the area has been done around breeding of virus resistant fishes. Virus-resistant cells have been injected into the eggs of grass carp, loach and white crucian carp, and a few eggs have developed to embryos or fry stage.

### 4.2.8. Cloning

- **Gene** tically identical copies of certain cells and organisms are called " **clones** ". In vertebrates, monozygous identical twins (mammals), parthenogenetic progenies (Pisces, Amphibia, Reptilia), individuals produced by nucleus transplantation, genetically homozygous individuals etc., are all considered to be **clone** s. Since the various classes of vertebrates are vastly different phylogenetically, there are large number of different methods available for cloning.
- *Embryo splitting* in mammals is a common method of producing a limited number of clones. Application of the same method to fish is however not possible today, because of the *polylecithal* and *telolecithal* type of ova.
- On the other hand, there are methods of cloning applicable only in vertebrates with external embryogenesis (fish) where efficient ***in vitro*** fertilization systems exist. Production of clones by two - step gynogenesis: Meiotic and mitotic gynogenesis. Through mitotic gynogenesis 100% genetically homozygous clones are produced in common carp, zebra fish.
- **Cloning by a combination of andro-and gynogenesis**
- The production of viable diploid progeny by androgenesis is much more difficult than through gynogenesis for two reasons.
- 1. It is quite difficult to perform the elimination of female pronucleus and polar bodies without damaging the **cytoplasm** .
- 2. The production of diploid progeny derived from the male pronucleus is also cumbersome since there is no partner **genome** integrating, like the second polar body in gynogenesis. This is the reason why the first mitotic division has to be inhibited in androgenesis (endomitosis) for restoring diploidy. This second step of androgenesis cytologically corresponds to the whole of mitotic gynogenesis.

- The possible **genotype** s produced as a result of successful diploid androgenesis are XX female and YY super male. In both cases homozygous individuals are produced, which could be used for cloning of carp. In the case of common carp female suppresses the male in growing intensity

### 4.2.9. DNA – based diagnostics

Molecular biology has been used to design a new **gene** ration of diagnostic tools, the PCR ( **Polymerase** Chain Reaction) and Gene **Probe** s. The key to DNA-based diagnostics is the generation of the target pathogen through **recombinant DNA technology** . This is done by purifying the infectious agent of interest and isolating its nucleic acid. The isolated DNA fragment has to be **sequence** d. Once the adequate genetic information (sequence information) is generated, the information can be used in PCR or gene probes.

Presently, PCR methods are available for the detection of many pathogens of shrimp:

*Vibrio vulnificus* (Hill *et al.,* 1991);

*V. parahaemolyticus* (Karunasagar *et al.,* 1997);

*V. penaeida* (Genmoto *et al.,* 1996);

MBV (Lee *et al.* 1993);

IHHNV (Lightner *et al.,* 1994);

rod shaped nuclear virus of *P. japonicus* (Takahashi *et al.* 1996), and BP (Wang *et al.* 1996).

PCR has been used to detect pathogenic bacteria and **viruses** in hatchery and aquaculture situations (Winton, 1992).

Nucleic acid probes are segments of DNA or RNA that have been labeled with **enzymes** , antigenic substances, chemiluminiscent substances or radioisotopes. Probes can be directed against either DNA or RNA targets.

Probes bind with complimentary sequences of pathogenic DNA during the detection process providing a signal (like colour change) that can be identified or measured.

- Today, non-radioactive probes (e.g. digoxigenin (DIG) labeled probes) are gaining importance due to their high level of sensitivity and safety compared to radioactive probes.

- *In-situ* hybridization and dot blot hybridization are gene probes being used in aquatic disease diagnostics. However, PCR has definite advantages over gene probes in its sensitivity for direct detection in clinical specimens.

Nucleic acid hybridization reaction consists of four components;

i) the probe,

ii) the target DNA/RNA (in the sample),

iii) the reporter molecule (the label on the probe), and

iv) the hybridization method.

- Hybridization can be performed in solutions or on solid support (dot-blot) or even on sections of tissue fixed on slides (*in-situ* hybridization).
- *In-situ* hybridization has the advantage in that non-specific tissue effects which may result in false positive diagnosis in dot-blot assay can be distinguished from specific histological lesions (Lightner, 1996).
- Presently, in shrimp disease diagnosis, hybridization probes are available for many viruses such as Infectious Hypodermal and Haematopoeitic Necrosis virus (IHHNV), Hepatopancreas Parvo-like Virus (HPV), *Baculovirus Penaei* (BP) and *Monodon baculo virus* (MBV).

### 4.2.10. Advantages of molecular methods

- Highly sensitive and rapid in diagnosis.
- Detection of non-culturable agents, the goal of the **DNA probe** technology is to eliminate the need for routine viral, bacterial and fungal cultures.
- DNA amplification can assist in detecting the pathogens that are present in low numbers and also in handling a tiny volume of specimen.
- Can be used to detect the latent infection and thereby identify the reservoir hosts of infection that is significant in the epizootiology.
- Can be used to differentiate antigenetically similar pathogen.

### 4.2.11. Disadvantages

- These methods are cost-intensive procedures.
- These tests cannot detect unsuspected samples.
- Molecular methods will have difficulty in detecting new pathogens, as the exclusive use of these would overlook such infections.

#### 4.2.12. Immunological techniques

### 4.2.12.1. Enzyme immunoassays

The interaction of an **antibody** with an antigen forms the basis of all immunochemical techniques. Immunoassays are both qualitative and quantitative. A labelled antibody/ /antigen is used to visualize the immune reaction. The Enzyme-Linked Immunosorbent Assay (ELISA), also known as the Enzyme Immuno Assay (EIA), has become a widely-used serological technique.

There are two basic methods.

i) Direct ELISA

ii) Indirect ELISA

**i) Direct ELISA**

- Coat the ELISA plate wells with antigen and incubate (4°C) it overnight followed by washing.
- Block the uncoated sites with milk powder or BSA, wash to remove excess of blocking agent.
- Add antibody which were raised against the antigen and conjugated with enzyme followed by washing to remove unbound antibody.
- Add substrate and read the colour developed under spectrophotometer.

**ii) Indirect ELISA**

- Coat the ELISA plate wells with antigen and incubate (4° C) it overnight followed by washing.
- Block the uncoated sites with milk powder or BSA, wash to remove excess of blocking agent.
- Add primary antibody which were raised against the antigen followed by washing to remove unbound antibody.
- Add secondary antibody conjugated with enzyme followed by washing to remove unbound antibody.
- Add substrate and read the colour developed under spectrophotometer.

A microtitre plate with numerous shallow wells is used in both procedures. There are three **enzymes gene** rally used for colour development with the second antibody. The earliest used was *alkaline phosphatase* and the commonest now is *horseradish peroxidase*. The third is *β-galactosidase*, from *E. coli*, which is active at the higher pH values preferred for antigen absorption.

The substrates generally employed for alkaline phosphatase and β-galactosidase are p-nitrophenylphosphate and o-nitrophenlbeta-D-galactopyranoside, respectively, with colour development being detected at 405 and 420 nm. In the case of horseradish peroxidase, o-phenylenediamine (OPD) or tetramethyl benzedine (TMB) are most common.

A more sensitive ELISA detection system may be obtained using fluorogenic substrates for alkaline phosphatase or betagalactosidase. Most ELISA are read in a spectrophotometer adapted for microtitre plates.

Crawford *et al.* (1999) developed an Enzyme-linked Immunosorbent assay for detection of antibodies to Channel Catfish Virus (CCV) in Channel catfish.

Sharif (1999) developed and standardized an ELISA for diagnosis of *Aeromonas hydrophila* infection in fish.

### 4.2.12.2.Dot immunobinding assay

- In the dot immunobinding assay the antigen is attached to nitrocellulose paper in a series of dots and is for screening on a limited budget.
- It is claimed to be equally sensitive to, or more sensitive than, ELISA assays.
- It is similar in principle to the ELISA, except in the use of nitrocellulose paper.

The original method involved the application of dots of the antigen to nitrocellulose sheets followed by cutting up of the sheets so that square pieces of paper containing the dot were put in to microtitre wells for incubation with the antibodies.

A variation of this involved the inversion of the microtitre plates containing the antibodies over the sheets with the matrix of dotted antigen with the tight seal for the **antibody** -antigen incubation.

The dot-ELISA is a sensitive assay for detecting or quantifying antigen or antibody. Further, it needs less expensive equipment, is time-saving and also provides a permanent record of the assay (Pappas, 1988).

### 4.2.12.3. Western blot ting

Western blotting is a technique by which **protein** s can be transferred from a polyacrylamide gel to a sheet of nitrocellulose so that a replica of the original gel pattern is obtained. A wide variety of analytical procedures can then be applied to immobilized protein. In this technique, a sheet of nitro-cellulose is placed against the surface of a SDS-PAGE protein fractionation gel and a current applied across the gel (at right angles to its face). This causes the proteins to move out of the gel and intro the nitrocellulose, where they bind firmly by non-covalent forces.

The technique involves three steps:

- protein separation by SDS-PAGE,
- blotting and

- immunoassay.
- To detect a specific protein, an **antibody** to that protein must be available. An antibody can either be produced for the protein of interest or some times purchased commercially. The nitrocellulose membrane itself has many non-specific sites that can bind proteins, including antibodies. These sites must be blocked with a non-specific protein solution such as re-hydrated milk. The primary antibody is added in the milk solution and binds to the protein of interest. The antibody protein complex is detected using a secondary antibody that has a label attached to it. Often a reporter enzyme such as alkaline phosphatase is linked to the secondary antibody, and the addition of lumiphos or X-phos to the blot allows detection of the protein band.

**Application**

i) screening of **hybridoma clones** .

ii) used as a diagnostic tool for various pathological conditions.

iii) ease of processing for **autoradiography** .

iv) include **hormone** -receptor, cyclic AMP-receptor and protein-nucleic acid interactions can be analysed.

v) used to distinguish species of piscine trypanosomes.

**4.2.12.4.Latex agglutination test**

Microsphere or latex agglutination tests (LATs) have been used since 1956, when Jacques Singer of Montefiore Hospital (Bronx, New York) developed a test for detection of reheumatoid factor.

Latex refers to the microscopic polymeric particles, which act as the base for various immunoassays and tests. They are made of polystyrene by the same emulsion polymerization process used for making synthetic rubber or latex. The particles are tiny, referred to as microspheres (diameter 0.015-40 mm), uniform (coefficient of variation 1-3 per cent), solid, and perfect, usually hydrophobic spheres.

Microsphere-based diagnostic tests (qualitative, yes/no results) and assays (quantitative results) are usually based upon the specific interaction of antigen (Ag) and **antibody** (Ab).

Sub-micron sized polystyrene (PS) microspheres, are used for solid support; Ab or Ag can be adsorbed to them.

These ‘sensitized’ microspheres then act to magnify or amplify the reaction, which takes place when they are mixed with a sample containing opposite reactant.

In simple particle agglutination, a positive test results when a drop of uniformly-dispersed milky-appearing Ab coated beads on a glass slide reacts with Ag in a drop of sample (whole blood, serum, antigen) to cause particle agglutination, i.e. clumping of microspheres, to look like curdled milk.

Alternatively, Ag-coated particles are agglutinated by a positive sample of Ab. Latex reagents are portable, useful every-where, rapid and efficient. Ideal for point-of-care use (field, on-site and ambulance), they can be run quickly and simply (2-3 min from the sample preparation), and diagnosis and treatment can commence promptly, before the advent of severe damage.

Since they can be run quickly and easily without instrumentation, they can replace other immunoassays like Radio-immunoassay (RIA) and Enzyme Linked Immunosorbent Assay (ELISA).

Ascencio (1990) developed a rapid particle agglutination assay using latex beads coated with connective tissue and serum **protein** s. This was evaluated for its ability to identify fibronectin, collagen cell surface receptors on *Vibrio* and *Aeromonas* **strain** s isolated from diseased fish, human infection and environment.

## Chapter 2: Development of Vaccines

### 4.3.1. Introduction

Vaccination is one of the important means of controlling disease. In 1798, Edward Jenner worked on small pox. He employed the term ‘vaccine’ (vaccination for protective inoculation). Pasteur extended Jenner’s findings to other infective diseases such as anthrax, rabies and chicken cholera. By ‘vaccination’ it is possible to induce active immunity to diseases. Immunisation is brought about by the use of killed or weakened(attenuated) bacteria. The immune system recognizes and begins to produce antibodies.

Control of diseases by vaccination has a number of advantages over chemotherapeutic methods.

- Vaccination is preventive measure. The use of vaccines has entered in the field of aquaculture recently. Because of the intensive culture systems, many industries have resorted to the routine use of vaccines which confer a high degree of protection when correctly used.
- Their use in salmon, trout, Mediterranean sea bass and even in shrimp and lobsters is now a standard part of husbandry in all important areas for fish culture in Scandinavia, North and South America and Asia.
- The concept of vaccinating fish on a commercial scale has now been realized with respect to Enteric Red Mouth and Vibriosis.

- Fish immunization began in 1942, with the successful oral immunization of trout against bacterium *Aeromonas salmonicida* by Duff.
- Fish vaccines in **gene**ral, fall into three major categories, namely, killed whole cell vaccine, live-attenuated vaccine and recombinant DNA based vaccines.
- Efficacy of these vaccines has been appreciably improved using adjuvants,immuno stimulants or vaccines carriers.
- However, it is still affected by the routes of vaccine administration. In general, injection is better than immersion and oral administration.

### 4.3.2. Mode of preparation of fish vaccines

- The bacterial fish vaccines may be categorized as follows.
- i) Chemically or heat inactivated whole cells. These vaccines may be mono or polyvalent.
- ii) Inactivated soluble cell extracts. i.e. Toxoids.
- iii) Cell lysate
- iv) Attenuated live vaccines, possibly **gene** tically engineered cells. There is a perceived risk that the vaccine **strain** may revert to pathogenic mode.
- v) Purified sub-cellular components, e.g. LPS. These vaccines require a detailed understanding of microbial biochemistry.

### 4.3.3. Methods of vaccine inactivation

- There are several methods of inactivating bacterial cells for incorporation into fish vaccines.
- i) Chloroform (3% v/v)
- ii) Formalin (0.2-0.5% v/v)
- iii) Phenol (0.3-3.0%)
- iv) Heat (56° C or 100° C)
- v) Sonication
- vi) Lysis with NaOH at pH 9.5 or with SDS.
- Commercially, the use of formalin has given encouraging results.

### 4.3.4. Killed whole cell vaccines

Killed whole cell vaccine is a suspension of heat or chemical killed pathogens that are able to induce specific protective immune response against those pathogens when administered into the host.

- These have been of great use in controlling some of the important fish bacterial pathogens such as, *V. anguillarum, V. salmonicida, V. ordalli, Y. ruckeri,*and *A. salmonicida*.
- All these killed vaccines are formalin inactivated whole cell vaccines administered with orwithout adjuvants and are commercially available.
- These bacterial vaccines are highly immune protective, and are cheap to produce, but are not known at present as to what specific antigens of these vaccines are involved in offering protection.
- Although in many cases it is believed that the protective substances are lipopolysaccharides.

Killed vaccines have been developed for some pathogenic fish **viruses** such as infectious pancreatic necrosis virus (IPNV), infectious haematopoietic necrosisvirus (IHNV), viral haemorrohagic septicaemia virus (VHSV) and spring viremia of carp virus (SVCV).

- Injection of rainbow trout fry with the inactivated IPNV offers good protection in rainbow trout but when administered in brook trout with Freund's complete adjuvant it induces strong humoral response with poor protection.
- Successful use of killed VHSV in rainbow trout has also been recorded.
- Formalin-inactivated IHNV has been found to protect rainbow trout against lethal IHNV when immunized at high concentration.
- Although all these above vaccines look promising at laboratory scale none of them has been commercialized.
- It is only the killed vaccine of spring viremia of carp virus (SVCV) that was commercially available for some years. This vaccine comprises of two inactivated **strain** s of SVCV emulsified in oil.

Disadvantages of using killed virus vaccines

- high cost of their production in cell culture, and
- their cumbersome method of purification and
- delivery.
- In **gene** ral, killed vaccines alone trigger only the humoral immune response and not the cell-mediated immune response. Further, this induces protective immunity, which fades away over time and needs to be given in booster doses.

### 4.3.5. Live–attenuated vaccines

Live-attenuated vaccine is a suspension of attended live pathogens that are able to replicate inside the host and induce protective immune response but unable to cause disease.

- They mimic the actual infection by pathogens and hence a small dose of vaccine is enough to induce long lasting protective immune response.
- These live attenuated vaccines can induce both humoral and cell-mediated immune responses.
- These are strong stimulants of cell-mediated immune response. These preferentially enhance T cell prolife rative response relative to B cell responses.
- Some of the conventional live viral vaccines have been produced against VHSV, IHSV and IPNV.
- Avirulent **strain** s of IHNV are also used as live vaccines.
- Use of VHSV- attenuated strains obtained through serial passage of VHSV in carp-cell line under progressive increase of temperature has been used as live vaccine.
- Protection of goldfish against some common ectoparasites has been observed by intraperitoneal andimmersion immunizations with live tomites of *Ichthyophthirius multifiliis* and *Tetrahymena pyriformis*.

Although some of these vaccines are found useful as live vaccines in laboratory, so far none of them has been licensed for field trial. This is because of some of the possible disadvantages that might be associated with this type of vaccines, such as apprehension of such vaccine strains becoming virulent in non-target species,possibility of reversion to pathogenic state and problems associated with residual virulence.

### 4.3.6.Recombinant DNA-based vaccines

**Recombinant DNA technology** has been widely used in development of novel vaccines that are now collectively termed as ‘recombinant DNA-based vaccines’ or ‘new **gene** ration vaccines’. Different types of vaccines based on recombinant DNA technology have been developed which include:

(i) recombinant immunogenic **protein** vaccines or epitopes purified from **vector** s carrying the gene of interest produced in prokaryotic or eukaryotic expression systems,

(ii) **peptide** vaccines,

(iii) live vaccines produced by defined genetic manipulations and microbial vectors carrying gene coding for immunogenic protein, and

(iv) Genetic vaccines (DNA vaccines and RNA vaccines)

(v) **Subunit vaccine** s.

### 4.3.7. Recombinant protein vaccines

Production of a recombinant protein vaccine starts with identification of the immunogenic subunit or protein from the pathogen of interest and verification of its immunogenicity *in vivo* and ***in vitro*** .

Once, the immunogenic proteins or subunits of pathogen are identified, the **gene** (s) involved in coding for them can be introduced into a **vector** , over-expressed in expression hosts and can be used as recombinant protein vaccines. The vector systems usually used to express recombinant proteins are **viruses** or bacterial **plasmid** s. Expression systems commonly used are prokaryotic and eukaryotic cells.

- Prokaryotic expression system comprises of bacteria such as, *Escherichia coli*, and the eukaryotic expression system comprises of yeast, insect cells and mammalian cells.
- Some inherent advantages and disadvantages exist with both of these expression systems.
- The major problem with the prokaryotic systems (such as bacteria) is that, they lack the signals required for proper post-translational modification and hence there lies the signals required for proper post translational modifications and hence there lies the problem of improper folding and lack of glycosylation. This leads to production of proteins of unpredicted antigenicities. In some cases, production of proteins will be in the form of inclusion bodies that need to be treated biochemically before being used as vaccine. This biochemical treatment of denaturation and renaturation of recombinant protein reduced its immunogenicity.
- Obvious advantages of prokaryotic expression system are, high level expression of recombinant protein (often more than 30%), well studied genetic and fermentation system of *E. coli* and easy scaling up of vaccine production.
- In the case of eukaryotic expression system, although the problem of folding and glycosylation does not exist, the final yield of expressed protein remains low, and hence the scaling up of the production process is difficult.

Both prokaryotic as well as eukaryotic expression systems have been used to produce fish viral, bacterial and parasitic antigens, and prokaryotic system is most widely used.

- For example, purified glycoproteins from IHNV and VHSV have been used as **subunit vaccine** s in fish and shown to be immunoprotective, and further these two proteins have been used widely for recombinant vaccine production.
- Similarly, an RNA-free subunit vaccine prepared from grass carp haemorrhagevirus (GCHV) treated with 1% NP40 in low salt solution has been shown to induce more than 80% protection in carp.

### 4.3.8. Peptide vaccines

**Peptide** vaccines comprise of synthetic peptides that are able to induce protective immune response when administered into the host.

i) To produce peptide vaccines it is necessary to identify immunogenic regions, also known as ‘epitopes’ on the antigenic **protein** .

- The term epitope refers to a stretch of 6-8 **amino acids** on antigens that specifically binds to antibodies or to receptors on immune T cells.
- Those epitopes that bind to the **antibody** produced by specific **B cells** are called as B-cell epitopes while those recognized by receptors on the surface of activated T-cells are termed as T-cell epitopes.
- Monoclonal antibodies are indispensable to identify the B-cell epitopes.

ii) A region with high **sequence** variability among several **strain** s of a pathogen is also chosen as a candidate for synthetic peptide vaccine.

Epitope mapping and use of peptide vaccines against fish pathogens are still in its infancy. Some of the B-cell epitopes have been identified on some fish viral proteins such as IHNV glycoprotein.

Synthetic peptide vaccines emulsified with Freund’s complete adjuvant has induced poor neutralizing antibodies than that of the native virus fish sera, which indicates that peptides alone are less immunogenic than the native protein.

- Synthetic peptide vaccines offer the advantage of safety, purity and low cost as compared to live or inactivated vaccines.
- It is now possible to induce virus neutralizing antibody response using peptides of specific amino acid sequences.
- The peptides can be chemically synthesized in pure form or made by bacterial expression using rDNA technology. In the latter case, the peptides may be fused into other expressed proteins (fusion peptides).

### 4.3.9. Gene tically modified live vaccines

Pathogens with defined genetic manipulations or microbial **vector** s carrying the gene coding for immunogenic **protein** can be used as live vaccines. Live vaccines replicate inside the recipient host resembling the natural infection and thus induce strong immunity. This kind of vaccine is reported to be highly immunogenic than the non-replicating vaccine products.

Selection of a stable non-pathogenic mutant usable as live vaccine is a complex process in the sense that it involves tedious procedure of growing **viruses** in

different culture conditions or introducing targeted mutations, followed by *in vivo* and ***in vitro*** assays.

Some important methods of selection of attenuated mutants are,

- adaptation to heterologous cell line,
- adaptation to elevated temperature and
- selection of neutralizing monoclonal **antibody** escape mutants. The rationale behind selection of **strain** s adjusted to such extreme conditions is that these strains are believed to be altered genetically hence resulting alteration of their virulence. **Nucleotide sequence** analysis of such strains can confirm the position of mutation.
- Further, *invivo* and *in vitro* analysis can reveal their phenotypic variation hence aiding in election of such strains as candidates for live vaccine.
- Defined genetic alterations resulting in mutants with desired phenotype can be achieved using site directed mutagenesis technique also.
- Live vaccines have been used against some of the fish bacterial pathogens such as *A. salmonicida* and *A. hydrophila*.
- Several techniques such as homologous recombination,chemical mutagenesis and transpos on mutagenesis are used to produce mutant bacteria those are a virulant and capable of being used as live vaccines.

### 4.3.10. Gene tic vaccines or Nucleic acid vaccines

- Genetic vaccines consist only of DNA (as **plasmid** s) or RNA (as mRNA), which is taken up by cells and translated into **protein** . In case of gene-gun delivery, plasmid DNA is precipitated on to an inert particle (generally gold beads) and forced into the cells with a helium blast. Transfected cells then express the antigen encoded on the plasmid resulting in an immune response.

#### 4.3.10.1. DNA vaccines

DNA vaccines consist of a suspension of bacterial plasmids carrying the gene coding for the immunogenic protein under the control of eukaryotic promoter.

- The basic attributes of a DNA vaccine include an origin of replication suitable for producing high yields of plasmid in *E. coli*, an **antibiotic** - resistant gene to confer antibiotic-selected growth in *E. coli*, a strong enhancer/promoter and an mRNA transcript termination/polyadenylation **sequence** for directing expression in mammalian cells.
- The plasmids hence **construct** ed are grown in *E. coli*, purified and suspended in saline and introduced into the host either by intramuscular injection or using a gene gun.

- DNA vaccines have been used in fishes with very encouraging results. Strong expression of **reporter gene** s in muscle cells following intramuscular injection of plasmid constructs carrying gene of interest and reporter gene have been reported.
- When plasmids carrying luciferase gene under the control of cytomegalovirus immediate early gene promoter is injected to rainbow trout at a dose of 50µg of DNA, maximum activity is seen at 5 to 7 day post-injection and the activity of luciferase remains for 115 days.
- Combined injection of plasmids carrying VHSV and IHNV glycoprotein genes shows plasmid DNA to remain in the muscle cells up to 45 days.

DNA immunization induced specific as well as non-specific immune response in the recipient host. High level of protection in clinical animal model has been observed due to the generation of specific antibodies and priming of T-cell responses. Significant protection of rainbow trout is observed against IHNV challenged following the injection of construct encoding the IHNV G protein.

Apart from introducing a part of the **genome** of pathogen coding for immunogenic protein, it is possible to introduce a gene coding for an **antibody** that can target and destroy the pathogen.

### 4.3.11. Advantages of DNA vaccines

DNA vaccines overcome almost all the drawbacks of all other form of vaccines. Major advantage of DNA vaccines over recombinant protein vaccine lies in its ability to induce production of native form of protein with appropriate post-translational modifications. This has been shown in the case of DNA immunization of rainbow trout. Upon injection of plasmids carrying VHSV G protein gene, the expressed G protein is recognized by specific monoclonal antibodies.

Additionally, DNA vaccines are able to induce long lasting immune response and are economical and safe. Practical application of DNA vaccine in fish does not seem to be encouraging because most of the important fish pathogens, especially the **viruses** those affect fish at a very young age. This makes it difficult for one to administer vaccine to small fish through injection route, which is so far the only method of introducing the DNA vaccines. However, the present methods of administration of DNA vaccine, such as use of injection machines are still useful for immunizing broodstocks of fairly large fish so as to ensure that immunity is passively transferred from mother to offspring as this being demonstrated in controlling Ich. Therefore, it is difficult to use DNA vaccines for individual fish on a large scale in intensive aquaculture unless one can introduce DNA vaccine to fish orally or through gill filaments via aquatic medium.

#### 4.3.11.1. RNA vaccines

Genetic vaccination through the delivery of RNA has also been investigated, but to lesser extent than DNA vaccination. RNA expression is short-lived, and is thus less effective in inducing an immune response. The preparation and administration of RNA is trouble some because of the low stability of the RNA. One advantage of the RNA strategy is that there is no risk of integration of the delivered gene into the host genome.

### 4.3.12. Subunit vaccines

Subunit vaccines are produced by **genetic engineering** . They are purified single proteins from the surface of a pathogen which can be produced cheaply in fermenters.

The great advantage of subunit vaccines is that they contain no live, potentially infectious organisms. The subunits are advantageous because the immune system of the animal is challenged with only one antigen, thereby omitting other components of the virion that might adversely affect the immune response.

The major drawback with subunit vaccine is that the antigenic mass cannot be greater than the amount injected. There is no amplification of the antigen. The first step in the production of recombinant subunit vaccine is the isolation of immunogenic genes, which are amplified by **cloning** .

The specific genes of virions are purified from the preparation of DNA or **cDNA** in case of RNA viruses. The DNA is amplified by cloning and cleaved with restriction **endonuclease** s to small fragments.

The DNA fragments which code for immunogenic proteins are identified and used for the preparation of recombinant vaccines.

### 4.3.13. Vaccine delivery system

A number of methods of administering vaccines to fish have been tried with varying degrees of success. These include:

i) Injection

ii) Oral uptake via food

iii) Immersion in a solution/suspension of the vaccine

iv) Bathing in a very dilute preparation of the vaccine for prolonged periods

v) Spraying or showering the vaccine into fish

vi) Hyposmotic infiltration, and

vii) Anal intubation

It can be administered by injection, by immersion or by spraying directly onto the fish according to what suits an individual farm's preference.

- For small fish (1.5 to 5 gms) by direct immersion in diluted vaccine (1:10) for 30 secs.
- For larger fish (70-100gms) sprayed with vaccine or immersion for 3-5 secs. Stress should be avoided at the time of handling. Maintain the vaccine solution at the same temperature on the holding tanks, oxygenating the vaccine solution during the vaccination procedure, etc.
- Oral vaccination of fish using Artemia as the vaccine delivery system can also done. When vaccine is given through oral route there is possibility of Ag being degraded by the digestive **enzymes** in the stomach. New approach involves first feeding the vaccine (a killed bacterial suspension) to the Artemia, and then feeding the Artemia as the first live food to the fry of the species of interest. It is thought that the vaccine becomes incorporated into the lipids of the Artemia and this protects it from the digestive degradation of the fish.
- Immunity in vaccinated animals tends to change with time following vaccination.
- Booster vaccination can be given. Duration of protection depends upon the method of vaccination,the size of the fish, their health status at the time of vaccination and the antigen used to vaccinate them.
- Vibrogen -2 vaccine is produced by Aquatic Health Limited, Greece. The AHL, Canada has developed another vaccine called Lipogen Triple bacterin (a combimnation furunculosis + vibriosis+ hitra bacterin) to protect against furunculosis.

- **Gene** tic vaccines can be delivered into the host by several routes and methods.
- The main methods of **plasmid** -DNA delivery is by needle injection or by gene-gun.
- While needle injection requires relatively large amounts of plasmid (50-100 µg), the amount of plasmid required for gene-gun immunization has been titrated down to a few nanogram. When delivered by gene-gun, the plasmid solubilizes when the plasmid coated gold bullet penetrates the cells and thus, plasmid is directly deposited into cells transfecting upto 20% of the cells in the target area. The gold particles directly penetrate due to the force of delivery, thereby increasing the rate of **transfection** without having to rely on the uptake of DNA by the host cell itself.

### 4.3.14. Environmental, ethical and regulatory aspects of fish immunization

Preventive immunization coupled with good management is obviously the most suitable means of fish disease control in intensive aquaculture.

- An ideal vaccine suitable for large-scale usage should be highly immunogenic, should offer a long-term protection, be cost effective, easy to produce and deliver and should be safe. It should meet all the safety and regulatory criteria before being used for field application.
- Although some vaccines meet many of the important attributes necessary for a good vaccine, the problem of environmental, ethical and regulatory aspects of field application still remain as an obstacle for their large-scale usage.
- This is obvious in the case of live-attenuated vaccines (both conventional and **gene** tically engineered) and DNA vaccines.
- Live vaccines, although are highly immunogenic, remains unattractive for long time for field application because of the apprehension of their reversion to pathogenic state and the chance of the vaccine **strain** becoming pathogenic to non-target species.
- Additionally, there is fear of shedding and persistence of live vaccine strains in tissues.

DNA vaccines are argued to pose many dangers to target animals such as:

- potential integration of **plasmid** DNA into the **genome** of the host cells,
- potential induction of immune tolerance or of autoimmunity and
- the potential induction of antibodies to the injected plasmid DNA.

### 4.3.15. Conclusion

Recent advances in the field of molecular biology have profoundly affected the development of fish vaccines.

- Antigens obtained from many fish pathogens with the potential of being used for vaccine have been identified.
- **Gene** tically engineered vaccines have been increasingly employed against many of the fish pathogens.
- DNA vaccine, the most recent of all vaccines have been shown to be highly efficient against some fish viral diseases.
- Field scale efficacy study of some of these vaccines is being undertaken.
- Although great efforts have been carried out on the development of efficient vaccines against various fish pathogens, the delivery systems of the vaccine to fish is equally important. One has to make sure that the vaccine can be effectively delivered and it is suitable for intensive aquaculture in terms of cost effectiveness.
- So far, there is only one recombinant **protein** vaccine for commercial use in aquaculture, which is against the IPNV of trout.

- It is expected that some more genetically modified vaccines may be commercialized in the near future.

**Unit 5: Marine biotechnology**

- **Chapter 1: Bioactive compounds from marine organisms**
- **5.1.1. Introduction**
- Marine bioactive compounds are organic compounds produced by microbes, sponges, gorgonians, soft and hard corals seaweeds, and other marine organisms. These products are the current interest of industry for new drugs and chemicals.
- Marine microorganisms form highly specific and symbiotic relationships with filter-feeding organisms like sponges, alcyonarians, ascidians and marine plants. The host organism synthesizes these compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment.
- A diverse array of bioactive compounds can be isolated from the extracts of marine organisms. Many of them have novel chemical structures which may lead to the development of entirely new drugs and therapeutic agents.
- · Anti-cancer agents have been isolated from algae, sponges, jellyfish, corals, shark cartilage and shellfish.
- · The pacific oyster contains a substance which may help diabetics by promoting the secretion of insulin.
- · Chemicals found in sponges may be used to treat yeast and fungi.
- · A poison emitted by the cone snail has been found to prevent brain damage in animals after a head injury or stroke, and offers great promise in future applications in humans. The bioactive substances extracted from different marine organisms is discussed in this chapter.

**5.1.2. Marine bacteria**

Many tropical marine organisms have little commercial value, but are increasingly important as resources in the search for new drugs. By screening these marine organisms, high incidences of **cytotoxic** ity, antiviral activity, antibacterial and anti-carcinogenic and other biological activity have been reported.

It has been demonstrated that marine bacteria produce anti-microbial substances.

- The first documented identification of a bioactive marine bacterial metabolite was the highly brominated pyrrole **antibiotic** , isolated from a bacterium obtained from the surface of the Caribbean Sea grass *Thalassia*. Subsequently, this unique metabolite was identified by x-ray crystallographic methods, which composed of more than 70% bromine by weight. The metabolite exhibited impressive ***in vitro*** antibiotic properties

against Gram-positive bacteria. However, it was inactive for Gram-negative bacteria and animal assays.

- As more evidence is obtained, it is becoming abundantly clear that bacteria form highly specific, symbiotic relationships with marine plants and animals. Experience in this area arose from a study of the pathogen resistance of the estuarine shrimp *Palaemon macrodactylus*. The eggs of *P. dactylus* possess significant bacterial epibionts, which, when removed by treatment with antibiotics, leads to the rapid infestation of the eggs by pathogenic fungi, especially of *Lagenidium callinectes.* It could be due to the anti-fungal agents produced by bacteria.
- **5.1.3. Marine fungi**
- Although terrestrial fungi have represented a major biomedicinal resource (e.g., penicillin from *Penicillium*), studies to develop the biomedicinal potential of marine fungi were less.
- · The isolation of a small lactone, leptosphaerin from *Leptosphaeria oraemaris* demonstrated that marine fungi may form important resource for unique metabolites.
- · Later, the useful chemical, Gliovictin was isolated from marine fungus, *Asteromyces cruciatus*.
- · Since then more than twenty useful bioactive compounds have been derived from marine fungi.

### 5.1.4. Marine microalgae

Marine microalgae are relatively unexploited but are rich resources for bioactive compounds. Toxins initially isolated from fish or shellfish were found to originate from microalgae, especially dinoflagellates. These toxins are useful tools to investigate the structure and function of ion channels on cell membranes or to elucidate the mechanism of tumor promotion based on their specific inhibitory action against protein phosphatases.

The number of antifungal or antitumoral substances of microalgal origin is rapidly increasing. More importantly, structural similarities have been found between many bioactives found in marine invertebrates and those in freshwater blue-green algae. The similarities point to a great potential of marine blue-greens, the least explored resource, for producing bioactive compounds of medicinal value.

Microalgae are significant resource for bioactive metabolites, particularly cytotoxic agents with applications in cancer chemotherapy.

- From the marine microalgae such as from the blooms of *Phaeocystis sp*., antibiotic substances were listed. *Phaeocystis pouchetii* is reported to produce chemicals such as Acrylic acid, which constitutes about 7.0% of the dry weight. The antibiotic substances thus produced are transferred

throughout the food chain and found in the digestive tract of Antartic penguins.
- Production of ß carotene and vitamins by the halotolerant alga *Dunaliella sp.*, is documented.
- Microalgae produce incredibly potent alkaloidal neurotoxins such as saxitoxin and polyketide neurotoxins such as the brevetoxins.

### 5.1.5. Marine macroalgae

Of the total marine algae so far evaluated, about 25% showed one or the other biological activity.

- The metabolites of green algae were reported to contain 1,4 –diacetoxic butadiene moiety, which exhibited icthyotoxic property.
- Among the red algae, halogenated lipids have been isolated, particularly from the *Laurencia sp.*
- The rare chemical prostaglandin was also reported to occur in *Gracilaria pichenoids*.
- *Ulva* meal supplementation was found to provide disease resistance to red sea bream in Japan. Similar results were also reported from Japan on the use of *Ulva* meal supplementation towards disease resistance and high growth rate in black sea bream.
- The polysaccharide fractions from marine algae, *Porphyra yezoensis* was found to stimulate the *in vivo* and *in vitro* murine phagocytic function.
- The purified fractions gave stronger phagocytic activity. Some of the macro algal crude extracts indicated their potential therapeutic nature when challenged with potential pathogens among fish and shellfish.

### 5.1.6. Marine sponges

The wider biosynthetic capability of sponges could be attributed to their biological association with other symbionts.

- About 38% of the sponge body comprises of microorganisms.
- A wide variety of secondary metabolites were isolated from sponges and these have been associated with antibacterial, antimicrobial, antiviral, antifouling, HIV-protease inhibitory, HIV reverse transcriptase inhibitory, immuno-suppressent and cytotoxic activities.
- In addition to potential anticancer applications, the bioactive compounds of sponges have a myriad of activities ranging from antibiotic activity including anticoagulant, antithrombin, anti-inflammatory, as well as imunomodulatory activities.
- Chemicals found in sponges may be used to treat yeast and fungi.
- The fact that the psammaplins have been isolated from a diversity of sponge “sources” and that brominated aromatic amino acid derivatives are common

in marine bacteria suggests that these metabolites may actually derive from biosynthetic pathways of microorganisms living in association with sponges.

Presence of specific symbiont morphologies of bacteria within specific sponges has been reported. These specific bacteria, which live symbiotically with sponges, passed through their feeding chambers without being digested. This suggested some sort of encapsulation or recognition process.

- In the demosponge, *Halichondria panicea,* an association with the microbe *Pseudomonas insolita* was suggested to be lectin-based. In the case of halichondrin, the exciting anticancer potential of this "sponge" metabolite has fueled an innovative chemical synthesis approach.
- Marine animals have yielded cardiovascular-active substances, and these include histamine and N-methylated histamines of sponges, viz. *Verongia fistularis*; asystolic nucleosides from the sponge, *Dasychalina cyathina*; and the nucleoside, spongosine, isolated from *Cryptotethya crypta*.

### 5.1.7. Sea Anemones

In a 1977 conference on "Drugs and Food from the Sea : Myth or Reality," "researchers described cardiotonic **polypeptide** s from sea anemones.

- The sea anemone, *Anemonia sulcata* is a well-known natural source of supply of biologically active poly **peptide** s. So far, five toxins, ATX I, II, III, IV and AS V, several polyvalent protease inhibitors, an elastase inhibitor, two blood pressure-depressive polypeptides have been isolated from it.
- The sea anemone toxins (especially toxin II of *A. sulcata*, ATX II) are very important tools in neurophysiological and pharmacological research.
- **5.1.8. Ascidians**
- Ascidians synthesize bioactive substances which are **cytotoxic**. Samples of the species *Cystodytes dellechiajei*, *Euherdmania* sp., and an unidentified species belonging to the Holozoidae family were extracted in methanol 5:1 (v/w). The extracts were tested for cytotoxicity using the brine shrimp lethality assay, sea urchin egg development assay, hemolysis assay.

### 5.1.9. Tunicates

Rinehart *et al*. (1981) have described antiviral and antitumor depsipeptides from a Caribbean tunicate.

- The extracts prepared from the sea squirt of the family Didemnidae, inhibit growth of DNA and RNA **viruses** , as well as L1210 leukemic cells.
- The tunicate of the *Trididemnum* genus, when extracted with methanol-tolulene (3:1), showed activity against herpes simplex virus, type I, grown in

CV-1 cells (monkey kidney tissue), indicating that the extract inhibited the growth of the virus.

- This antiviral activity may also involve antitumor activity. When tested against other viruses, essentially all extracts of the tunicate collected at a number of sites showed activity in inhibiting both RNA and DNA viruses. The suggestion that the extracts might also have antitumor properties was evidenced from their high potency against L1210 murine leukemic cells.
- Didemnin B, a cyclic antiproliferative depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum*, was the first marine natural product to enter clinical trial as an antitumor agent. It showed antitumor activity against a variety of models and has been investigated in phase II clinical trials for the treatment of breast, ovarian, cervical, myeloma, glioblastoma/astrocytoma, and lung cancers. Didemnin B inhibits the synthesis of RNA, DNA, and **protein** s and binds noncompetitively to palmitoyl protein thioesterase.
- **5.1.10. Sea Hares**
- In the early 1970s, the extremely potent anticancer properties were reported from the extracts of sea hare *Dolabella auricularia*. However, due to the vanishingly small abundance of the active principle ( ~ 1.0 mg/100 kg of collected organism), the structure elucidation of dolastatin 10 took nearly 15 years to complete. The low concentrations of dolastatin 10 in sea hares implicates a cyanobacterial diet as the origin of this bioactive secondary metabolite, and this was subsequently confirmed by direct isolation of dolastatin 10 from field collections of the marine cyanobacterium *Symploca*.

### 5.1.11. Marine Toxins

A toxin is a substance possessing a specific functional group arranged in the molecule (s) and showing strong physiological activity. A toxin has the potential to be applied as a drug or pharmacological reagent. Furthermore, even if direct use as a drug is not feasible because of potent of harmful side effects, the toxin can serve as a model for synthesis or improvement of other drugs. Many attempts have been made to develop useful drugs from the sea by screening for anticarcinogenic, **antibiotic** , growth-promoting (or inhibiting), hemolytic, analgetic, antispasmodic, hypotensive, and hypertensive agents. Marine toxins show great promise not only as pharmacological reagents, but also as models for the development of new synthetic chemicals.

- **Tetrodotoxin,** the main action of which is paralysis of peripheral nerves, is a valuable pharmacological reagent. Because it inhibits specifically the sodium permeability of nerve membranes, it has been valuable for elucidating the excitation mechanism.
- Insecticide developed from **nereistoxin** are widely marketed. Fishermen are familiar with the fact that flies die when they come into contact with the dead marine annelid, *Lumbrinereis* (*Lumbriconereis*) *brevicirra*, commonly

used as bait. A new insecticide was developed from nereistoxin that was active against the rice stem borer and other insect pests, it does not appear to be toxic to warm blooded animals, and resistant **strain** s of insects do not readily develop.

- Recently **ciguatoxin, palytoxin** and **halitoxin** have also been investigated and provide interesting new information.
- Ciguatera is a human disease caused by the ingestion of a wide variety of coral reef fishes that contain toxins accumulated via the marine food web. The principal toxin of ciguatera poisoning is a heat-stable, lipid-soluble compound named ciguatoxin. Origin of the toxin is not yet fully understood but may be derived by the fish from ingestion of toxic tropical red tide dinoflagellates such as *Pyrodinium bahamense*.
- Halitoxin, a toxic complex of several marine sponges of the genus *Haliclona*, has been isolated, partially purified, spectrally characterized, and chemically degraded, yielding a proposed chemical structure for the toxin. The toxin has proved to be a complex mixture of high molecular weight and toxic pyridinium salts, and can be isolated from the sponges, *Haliclona rubens*, *H. viridis*, and *H. erina*. The sponge extracts are toxic for fish and mice.
- **Lophotoxin** , a new neuromuscular toxin isolated from several Pacific gorgonians of the genus *Lophogorgia*, has been isolated and purified. Originally discovered during a search for chemical defense adaptations of marine organisms, a variety of horny corals or gorgonians (sea fans and whips) in tropical or subtropical waters were studied; and **cytotoxic** , ichthyotoxic and antibacterial activity was noted. Lophotoxin inhibits nerve-stimulated contraction without affecting contraction evoked by direct electrical stimulation of the muscle. The data suggest that epoxylactone and furanoaldehyde groups may be responsible for the potent biological properties of lophotoxin.
- **Palytoxin** , an extremely poisonous, water-soluble substance from marine coelenterates belonging to the genus Palythoa. Palytoxin influences calcium and potassium ion transport in nerves and the heart. Animals undergo paralysis and heart failure. Palytoxin is synthesized by a marine *Vibrio* sp. growing symbiotically with the coelenterate *Palythoa* and apparently related to *Vibrio cholerae*. Toxins from marine animals that are well known have been summarized and are listed in Table 1.

Several marine organisms have provided useful drugs : liver oil from some fish provides excellent sources of vitamins A and D; insulin has been extracted from whales and tuna fish; and the red alga, *Digenia simplex*, has long been used as an anthelminthic.

**Table 1. Marine Toxins**

| Toxin | Source | Dose and Route | Mechanism of action | Comment |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Cephalotoxin | *Octopus vulgaris* | 150-300mg subcutaneous (in the dog) | Lowers blood pressure, arrests heart and respiration; neurotoxicologically similar to saxitoxin, tetrodotoxin | Human deaths have occurred from octopus bites |
| Ciguatoxin | Coral reef organisms | | Neurotoxic effects; nausea, vomiting, diarrhea; recovery after several weeks | Occurs in moray eel, barracuda, several fish species off Ryukyu Islands |
| Halitoxin | *Haliclona viridis* | 270mg interperitoneal (in the mouse) | Blocks nerve-muscle junction; effective against cancer cells | |
| Holotoxin | *Holothuria tubulosa* | 5-15mg intravenous (mouse) | Cardiotoxic | A toxin of the sea cucumber |
| Nereistoxin | *Lumbriconereis heteropoda* | 33 mg intravenous (mouse) | Causes muscle paralysis, halts respiration | Used as an insecticide in Japan |
| Palytoxin | *Palythoa spp.* | 100µg intraperitoneal (mouse) | Constricts coronary artery | Effect in man occurs upon ingestion of the file fish |
| Saxitoxin, | *Saxidomus giganteus* | 10µg intraperitoneal (mouse) | Causes neuromuscular junction paralysis | Effect in man occurs upon ingestion of clams and muscles. |
| Tetrodotoxin | Puffer fish | 8-20µg gastrointestinal (mouse) | Neurotoxic; causes respiratory paralysis | Used in clinical trials for pain in neurogenic leprosy |

**Unit 6: Environmental Biotechnology**

**Chapter 1: Waste water treatment**

**6.1.2.Effect of aquaculture wastewaters**

The major impact on the receiving water bodies are eutrophication, silting, oxygen depletion and toxicity of ammonia and sulfide. High organic load increases the oxygen demand in water bodies. This eventually reduces dissolved oxygen levels in aquaculture systems. The urine and faeces from the aquatic animals can cause high content of ammonia nitrogen and an increase of BOD (biochemical oxygen demand). Ammonia is the main nitrogenous waste that is produced by fish via metabolism and is excreted across the gills. Nitrite is a naturally occurring intermediate product of the nitrification process. The nitrate ion ($NO3^-$) is the most oxidized form of nitrogen in nature and is relatively non-toxic to fishes. However, when nitrate concentrations become excessive and other essential nutrient factors are present, eutrophication and associated algal blooms can become a serious environmental problem.

Excess nitrogen and phosphorous content lead to eutrophication and algal bloom, especially of toxic species produced by high levels of nutrients. This can cause environmental hazards including mortality of fish and severely reducing water quality.

### 6.1.3. Treatment of aquaculture wastewater

#### 16.1.3.1. Removal of organic matter

Removing of organic matter from wastewater can be accomplished by two main processes that are aerobic and anaerobic. Aerobic process is suitable for the wastewater if the concentration of BOD is less than 1000 mg/l and anaerobic process is suitable if the concentration of BOD is more than 1000 mg/l.

#### 6.1.3.2. Oxidation and synthesis

Oxygen for above reaction is supplied from the air by air diffuser or surface aeration in which surface aeration is preferred in aquaculture pond. The function of aeration is to supply the oxygen for aqua-livings and microorganism to decompose organic matter.

#### 6.1.3.3. Nitrogen removal

Ammonia is the principal excretory product of most aquatic organisms. Inputs of ammonia cannot be eliminated from the water body. But ammonia is toxic, acutely and chronically, to fish and invertebrates. Ammonia should be maintained below 0.1 mg/l (total ammonia). The most efficient way to do this is by the establishment of a **biological filter**. **A biological filter is a collection of naturally occurring bacteria, which oxidize ammonia to nitrite, and other bacteria, which then convert nitrite to nitrate.**

Nitrite is formed either by the oxidation of ammonia (nitrification) or the reduction of nitrate (denitrification).

Nitrite is toxic to fish and some invertebrates and should be maintained below 0.1 mg/l.

Nitrate is the end product of nitrification. The vast majority of aquaculture ponds accumulate nitrate as they do not contain a denitrifying filter. In **gene** ral, nitrate should be maintained below 50 mg/l (measured as $NO_3$-N) but it is not a critical water quality factor. The most common ways to reduce nitrate are water changes and growing live plants.

- The **enzymes** namely Ammonia monooxygenase (AMO) and Hydroxylamine oxidoreductase (HAO) are involved in the oxidation of ammonia to nitrite.
- *Nitrobacte* r sp. is facultatively mixotrophic and capable of growing anaerobically with nitrate as electron acceptor, producing nitrite, nitric oxide and nitrous oxide and then to nitrogen gas.

### 6.1.3.4. Nitrification

Nitrification involves the two step conversion of ammonia to nitrite and nitrite to nitrate by autotrophic aerobic microorganisms which are *Nitrosomonas* sp. and *Nitrobacter* sp. The process for ammonium oxidizing bacteria is

*Nitrosomonas*

$$NH_4^+ + {}^3/_2O_2^+ \rightarrow NO_2^- + 2H^+ + H_2O + \text{Energy}$$

*Nitrobacter*

$$NO_2^- + 1.5O_2^+ \rightarrow NO_3^- + \text{energy}$$

Both *Nitrosomonas* sp. and *Nitrobacter* sp. are chemoautotrophic and obligate aerobes. Thus, they require no organic growth factors and are capable of growing in completely inorganic media using carbon dioxide as the sole source of carbon. The inorganic energy sources for the two species are $NH_3$ and $NO_2$ respectively.

**Autotrophic denitrification**

An alternative to heterotrophic biological denitrification is autotrophic denitrification which uses inorganic substance as electron donor, these substance include hydrogen and sulphur which utilize inorganic carbon compounds ($CO_2$ , $HCO_3$) as their carbon source.

Autotrophic denitrification with sulphur uses *Thiobacillus denitrificans*. This bacterium can reduce nitrate to nitrogen gas while oxidizing elemental sulfur or

reduced sulphur compounds ($S^{2-}$, $S_2O_3^{2-}$, $SO_3^{2-}$) to sulphate, thereby eliminating the need for organic compounds.

**3. Phosphate removal**

Phosphorus is released from bacterial biomass in the anaerobic stage and is assimilated by these bacteria in excess as polyphosphate (Poly P) during the aerobic stage.

In the aquaculture systems, stable ortho phosphate concentrations were found throughout the culture period. Phosphorus immobilization took place in the anoxic treatment stages of the system where it accumulated to up to 19 % of the sludge dry weight.

**6.1.3.5.Denitrification**

Biological denitrification occurs naturally when certain bacteria use nitrate as terminal electron acceptor in their respiratory process, in the absence of oxygen. Denitrification consists of a sequence of enzymatic reaction leading to the evolution of nitrogen gas. The process involves the formation of number of nitrogen intermediates and can be summarized as follows

$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$

**6.1.3.5.1.Heterotrophic denitrification**

Under oxygen-limited or anoxic conditions, denitrification is usually realized by heterotrophic bacteria in the presence of a suitable electron donor. Electron donors that are often used include

- COD in the influent wastewater

- the COD produced during endogenous decay

- an exogenous source such as acetate, methanol and ethanol.

**6.1.3.5.2.Autotrophic denitrification**

An alternative to heterotrophic biological denitrification is autotrophic denitrification which uses inorganic substance as electron donor, these substance include hydrogen and sulphur which utilize inorganic carbon compounds ($CO_2$ , $HCO_3$) as their carbon source.

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### 6.1.3.6. Phosphate removal

Phosphorus is released from bacterial biomass in the anaerobic stage and is assimilated by these bacteria in excess as polyphosphate (Poly P) during the aerobic stage.

In the aquaculture systems, stable ortho phosphate concentrations were found throughout the culture period. Phosphorus immobilization took place in the anoxic treatment stages of the system where it accumulated to up to 19 % of the sludge dry weight.

### 6.1.4. Recent studies on treatment of aquaculture waste water

Recently, the concerns of treatment of aquaculture waste water has been increased. So aquaculture wastewater must be treated properly and recirculated back to the system.

- Removal of organic matter and nitrogenous substance in aquaculture wastewater by combining both aerobic and anaerobic biofiltration for nitrification and denitrification in an aquaculture unit with an aerobic trickling filter (for nitrification) and two anaerobic fluidized bed columns (for denitrification) can be done.
- Carbon source for denitrification is the organic carbon produced in the fish culture units (fish feces and unutilized feed) and external organic compound (methanol).
- The maximum removal rate of ammonia by trickling filter was 0.43 g $NH_4$ – N/ $m^2$ / day and maximum nitrate removal rates was around 432 g $NO_3$ – N / $m^2$ / day.

Treatment of aquaculture wastewater can be accomplished by **construct** ed **wetland**. The studies have demonstrated that constructed wetlands can efficiently remove the major pollutants from

- catfish, shrimp and milkfish pond effluents,
- including suspended solids, organic matter, nitrogen, phosphorus and phytoplankton.
- Accordingly, a constructed wetland was technically and economically feasible for managing water quality of an intensive aquaculture system.
- It can improve the water quality and provide a good culture environment.

### 6.1.1 Characteristics of aquaculture wastewater

In aquaculture system, especially in extensive culture the primary source of nitrogen and phosphorous in the pond water is derived from metabolic waste and uneaten feed.

- Only about 30% feed N and P are retained by salmonid fed, even if they consume all of the feed fed.
- The pollutant load discharged into the environment from aquaculture systems has been calculated and found that one ton of produced fish **gene** rates 0.8 kg of nitrogen/day and 0.1 kg of phosphorous/day.
- In intensive shrimp culture, 11.56% nitrogen and 14.11% phosphorous of nutrient input remained in water body; 19% and 36.21% accumulated in sediment.
- Shrimp stocking densities of 30-50/$m^2$, the average harvest of 5 tons to 6 tons/crop would require 10-12 tons of feed, assuming a food conversion ratio of 2.
- However, only about 20% of the feed is incorporated into shrimp biomass, so approximately 8-10 tons of feed ends up as uneaten food and excreted matter of shrimp.

### 6.1.5. Bioremediation in aquaculture systems

The recent approach to improve water quality in aquaculture is the application of microbes/ **enzymes** to the ponds, known as ‘bioremediation’.

- Bioremediation involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and get rid of undesirable waste compounds and there by toxic effect.
- When macro and micro organisms and/or their products are used as additives to improve water quality, they are referred as bioremediators or bioremediating agents (Moriaty, 1998).
- The isolation and development of indigenous bacteria are required for successful bioremediation.
- The administration of beneficial bacteria in the culture water has emphasized two advantages: bioremediation for controlling water quality and biocontrol with the goal of being antagonistic to pathogens.

#### 6.1.5.1. Bioremediation of organic detritus

The dissolved and suspended organic matter contains mainly carbon chains and is highly available to microbes and algae. A good bioremediator must contain microbes that are capable of effectively clearing carbonaceous wastes from water. Additionally, it helps if these microbes multiply rapidly and have good enzymatic capability.

- Members of the genus *Bacillus, B. subtilis, B. icheniformis, B. cereus, B. coagulans,* and of the genus *Phenibacillus, P. polymyxa,* are good examples of bacteria suitable for bioremediation of organic detritus. However, these are not normally present in the required amounts in the water column, their natural habitat being the sediment. When certain *Bacillus* **strain** s are added to the water in sufficient quantities, they can make an impact.They compete with the bacterial flora naturally present for the available organic matter, like leached or excess feed and shrimp faeces.
- As a part of bio-augmentation, the *Bacillus* can be produced, mixed with sand or clay and broadcasted to be deposited in the pond bottom.
- *Lactobacillus* is also used along with *Bacillus* to break down the organic detritus. These bacteria produce a variety of enzymes that break down **protein** s and starch to small molecules, which are then taken up as energy sources by other organisms. The removal of large organic compounds reduces water turbidity.

### 6.1.5.2. Bioremediation of Nitrogenous compounds

Nitrogen applications in excess of pond assimilatory capacity can lead to deterioration of water quality through the accumulation of nitrogenous compounds (e.g., ammonia and nitrite) with toxicity to fish and shrimp.

- Bacteriological nitrification is the most practical method for the removal of ammonia from closed aquaculture systems and it is commonly achieved by setting of sand and gravel bio-filter through which water is allowed to circulate.
- The **ammonia oxidizers** are placed under five **gene** ra, *Nitrosomonas, Nitrosovibrio, Nitrosococcus, Nitrolobus* and *Nitrospira,* and **nitrite oxidizers** under three genera, *Nitrobacter, Nitrococcus* and *Nitrospira.*
- There are also some heterotrophic nitrifiers that produce only low levels of nitrite and nitrate and often use organic sources of nitrogen rather than ammonia or nitrite.
- Nitrifiers in contaminated cultures have been demonstrated to nitrify more efficiently.
- Nitrification not only produces nitrate but also alters the pH slightly towards the acidic range, facilitating the availability of soluble materials.
- The vast majority of aquaculture ponds accumulate nitrate, as they do not contain a denitrifying filter.
- Denitrifying filters helps to convert nitrate to nitrogen. It creates an anaerobic region where anaerobic bacteria can grow and reduce nitrate to nitrogen gas. Nitrate may follow several biochemical pathways following production by nitrification.

$$NO_3 \rightarrow NO_2 \rightarrow NO\ N_2O \rightarrow N_2$$

Unlike the limited species diversity of bacteria mediating nitrification, at least 14 genera of bacteria can reduce nitrate. Among these, *Pseudomonas*, *Bacillus* and *Alkaligenes* are the most prominent numerically.

### 6.1.5.3. Bioremediation of Hydrogen Sulphide

Sulphur is of some interest in aquaculture because of its importance in anoxic sediments.

- In aerobic conditions, organic sulphur decomposes to sulphide, which in turn get oxidized to sulphate.
- Sulphate is highly soluble in water and so gradually disperses from sediments.
- Sulphide oxidation is mediated by micro organisms in the sediment, though it can occur by purely chemical processes. Under anaerobic conditions, sulphate may be used in place of oxygen in microbial metabolism. This process leads to the production of hydrogen sulphide gas. The $H_2S$ is produced by a series of microbially mediated reductions.

$SO_4^{2-} + 4H_2 + 2H\ H_2S + 4H_2O$

Organic loading can stimulate $H_2S$ production and reduction in the diversity of benthic fauna.

- $H_2S$ is soluble in water and has been suggested as the cause of gill damage and other ailments in fish.
- Unionised $H_2S$ is extremely toxic to fish at concentrations that may occur in natural waters as well as in aquaculture farms. Bioassays of several species of fish suggest that any detectable concentration of $H_2S$ should be considered detrimental to fish production.

The photosynthetic benthic bacteria that break $H_2S$ at pond bottom have been widely used in aquaculture to maintain a favourable environment.

- They are purple and green sulphur bacteria that grow at the anaerobic portion of the sediment-water interface. Photosynthetic purple non-sulphur bacteria can decompose organic matter, $H_2S$, $NO_2$ and harmful wastes of ponds.
- The green and purple sulphur bacteria split $H_2S$ to utilize the wavelength of light not absorbed by the overlying phytoplankton. The purple and green sulphur bacteria obtain reducing electrons from $H_2S$ at a lower energy cost than $H_2O$ splitting photoautotrophs and thus require lower light intensities for carrying out photosynthesis. The general equation of this reaction is as follows.

$CO_2 + 2H_2S \rightarrow (CH_2O) + H_2O + 2S$

Chromatiaceae and Chlorobiaceae are the two families of photosynthetic sulphur bacteria that favour anaerobic conditions for growth while utilizing solar energy and sulphide. Chromatiaceae contain sulphur particles in cells but Chlorobiaceae precipitate them out.

$S + CO_2 + 3H_2S \rightarrow (CH_2O) + H_2SO_4$

$CO_2 + NaS_2O_3 + 3H_2O \rightarrow 2(CH_2O) + NaS_2O_4 + H_2SO_4$

- The family Rhodospirillaceae is not of any use for $H_2S$ removal as they mainly utilize organic material, such as lower fatty acid, as source of hydrogen. But they can be used as efficient mineralizers at pond bottom as they grow in both aerobic and anaerobic conditions as heterotrophic bacteria even in the dark without utilizing solar energy.

**Rhodospirillaceae**

*Rhodospirillum, Rhodopseudomonas, Rhodomicrobium*

**Chromatiaceae**

*Chromatium,Thiocystis, Thiosarcina, Thiospirillum, Thiocapsa, Lamprocystis, Thiodictyon, Thiopedia, Amoebobacter, Ectothiorhodospira*.

**Chlorobiaceae**

*Chlorobium, Prosthecochloris, Chloropseudomonas, Pelodictyon, Clathrochloris.*

For bioremediation of $H_2S$ toxicity, the bacterium that belongs to Chromatiaceae and Chlorobiaceae can be mass cultured and can be applied as pond probiotic. Being autotrophic and photosynthetic, mass culture is less expensive and the cultured organisms can be adsorbed on to the sand grains and applied so that they may reach the pond bottom to enrich the hypolimnion and ameliorate $H_2S$ toxicity.

### 6.1.5.4 . Bioremediators as disease controlling agents

Most probiotics proposed as biological control agents in aquaculture belong to the Lactic Acid Bacteria (*Lactobacillus, Cornybacterium,* etc.), *Vibrio* (*V. alginolyticus*), *Bacillus,* and *Pseudomonas.*

Beneficial microbes, such as non-pathogenic isolates of *V. alginolyticus* can be inoculated into shrimp culture systems to suppress the pathogenic vibrios like *V. harveyi, V. parahaemolyticus* and *V. splendens* and reduce the opportunistic invasion of these pathogens in shrimps.

The role of bioremediators is presented in Table 1.

**Table.1. Bioremediators and their Role**

| | |
|---|---|
| *Bacillus* sp. | Mineralization and breakage of proteins |
| *Nitrosomonas* sp. | Oxidation of ammonia |
| *Nitrobacter* sp | Oxidation of nitrites |
| *Aerobacter* sp. | Reduction of organic matter |
| *Cellulomonas* sp. | Breakage of plant material |

### 6.1.5.5 Bioremediation of aquaculture effluent using microbial mat

The conventional bioremediation technologies applied to remove the pollutant nutrients are impractical for sensitive areas, gene rally costly to operate for developing countries, often lead to secondary pollution and to incomplete utilization of natural resources.

- Bioremediation using microbial mats is the latest concept.
- Microbial mats are laminated heterotrophic and autotrophic vertically stratified communities typically dominated by cyanobacteria, eukaryotic micro algae like diatoms, anoxygenic phototrophic bacteria and sulphate reducing bacteria.
- The microbial communities convert the organic pollutants into the non – harmful products on a useful time scale.

Bioremediation of shrimp ( *Litopenaeus vannamei*) culture effluent

The treatment concept relies on the immobilization of natural marine microbial consortium on glass wool to mitigate the levels of dissolved nitrogen from a shrimp culture effluent.

The treatment via construct ed microbial mats is a technically feasible method for simultaneously reducing effluent nutrient loading (especially nitrate and ammonia) and for reducing organic loading (especially $BOD_5$) of shrimp culture effluents.

Michel and Garcia (2003) developed an efficient *ex-situ* bioremediation method for shrimp (*L. vannamei*) culture effluent using constructed microbial mat.

Collection and construction of microbial mat

- The natural microbial mats were collected from the fish / shrimp culture ponds.
- The collectors were used for collecting microbial mat in semi- intensive pond using substrate of polyester membrane.

- The collected mats were kept vertically suspended in the water column at different representative sites in the pond.
- After 35 days, the fully colonized collectors were recovered and placed in properly sealed polyethylene plastic bags in an oxygen rich atmosphere at $27^{o}$C and transferred to the laboratory.
- The species components of the different microbial mats were analyzed. The microbial mat was constructed by different immobilization methods like glass wool, plastic mesh sheet and low density polyester support. The constructed mats were inoculated in a two different prototype bioreactors .

Inoculation of microbial mat

The constructed mats of three different immobilized supports were suspended in these two different prototype bioreactors, where in aquaculture effluent waste water was inoculated.

- The initial water quality parameters such as dissolve oxygen, ammonia nitrogen ($NH_3$ – N), Nitrite nitrogen ($NO_2$-N), Nitrate nitrogen ($NO_3$ –N) and Phosphate phosphorus ($PO_4$ –P) can be analyzed.
- After 35 days of inoculation, the final water quality parameters such as dissolved oxygen, ammonia nitrogen ($NH_3$ – N), Nitrite nitrogen ($NO_2$-N), Nitrate nitrogen ($NO_3$ –N) and Phosphate phosphorus ($PO_4$ –P) can be analyzed.
- The concentration of ammonia nitrogen, nitrate nitrogen and $BOD_5$ will be decreased significantly in the microbial mat treatment when compared to the control. Nitrifying activities in the reactors will be increased significantly through 20 days.
- The photoautotrophic (top of the mat) and heterotrophic community will be dominated by diatoms (*Nitzchia* sp. and *Navicula* sp.) and filamentous forms of cyanobacteria (*Microcoleus chthonoplastes*, *Spirulina* sp., *Oscillatoria* sp., *Schizothrix* sp., *Calothrix* sp. and *Phormidium* sp.) as well as green algae comprised by *Chlorella* sp. and *Dunaliella* sp.
- Mixed microbial mats containing filamentous cyanobacteria (*Oscillatoria* sp.) as the dominant species have been shown to remove nitrogenous compounds and other toxic chemicals from polluted sites.
- The efficiencies of 97% of ammonia nitrogen and 95% of nitrate nitrogen removal will be observed over a 20 days period of culture.

Microbial mats are able to degrade a variety of organic compounds, including those found in fish wastes. Mats spontaneously form in estuarine and fresh water environments and can be easily cultured for various specialized uses. Microbial mats are rich in nitrogen and can possibly be used as feed for young tilapia and thus if tilapia are fed microbial mats there is the possibility of a complete recycling of all nitrogen and carbon.

Advantages associated with the constructed microbial mat

1. The isolated microorganisms required for the construction of the mats attach effectively to the polyester carrier

2. The wastewater and faeces of the shrimp are suitable substrates for the development of nitrification in the continuous and self-sufficient growth of indigenous microorganisms in the mats

3. Additional oxygen can be supplied to the water column by the photosynthetic components of the mats for the degradation of the organic matter and the nitrification process;

4. The use of the constructed microbial mats represents an effective and economical *in situ* model of environmentally clean land-based shrimp production and represents an additional source of nutrition for *L. vannamei*. The advantages of this support over other immobilization supports (e.g., polymeric matrices, bead) include a lower cost, easy operation, and durability. The water exchange can be reduced without sacrificing growth or survival and, consequently, the total amount of nutrients and BOD discharged into adjacent water bodies may be minimized.

Bioremediation using aquatic plants

· Seaweed (*Gracilaria fisheri),* is capable of assimilating $NH_3$, $NO_2$, $NO_3$ and $PO_4$ from shrimp-farming effluents.

· Other seaweed, such as, red seaweed *(Gracilaria salicornia),* green seaweed *(Caulerpa macrophysa),* and brown seaweed *(Sargassum polycystum),* also assimilate waste nitrogen ($NH_3$ and $NO_3$-) from shrimp pond effluent efficiently.

· The maximum absorption rates of all seaweeds were found within the first 24 h of experiments with 1 g/l stocking density. *C. macrophysa* has higher growth rate as well as higher efficiency than the other two species

· The Red algae *Gracilaria lameneiformis* has high nutrient bioremediation efficiency and assimilative capacity, and its co – culture with fish could be an effective measure to reduce nutrient loading in coastal fish culture.

· Besides seaweed, fresh water aquatic plants such as coontail *(Ceratophyllum demersum)* and Duck weed (*Lemna* sp.) can efficiently assimilate ammonia nitrogen.

### 6.1.5.6. Wastewater related from seafood processing plant

**Liquid waste**

The large amount of liquid waste is preferably treated in a pond or aerated lagoon systems. Problems have been observed in some of these wastewater treatment plants with the appearance of a red colour (photosynthetic bacteria) and bad smell.

- The discharge of red effluent into streams would most certainly have an impact on the quality of natural waterways or reservoirs.
- Use of activated sludge is becoming more popular for treatment.
- Dissolved air flotation has also been implemented in some factories before the activated sludge to remove oil, grease and **protein** .

**Clean technologies based on the use of microorganisms**

The most useful microbial process for the treatment of the liquid waste is the anaerobic digester system.

- The effluent from the biodigester may then be transferred into a shallow pond for the production of the alga *Chlorella* and photosynthetic bacteria.
- Both microorganisms have a high chlorophyll, carotenoid, protein, vitamin and fatty acids content, and therefore make an excellent feed for fish production in a second deeper pond, or could be sold to the large shrimp-farm industry.
- Liquid waste also contains a large variety of **enzymes** , in particular proteases, which could be recovered by simple biochemical purification steps.

Fish processing waste is one of the richest resource materials for further enzymatic and microbial process development. The introduction of all or part of the mentioned processes would not only eliminate the problems in the presently used wastewater treatment methods but would also undoubtedly raise the economic benefits, viability and sustainability of the industry in a clean ecological environment.

## Chapter 2: Biofilters in aquaculture

### 6.2.1.Introduction

Biofilters are devices to culture microorganisms that will perform a given task. Different types of organisms will perform different tasks. Designing and using biofilters is to create an environment that will promote the growth of the organisms that are needed.

Biofilters are used

- to maintain water quality in recirculating or closed systems and
- to improve water quality before water is discharged from a facility.

- It is however, a very important and essential component especially for recirculating aquaculture or aquarium systems.

Biofilters perform the following functions. The first three functions are performed by biological means and the last four are done by physical processes that do not depend on living organisms.

**Biological functions**

1. Remove ammonia

2. Remove nitrites

3. Remove dissolved organic solids

**Physical functions**

4. Add oxygen

5. Remove carbon dioxide

6. Remove excess nitrogen and other dissolved gasses

7. Remove suspended solids

In **gene** ral, there are three types of aerobic microorganisms that colonize biofilters for aquaculture.

a) **Heterotrophic bacteria** utilize the dissolved carbonaceous material as their food source.

b) ***Nitrosomonos* sp.** bacteria utilize ammonia as a food source and produce nitrite as a waste product.

c) ***Nitrospira* sp.** utilize nitrite as a food source and produce nitrates as a waste product.

*Nitrosomonos* and *Nitrospira* which grow relatively slow will grow and colonize the biofilter as long as there is a food source available. Heterotrophic bacteria grow about 5 times faster and will out compete the other two types for space if food is available. Since most aquaculture biofiltration systems are designed for the purpose of converting and removing ammonia from the water this presents a problem.

There are three ways to deal with this problem.

i) The first is to remove most of the carbonaceous BOD ( **biological oxygen demand** ) before the water enters the biofilter.

ii) The second method is to provide sufficient extra capacity (surface area) in the biofilter to allow all of the various bacteria to grow.

iii) Another method is to have a very long plug flow path through the biofilter. This allows different zones of bacteria to establish themselves in different parts of the biofilter.

There are 4 main types of aerobic biofilters and several subcategories of each.

I. Recirculated suspended solids (Activated sludge)

II. Aquatic Plant Filters

i) Unicellular (Microscopic)

ii) Multicellular (Macroscopic)

III. Fluidized Bed Filters

i) Sand Filters

ii) Bead Filters

IV. Fixed film

i) Rotating Biological Contactors (RBC)

ii) Trickling Filters

iii) Submerged Filters (with or without aeration)

a) Up flow

b) Down flow

c) Horizontal flow

d) Moving Bed

**Anaerobic filters**

Anaerobic filters can also be defined as biofilters but they are never the main biofilter used for maintaining water quality in the culture system.

**Disadvantages**

i) They are not capable of effectively cleaning the water to the level required.

ii) They operate too slowly.

**Advantages**

i) Convert the nitrates into $N_2$.

ii) Best suited for processing high strength waste.

- The sludge produced by the physical filter system is an example of a high strength waste.
- Processing plant wastes are another candidate for anaerobic digestion.
- In an integrated production/processing plant these two streams could be combined.
- The best feature of anaerobic systems is the production of *methane*.
- There are specially designed engines that can burn this gas to produce electricity. Using the gas to heat water is another obvious possibility. However, the capital cost of these systems generally limits their use to large operations.
- **6.2.2. General water quality maintenance principles**
- All aquaculture applications have different requirements for biofiltration. Different farmers may grow the same crop under different conditions. The biofilter is one of several components of the system used to maintain water quality. The functions that the biofilter must perform are determined by the presence and effectiveness of other components. Here are some other components and their effects on the system.

### 6.2.2.1. Aeration or oxygenating systems

- Aeration is always the first step when increasing carrying capacity over an open, lightly loaded system.
- Mechanical surface aerators, subsurface air bubblers and pure oxygen injection is the typical progression in terms of technology and complexity.
- All aerobic biofilters require oxygen to operate. If the biofilter does not provide its own oxygen, it will be limited to the oxygen carried in with the water.

### 6.2.2.2. Particulate Filters

- Once sufficient oxygen is provided, the next easiest way to improve water quality is to remove suspended solids.

- This is a more difficult task since particles come in all shapes, sizes and densities.
- Suspended solids consist primarily of uneaten food and feces which are slightly denser than water. Large particles, above 100 microns, will settle out quite easily. Particles above 50 microns can be filtered out with a screen. Particles below 10 microns are difficult to filter and are generally removed by some other means.

There are different types of particulate filters that can remove suspended solids. They generally fall into three broad categories.

- The first type are settling basins, tube settlers, plate settlers, swirl separators and similar systems that allow the particles to drop out of the flowing stream by gravity. They are relatively simple devices and they work well on large particles. Settling systems generally have very low pump head requirements.
- The second type are sand filters, sock filters, drum filters, disk filters, belt filters and similar systems that mechanically remove the particles from a flowing stream. These types of systems "screen" the particles. The size of particle removed is dependent on the size of the screen or sieve. Pump head requirements can vary from low to very high. Some biofilters such as bead filters claim to do both particulate filtration and biological filtration.
- The third type of particulate filter is the floatation or bubble seperator. These are commonly known as **protein** skimmers. In this device, air is bubbled into a column and the fine particles become attached to the surface of bubbles. The resulting froth or foam is collected and removed from the system. These devices require a certain amount of surfactant type compounds in the water in order to work properly. Although they are not typically designed for solids removal, some submerged biofilters will tend to collect fine particles due to the sticky nature of biofilms. This can be both a benefit and a maintenance problem. If the biofilter is not designed for easy cleaning, solids collection can represent a maintenance headache.

Removal of suspended solids is important since suspended solids comprise the majority of the BOD ( **Biological Oxygen Demand** ). The BOD not removed by the particulate filtration system must be removed by the biofilter before effective ammonia removal will occur. Thus the size of the biofilter is influenced by the effectiveness of the particulate filter.

The way that solids are removed is also important. The best systems remove solids quickly without degrading them in any way. If the solid particles are broken or reduced in size, it makes it easier for nutrients to dissolve into the water. These nutrients must then be removed by another part of the water treatment system or flushed out by water exchange. Time is also important because the longer solids are held in the system, the more degradation will occur. Floating bead filters are

particularly bad in this regard since they hold the solids for long periods of time before backflushing.

#### 6.2.2.3. Foam fractionators

Foam fractionators are very useful but sometimes optional pieces of equipment.

- They are good at removing small particles (under 10 microns) and surface active compounds.
- They are sometimes referred to as protein skimmers. Since proteins are nitrogenous compounds that degrade into ammonia, foam fractionators can reduce the load on the biofilters.
- They are definitely useful in systems where water clarity is important.
- Foam fractionators also add oxygen to the water as a secondary benefit.
- Unfortunately, foam fractionators do not always work well in fresh water.

#### 6.2.2.4. Ozone

- Ozone is a powerful oxidizer and sterilant.
- It is potentially harmful to fish, humans and most living organisms. It is definitely harmful to biofilters.
- It is used to improve water clarity and reduce disease transmission.
- Ozone should never be used directly before a biofilter.
- If ozone is used upstream of a biofilter, there should be sufficient retention time after the injection point to insure that no ozone residual enters the biofilter.

#### 6.2.2.5. UV light

- Certain wavelengths of UV (Ultraviolet) light can be used as a sterilant.
- UV light is often used with ozone.
- UV light and ozone are complimentary and synergistic.

#### 6.2.2.6. Carbon dioxide strippers

- Build up of $CO_2$ can be a serious problem in a heavily loaded, intensive recirculating system using pure oxygen.
- The choice of biofilter has a direct influence on the degree to which $CO_2$ is a problem.
- In general, any biofilter other than a trickling filter will have a $CO_2$ problem when pure oxygen is used rather than compressed air for aeration.
- Building a $CO_2$ stripper is not a difficult task but it must be included in the overall design of the system.
- In order to remove $CO_2$, there must be a large interfacial area between air and water. The interfacial area can be increased through the use of

subsurface aeration, mechanical surface aerators, spray systems or packed columns. Subsurface aeration is not very efficient and mechanical surface aerators are difficult to use in an intensive recirculating systems. Spray systems can be big energy users and they are not very efficient either. The best choice for intensive and space limited systems is the packed column. Packed columns can be either cross flow or counter flow systems. Packed columns for $CO_2$ stripping require fans to either force (push) air in or induce (pull) air through the packing.

- **6.2.3. Characteristics of the "Ideal" biofilter**
- The following are the features of a good biofilter.
- 1. Small footprint - The biofilter should occupy as little space as possible. It is common to have culture tanks and the biofilters under cover for protection and temperature control. Space allocated for biofilters takes away area that could be used for culture tanks.
- 2. Inert materials of **construct** ion - All materials used in the biofilters should be non- corrodible, UV resistant, resistant to rot or decay and **gene** rally impervious to chemical attack. In general, marine grade construction materials are required for reasonable working lifetimes.
- 3. Low capital cost - The biofilter must be inexpensive to purchase or build and cheap to transport to the farm location.
- 4. Good mechanical strength - The biofilter and its components must be tough enough to withstand the normal wear and tear of a industrial/agricultural environment.
- 5. Low energy consumption - The energy cost (usually electricity) to operate the biofilters should be as low as possible. The largest energy users are the pumps to move water and compressors to move air.
- 6. Low maintenance requirements - The biofilters should be self cleaning with little or no care required for the normal life of the crop.
- 7. Portability - The biofilters should be easily movable to facilitate changes in operation of the facility.
- 8. Reliability - Ideally the biofilters should have no moving parts that could fail at an inopportune time. If the biofilters does have moving parts, they should be rugged and designed for a continuous operating life of several years.
- 9. Monitorability - It should be easy to observe the operation of the biofilter to insure that it is operating correctly.
- 10. Controllability - It should be easy to change operating variables to assure optimum performance.
- 11. Turndown ratio - The biofilters should be able to work under a wide range of water flow rates and nutrient loading levels.
- 12. Safety - The biofilters should not have any inherent dangers to either the crop or the owner/operator.
- 13. Utility - The biofilters should accomplish all of the goals i.e. removal of ammonia, carbon dioxide, BOD, suspended solids, etc.

- 14. Scalable - A small system should work the same way as a large system. The performance per unit volume should be constant regardless of the size of the system.
- **6.2.4. Characteristics of real biofilters**
- **6.2.4. 1. Activated sludge systems**
- · Activated sludge is defined as a suspension of microorganisms, both living and dead, in a wastewater.
- · Activated sludge systems are not common in aquaculture systems.
- · Air is pumped into the basin to supply oxygen and mixing.
- · Pure oxygen may be supplied as an alternative to air but supplemental mixing is required.
- · Activated sludge systems are good at removing carbonaceous BOD in systems with high nutrient loadings.
- · They are commonly used in domestic waste water treatment systems.
- · Activated sludge systems are typically expensive to operate and do not provide the effluent water quality necessary for aquaculture.
- **6.2.4.2. Aquatic plant systems**
- Plants are not normally used for the primary biofilter in aquaculture systems. They do however provide a very good sink for the nitrates produced by a well functioning biofiltration system. The marriage of recirculating aquaculture systems and hydroponics are a good example of efficient use of resources.
- · In addition to commercially valuable plants grown in hydroponics systems, aquatic plants such as hydrilla, cattails, water hyacinths and duck weed can be used to absorb nitrates and phosphorus from waste water.
- · Unicellular plants (algae, diatoms etc.) are sometimes allowed to grow in the culture tanks.
- · Some species such as tilapia are tolerant of poor water quality and can use the algae as food.
- · Systems operated this way are sometimes called "green water" systems to distinguish them from the clear water systems that many species require.
- · Green water systems can be a very cost effective way to culture certain species but they are not recommended for beginners to aquaculture.
- · Management of these systems requires some experience and specific knowledge.
- **6.2.4.3. Fluidized bed sand filters**
- Regular sand filters such as the type used for swimming pool filters or potable water filters are virtually worthless as biofilters for aquaculture. The biofilm quickly fills the spaces between the grains and the pressure drop across the filter rises rapidly. Frequent back flushing is required and the active biological film is removed each time.
- In contrast, fluidized bed filters have been successfully used for aquaculture applications. A sand filter becomes fluidized when the velocity of the water flowing up through the bed is sufficient to raise the grains of sand up and

separate each grain from its neighbors. In hydraulic terms, the drag on each particle is sufficient to overcome the weight of the particle and the particle is suspended in the stream of water. The velocity required to fluidize the particle is a function of the shape, size and density of the particle.

- **Advantages**
- · They pack more biologically active surface area into a given volume than any other type of biofilter.
- · The best shape for a fluidized bed sand filter is a tall column. Thus they have a small foot print for a given capacity.
- · They are self cleaning and relatively tolerant of different nutrient loadings.
- **Disadvantages**
- · The fluidized bed sand filter has a relatively high energy requirement because of the high pressure drop necessary to fluidize the sand.
- · The pressure required to fluidize the bed varies depending on the amount of biofilm on the sand particle. As the biofilm builds on the sand particle the size of the particle increases while the density of the particle decreases. This means that the depth of the bed will tend to increase as the bed ages. It also means that the bed depth will fluctuate as the loading on the bed varies.
- · In order to prevent blowing the sand out of the tank, the tank must be oversized or the flow of water needs to be regulated.
- · Another potential problem is the uniformity of the water flow. In order to completely fluidize the bed, the water needs to be evenly distributed across the whole bed. Two things can happen if the flow is not uniform. One possibility is that the water will channel and short circuit through the bed. This means that the treatment capacity will plummet. Another possibility is that the short circuit will happen near the wall of the vessel and the abrasive sand will eat a hole through the wall of the vessel.
- Fluidized bed sand filters are limited to the oxygen carried in with the water. This means that the water entering the filter should have a high level of oxygen in order to insure a good level of treatment.

### 6.2.4.4. Bead filters

Bead filters are a relatively new type of biofilter. They are advertised as the complete solution to water quality for recirculating systems.

- They consist of a closed vessel partially filled with small beads of plastic. Usually the vessel is filled with water and the beads float at the top of the vessel.
- Water flows up through the bed of beads. The beads are small enough to trap most large suspended solids.
- In addition, the surface of the beads supports the growth of a biofilm. The small size of the beads means that they have a relatively large surface area per unit volume.

- The more sophisticated systems incorporate a mechanical stirring devices such as a propeller on a shaft.
- Periodically the water flow is shut off and the bed of beads stirred to dislodge the suspended solids.
- The solids are allowed to settle into the bottom of the vessel and then drained off.
- This ability to remove suspended solids and act as a biological filter is the main advantage to bead filters.

The difficulty in successfully operating bead filters lies in striking a balance between the competing functions.

- Too frequent washing to remove solids dislodges the biofilm and disrupts the nitrification process.
- If the beads are not washed enough however, the solids start to plug the bed.
- The other potential problem is the presence of large amounts of carbonaceous solids which tends to encourage the growth of heterotrophs at the expense of *N itrobacter* sp. and *N itrospira* sp.
- Another drawback to bead filters is their relatively high energy consumption due to their high pressure drop. Also, the water flow and pressure drop are not constant. As the bed of beads becomes loaded with solids, the pressure drop rises and the water flow decreases. This leads to cyclic rather than constant performance.

Since bead filters are not aerated, they are limited to the oxygen carried in with the water. In gene ral this is not a problem since retention times are low. Bead filter systems are probably suitable for small, lightly loaded systems where labor costs are low. At this time they are not available for large systems except as multiple units.

### 6.2.4.5. Biodisks or RBC (Rotating Biological Contactors)

· Rotating Biological Contactor units contain circular disks made of Styrofoam, high-density plastic, or other lightweight material.

· Oxygen is transferred from the atmosphere to the exposed film.

· RBC's were first used in domestic sewage treatment applications.

· There are several different types that are manufactured. A typical design consists of plates or disks that are attached to a horizontal shaft. The shaft is located at the surface of the water and it is turned at a very slow speed (1-5 rpm). A series of disks are partially submerged in the wastewater at all times. As they rotate, the biofilm attached to the surface of the disk is alternately exposed to air and then submerged in the water.

· Diffused aeration may be used to enhance performance.

· The process has similarities to trickling filters and the activated sludge process but the biofilm performance is the main feature of the process.

· The original designs used an electric motor to turn the shaft. There is a new design specifically for aquaculture that uses compressed air or pumped water to drive a paddle wheel in the center of the cylinder.

· These RBC's float in the water and do not require bearings or elaborate mechanical supports.

**Advantages**

· offer excellent treatment efficiencies.

· require very little energy to operate and can be located in the culture tank to save space if necessary.

· do not require additional oxygen and are not limited to oxygen contained in the incoming water.

· can remove dissolved BOD or ammonia depending on nutrient levels.

· They are biologically robust and handle shock loads well.

· It is easy to observe their operation and visually monitor the biofilm.

**Drawback**

· They only have one major drawback besides cost and that relates to reliability.

· If there is a power failure or the cylinder stops turning for any reason, the biofilm exposed to the air can dry out.

· When this happens, the cylinder will be unbalanced and become difficult to turn.

### 6.2.4.6. Trickling filters

Trickling filters are one of the oldest types of biological filters. Trickling filters filled with rock or coal were built in the late 1800's for sewage treatment.

- Trickling filters use a solid support media to provide surfaces on which bacteria grow and accumulate.

- In a trickling filter waste water is sprayed over a rock or synthetic media bed. Waste water is applied on an intermittent basis and collected in a sump underneath the media.
- Bacteria attach and grow on the media.
- Natural air currents supply oxygen.
- In some systems, air is forced into the filter with a fan. However, most filters rely on natural convection and diffusion to move air throughout the filter.

The first step in the design of a trickling filter is to pick the right packing or media. Over the years many different materials have been used for trickling filters but today, the best packing is structured media. Structured media is composed of sheets of rigid PVC that are corrugated and glued together to form blocks.

One of the advantages of structured media is it's flexibility and ease of use. Structured media can be used to build a convenient biofilter without a vessel. Since the vessel is typically the major cost of a biofilter, a biofilter with no vessel can be a real money saver. Structured media can be stacked on a frame work or any flat surface. It can be located over a culture tank or have its own water collecting sump. No sides are required because the packing is self supporting.

The most important requirement in the design of any trickling filter is a good water distribution system at the top. There are two common ways to do this. A pressure spray system with splash guards at the top is probably the simplest. The only drawback is the additional pressure drop required to operate the nozzle. The other system involves the construct ion of a shallow water distribution pan with several gravity flow target nozzles in the bottom of the pan. Here are some typical arrangements for a "vessel-less" trickling filters.

Designing a trickling filter is to balance the competing requirements on the design.

Advantages

- Trickling filters are rugged and easy to operate.
- They have the ability to treat a wide variety of nutrient levels. Properly designed systems can handle solids very well.
- One of the big advantages of a trickling filter is that the water can leave with more oxygen than it entered.
- Because trickling filters have a large - air water interface, they also act as strippers to remove $CO_2$, $H_2S$, $N_2$ or other undesirable volatile gases.

Drawback

- Energy cost required to pump the water to the top of the filter.
- A high narrow filter will save space but take more pumping energy.

- A wide low filter will use less energy but take up more space.
- In order to keep the energy costs to a minimum, the pumping head for the filter should be as low as possible. The maximum plan area covered by the filter is determined by the minimum water loading.
- In order to minimize the floor space used by the filter, the filter should be as tall as possible. The practical limitations are the height of the building, the head limits on the pump and the structural and stability considerations of the vessel.
- A taller filter will have a longer flow path for the water. This means a more complete treatment of the water with each pass.
- Taller filters will have higher specific water loadings. This means better flushing action, more turbulent water films and higher ammonium removal rates.

Trickling filters for industrial applications are sometimes 30 ft. tall. This is not practical for aquaculture systems. In gene ral, trickling filters for aquaculture are between 4 and 10 ft. tall.

### 6.2.4.7. Submerged bed filters

An under gravel filter is a classic down flow submerged bed filter. Submerged bed filters have been used extensively for small scale aquaculture and backyard water feature systems. These filters can be operated in up flow, down flow or cross (horizontal) flow.

The classic (old) systems

- Consisted of gravel with an under drain system.
- An improvement to these systems was the addition of air piping underneath. The air was used to 'bump' the filter to dislodge solids and restore full flow. The problems with these filters is the large size, low void fraction, tendency to plug and extremely high weight. In gene ral, these old gravel based systems are not suitable for modern aquaculture.

Modern submerged bed filters

- Are very efficient, have low head loss and are very easy to build and maintain.
- The key difference is the type of media and the water flow path.
- A modern submerged filter uses structured media in a cross (horizontal) flow mode. This type of biofilter probably comes closer to the ideal biofilter than any other type.

A typical installation would be configured similar to a raceway. The filter media is installed in a long trough. The length of the flow path can vary based on the

retention time required. By using a relatively high velocity, it is possible to insure plug flow. This is a big advantage over well mixed systems or systems with short retention times. If it is not possible to remove the entire BOD before the biofilter, one will establish different zones in the filter. As nutrients are absorbed or removed in the first sections of the filter, different types of organisms will establish dominance in the zones where they enjoy optimum conditions. There are a variety of ways to configure a raceway type system.

Submerged filters can operate with or without aeration. If the flow path is long and the nutrient loading is high, it is wise to have aeration in the filter. One of the easier methods is the traditional aeration system with large silica air stones.

There is always the possibility to install the submerged biofilter media in the culture tank. This has the advantage of saving the cost a separate vessel and associated piping. The big disadvantage to this system is that it is difficult to remove the suspended solids before the water enters the biofilter. Because there are too many different configurations to draw them all, here is a brief description of a few of the possibilities.

1. Air lift the water into one end of a filter designed as a raceway and air lift it back into the culture tank at the other end.

2. Pump the water into a particulate filter such as a rotary drum and then flow through the biofilter.

3. Locate tubes or columns of packing throughout the culture tank and induce a flow through them with air stones.

4. Locate the filter media around the walls of the culture tank and induce a flow up through the media with air stones.

Sometimes it is not possible to use a raceway type biofilter system. If existing tanks must be used, it might be easier to build a system with internal recirculation. The advantage of internal recirculation is that it increases the velocity of water past the media and adds oxygen to the water. Increasing the velocity helps insure a more even distribution of water throughout the filter media and reduces the possibility of dead zones that are not receiving nutrients and oxygen. It also helps to keep particles in suspension. Suspended solids tend to settle out in areas of low water velocity. This is a problem because accumulations of solids can become anaerobic and contribute to poor water quality. Here are a couple of examples of internal recirculation systems. The cone bottom tank is preferred over the flat bottom tank because any solids that settle out will be removed immediately.

### 6.2.4.8. Submerged filters

Submerged filters are excellent choices for small systems because they are very versatile. They can be located in a separate tank or in the culture tank. They can be horizontal flow, up flow or down flow. They can be aerated or not.

· The most important consideration for the design is the even distribution of water to the packing.

· It is very common for submerged filters to be designed as large, flat and thin sections of packing with water direction being up flow or down flow.

· There is typically no provision for distributing the water to all areas of the media.

· The length of the water path through the media is very short and the resistance to flow is very low.

· This is a recipe for disaster. The water flow will short circuit though a small section of the media and the rest of the biofilter will become anaerobic.

· Ideally the flow path through a submerged filter should be as long as possible. A long thin raceway is the best. This type of biofilter is known as a long path, plug flow submerged filter.

· Another possible alternative is the use of aeration to induce a circulating flow around a tank.

· The goal should always be to provide sufficient velocity through the media to insure a fresh supply of oxygen and nutrients to the bugs on the surface of the media.

## Chapter 3:Biofertilizers

### 6.3.1.Introduction

The term 'Biofertilizer' itself means 'Live Fertilizer'. 'Biofertilizer' is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant.

Contain live or latent beneficial microbes which help to

- fix atmospheric nitrogen,
- solubilize and mobilize phosphorus,
- translocate minor elements (Zinc, Copper, etc.,) to the plants,
- produce plant growth promoting **hormone** s, vitamins, **amino acids** and
- control plant pathogenic fungi.

Biofertilizers are extremely advantageous in

- Enriching the soil fertility and fulfilling the plant nutrient requirements by supplying the organic nutrients through microorganism and their byproduct.
- Biofertilizers do not contain any chemicals which are harmful to the living soil.
- Biofertilizers are eco-friendly organic agro-input and more cost effective than chemical fertilizers.
- Biofertilizers like *Rhizobium*, *Azotobacter*, *Azospirillum* and blue green algae (BGA) are in use since long time ago.
- *Rhizobium* inoculant is used for leguminous crops.
- *Azotobacter* can be used with crops like wheat, maize, mustard, cotton, potato and other vegetable crops.
- *Azospirillum* inoculants are recommended mainly for sorghum, millets, maize, sugarcane and wheat.
- Blue green algae belonging to **gene** ra *Nostoc, Anabaena, Tolypothrix* and *Aulosira* fix atmospheric nitrogen and are used as inoculants for paddy crop grown both under upland and low land conditions.
- *Anabaena* in association with water fern *Azolla* contributes nitrogen up to 60 Kg/ha/season and also enriches soils with organic matter.
- Other types of bacteria, so-called Phosphate solubilising bacteria like ***Pantoea agglomerans*** **strain** P5, and ***Pseudomonas putida*** strain P13 are able to solubilize the insoluble phosphate from organic and inorganic phosphate source.
- In fact, due to immobilization of phosphate by mineral ions such as Fe, Al and Ca or organic acids, the rate of available phosphate (Pi) in soil is well below plant needs.
- In addition, chemical Pi fertilizer is also immobilized in the soil immediately so that less than 20 percent of added fertilizer is absorbed by plants. Therefore, reduction in Pi resources, on one hand, and environmental pollutions resulted from both production and applications of chemical Pi fertilizer, on the other hand, have already demanded the use of new generation of phosphate fertilizers globally known as phosphate solubilizing bacteria or phosphate biofertilizers.

The significance of biological nitrogen fixation in aquatic ecosystems has brought out the utility of biofertilization through application of heterocystous blue–green algae and related members. This assumes great importance in view of the increasing costs of chemical fertilizers and associated energy inputs that are becoming scarce as also long– term environmental management.

*Azollae*, a free-floating aquatic fern fixing atmospheric nitrogen through the cyanobacterium. *Anabaena azolla*, present in its dorsal leaves, is one of the potential nitrogenous biofertilizers. Its high nitrogen-fixing capacity, rapid

multiplication as also decomposition rates resulting in quick nutrient release have made it an ideal nutrient input in farming systems.

*Azolla* is a heterosporous fern belonging to the family *Azollaceae* (*Salviniaceae)* with seven living and twenty extinct species. Proliferation of *Azolla* is basically through vegetative propagation but sexual reproduction occurs during temporary adverse environmental conditions with the production of both microsporocarp and megasporocarp.

Biofertilizers add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing phophorus, and stimulating plant growth through the synthesis of growth promoting substances. Biofertilizers can be expected to reduce the use of chemical fertilizers and pesticides.

### 6.3.2. Potentials of *Azolla*

Though *Azolla* is capable of absorbing nitrogen from its environment, *Anabaena* meets the entire nitrogen requirements of *Azolla–Anabaena* association.

- The mean daily nitrogen fixing rates of a developed *Azolla* mat are in the range of 1.0–2.6 kg/ha and a comparison with the process of industrial production of nitrogenous fertilizers would indicate the efficacy of biological nitrogen fixation.
- While the latter carried out by the enzyme nitrogenase, operates with maximum efficacy at 30°C and 0.1 atm, Haber-Bosch process employed by the fertilizer industry requires reaction of nitrogen and hydrogen to form ammonia at a temperature and pressure as high as 300ºC and 200 – 1000 atm respectively.

The normal doubling time of *Azolla* plants is three days and one kg of phosphorus applied results in 4-5 kg of nitrogen, through *Azolla, i.e*. about 1.5 – 2.0 t of fresh biomass.

- *Azolla* can survive in a wide pH range of 3.5 to 10.0 with an optimum of 4.5–7.0
- withstand salinities of up to 10 ppt.
- With a dry weight range of 4.8–7.1% among different species, the nitrogen and carbon contents are in the ranges of 1.96–5.30% and 41.5–45.3% respectively.

Added to these are its high rates of decomposition with mean daily loss rates of 1.36–4.57% of the initial weight and nitrogen release rates of 1.25% which make *Azolla* a potential biofertilizer in aquaculture systems.

*Azolla* is considered as a multifaceted and multipurpose bio-resource. It can be used as:

- green manure specially for rice;
- mosquito-controlling agent;
- Water purifier-capable of absorbing the nutrients from the eutrophic waters;
- poultry and duck feed; fish feed
- source of nitrogen;
- Antifungal agent
- natural biofertiliser; and
- source of hydrogen gas

### *6.3.2.1. Application of Azolla in aquatic system*

- *Azolla* is used in lake-agro ecosystem in inland fisheries and aquaculture, where water quality management is mandatory for prevention of water pollution and harvesting optimum production.
- The high organic loads associated with high BOD and COD results in eutrophication and render the water unsuitable for human and agricultural use.
- *Azolla* is a natural biophysical scavenger that absorbs and depletes the organic nutrient load of the eutrophic lake agro ecosystems rendering them oligotrophic and rejuvenated.
- In the lake agro ecosystem management, *Azolla* is useful in controlling eutrophication, maintenance of water quality and conservation of biological diversity.
- In addition, *Azolla* as already stated, is a powerful bio-agent capable of fixing atmospheric $N_2$ and releasing $H_2$.
- The biomass of the plant is useful for biogas production, as organic manure, poultry feed, and antifungal agent.
- As such, *Azolla* is not a bane but a boon if proper ecosystem management methods and ecofriendly technologies are used for sustainable development of the agro-lake ecosystems benefiting mankind.
- **6.3.2.2. Cultivation of *Azolla***
- It is necessary to cultivate *Azolla* separately for aquaculture and resort to periodic application in fish ponds.
- A system comprises a network of earthen raceways (10.0 x 1.5 x 0.3 m) with facilities for water supply and drainage.
- The operation in each raceway consists of application of *Azolla* inoculum (6 kg), phosphatic fertilizer (50 g single super phosphate) and pesticide (carbofuron dip for inoculum at 1.2 ppm), maintenance of water depth of 5–10 cm and harvesting 18– 24 kg in a week's time.
- The maintenance includes periodic removal of superficial earth layers with organic accumulation, dyke maintenance, application of bleaching powder for crab menace and algal blooms, etc.

- A unit of 0.1 ha area that can hold about 50 raceways is suitable for a family to be taken up as cottage industry in rural areas.
- *Azolla* can be cultured in puddles, drainage and shallow water stretches, at the outlets of ponds and tanks and hence prime agricultural land need not be used.
- **6.3.2.3. Applications in fish farming**
- *Azolla* is useful in aquaculture practices primarily as a nitrogenous biofertilizer.
- Its high decomposition rates also make it a suitable substrate for enriching the detritus food chain or for microbial processing such as composting prior to application in ponds.
- Further, *Azolla* can serve as an ingredient of supplementary feeds and as forage for grass carp too.
- Studies made on *Azolla* biofertilization have shown that the nutrient requirements of composite carp culture could be met through application of *Azolla* alone at the rate of 40t/ha/yr providing over 100 kg of nitrogen, 25 kg of phosphorus and 90 kg of potassium in addition to about 1500 kg of organic matter. This amounts to total substitution of chemical fertilizers along with environmental upkeep through organic manuring.
- It is observed that about 1 ton of *Azolla* biomass could be harvested every week from a water spread area of 650$m^2$, with a phosphorus input-nitrogen output ratio of 1:4.80.
- With an approximate water to land ratio of 1 : 0.5 the total land requirement of such an *Azolla* farm is 0.1 ha. For fertilizing 1ha of water area at the above suggested rate of 40t/ha/yr, about 550$m^2$ of water spread is required (1.5kg/$m^2$/week; 42t/yr) with the total area of 800$m^2$, accounting for 6% if the area to be fertilized.
- Larger production plots (20m x 5m) could be provided reducing the total land area required for mass cultivation. There would however be regional variations depending on the agro-climatic conditions, as some species of *Azolla* give poor yields during low temperatures in winter.
- *Azolla* is a new aquaculture input with high potentials in both fertilization and trophic enrichment. Studies are also being made with regard to reduction of land requirement and production costs through *in situ* cultivation in shallow zones or floating platforms in fish ponds, use of organic inputs like biogas slurry, etc. The costs may be reduced further if the *Azolla* culture system is managed by the farmer or by his household members. The technology would pave the way for economic, eco-friendly and environment – conserving fertilization in aquaculture.
- The suggested application rate of *Azolla* to meet the nutrient requirements in carp polyculture (and substitute for chemical nitrogenous fertilizer) is 40 tons/ha/year. This requires an area of about 800 sq m, a unit that could be taken up as a subsidiary farming activity. Along with the environmental

benefits, a saving of up to 30-35% over the traditional manuring practices is estimated.

## Chapter 4: Probiotics

### 6.4.1 Introduction

The term, probiotic, simply means “for life”, originating from the Greek words “pro” and “bios”. The concept of probiotics was first popularized at the turn of the 20$^{th}$ century by the Russian Nobel laureate Elie Metchnikoff.

Metchnikoff proposed that a normal, healthy gastrointestinal microflora in humans and animals provided resistance against “putrefactive” intestinal pathogens.

The word “probiotic” was introduced by Parker (1974) and defined as probiotics are “organisms and substances which contribute to intestinal microbial balance”.

Fuller (1989) revised the definition as “live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”.

Although application of probiotics in aquaculture seems to be relatively recent, the interest in such environment friendly treatments is increasing rapidly. The use of microbial probiotics in aquaculture is now widely accepted.

At present, a number of probiotics are commercially available and have been introduced to fish, shrimp and molluscan farming as feed additives, or are incorporated in pond water. According to the claims of the producers, these products are effective in supporting the health of aquatic animals and are also safe. On the other hand, there are doubts with regard to the **gene** ral concept of probiotics and to these claims. Thus, there is clearly a need to increase our knowledge of intestinal microbiology and the effective preparation and safety evaluation of probiotics.

### 6.4.2.The use of probiotics in aquaculture

The research on use of probiotics in aquatic animals is increasing with the demand for environment friendly aquaculture. As an alternative strategy to **antibiotic** use in aquatic disease management, probiotics have recently attracted extensive attention in aquaculture. However, the use of terrestrial bacterial species as probiotics for aquaculture has had limited success, as bacterial **strain** characteristics are dependent upon the environment in which they thrive. Therefore, isolating potential probiotic bacteria from the marine environment in which they grow optimally is a better approach.

The common organisms in probiotic products are *Aspergillus oryzae, Lactobacillus acidophilus, L. bulgaricus, L. plantarium, Bifidobacterium bifidium, Streptococcus lactis* and *Saccharomyces cerevisiae*.

Other bacteria that have been used successfully as probiotics belong to the genus *Vibrio* and *Bacillus*, and the species *Thalassobacter utilis.*

These products can be administered through water or incorporated in the feed. Probiotics have been particularly useful in the early stages of larval growth since the gut of the newly hatched larvae is sterile and administering probiotics through water at this stage helps to build up beneficial bacteria much faster than the normal course.

The benefits of the supplements include

- improved feed value,
- enzymatic contribution to digestion,
- inhibition of pathogenic microorganisms,
- antimutagenic and anticarcinogenic activity,
- growth promoting factors, and increase immune response.

Improved water quality has especially been associated with probiotics. It has been reported that use of *Bacillus* sp. improved water quality, survival and growth rates and the health status of juvenile *Penaeus monodon* and reduced the pathogenic vibrios. Recent research also shows that the use of commercial probiotics in *Penaeus vannamei* ponds can reduce concentrations of nitrogen and phosphorus and increase the shrimp yields.

The future application for probiotics in aquaculture looks bright. There is an ever-increasing demand for aquaculture products and a similar increase in the search for alternatives to antibiotics. The field of probiotics intended for aquacultured animals is now attracting considerable attention and a number of commercial products are available, particularly directed at shrimp larval culture.

Probiotics have also been used in a big way as pond cleaners in aquaculture. Probiotic bacteria directly uptake or decompose the organic matter or toxic material and improve the quality of water. The microbial cultures produce a variety of **enzymes** like amylase, protease, lipase, xylanase and cellulase in high concentrations than the native bacteria, which help in degrading waste. These bacteria have a wide range of tolerance for salinity, temperature, pH which usually exist in aquaculture operations.

The use of antibiotics in aquaculture is banned due to rejection of export consignments of marine products. Hence usage of probiotics is propagated to counter the effect of viral and bacterial infections in commercial aquaculture.

The pond probiotics also have a special blend of denitrifying bacteria that remove the algae's primary source of food, nitrogen from the water. This drastic reduction in nitrogen concentration makes it difficult for the algae to bloom. The balance between phytoplankton, zooplankton and beneficial bacteria during culture period play a crucial role in the maintenance of pond health. There is yet no definitive parameter to judge the efficacy of probiotics. A quick and easy microbiological testing kit would be very useful in evaluating pond health on a daily basis. The usage of probiotics has been a subject of intense research all over the world and has been accepted as an alternative for antibiotics.

### 6.4.2.1. Rationale for the use of probiotics in aquaculture

In order to understand the function and potential contribution of probiotics towards health and well-being, in-depth knowledge of the digestive tract as an ecosystem is required. The studies conducted in recent years confirm the importance of microbials in digestive tract. Indeed, a beneficial association of ‘‘lactic acid producing’’ microorganisms, particularly lactic acid bacteria (LAB), with the human host has been suggested more than 100 years ago.

The digestive tract of hydrobionts is an open system constantly contacting with the surrounding environment-water. Compared to water, digestive tract is an ecosystem far richer in nutrients and therefore more favourable for the growth of the majority of bacteria. Gastrointestinal bacteria (GIT) take part in the decomposition of nutrients, provide the macroorganisms with physiologically active materials, such as **enzymes** , **amino acids** , and vitamins.

The study conducted on the aerobic bacteria associated with the GIT of freshwater fish species revealed that selected bacterial **strain** s produced digestive enzymes, thus facilitating feed utilization and digestion. The same beneficial characters of probiotics added inthediet have also been reported.

It has also been proved that micro flora of the digestive tract of partial hydrobionts play an important role in the resistance to infectious diseases since they produce antibacterial materials preventing pathogenic bacteria from getting into an organism.

The initial major purpose of using probiotics is to maintain or re-establish a favorable relationship between friendly and pathogenic microorganisms that constitute the flora of digestive tract or skin mucus of hydrobionts.

Some bacteria used as candidate probiotics also have antiviral effects. Although the exact mechanism by which these bacteria do this is not known. Laboratory tests indicate that the inactivation of **viruses** can occur by chemical and biological substances, such as extracts from marine algae and extracellular agents of bacteria.

In addition, adhesion and colonization of the mucosal surfaces are possible protective mechanisms against pathogens through competition for binding sites and nutrients, or immune modulation.

Multiple ways exist in which probiotics could be beneficial and these could act either singly or in combination for a single probiotic. These include :

- inhibition of a pathogen via production of antagonistic compounds,
- competition for attachment sites,
- competition for nutrients,
- alteration of enzymatic activity of pathogens,
- immunostimulatory functions, and
- nutritional benefits such as improving feed digestibility and feed utilization.

It is often reported that a probiotic must be

- adherent and colonize within the gastro intestinal tract,
- it must replicate to high numbers,
- it must produce antimicrobial substances, and
- it must withstand the acidic environment of the gastro intestinal tract.

The effects of ecological factors on microbial growth and activity are of **gene** ral importance and also apply to the aquatic animals digestive tract flora and feed.

Moreover, microbial food webs are an integral part of all aquaculture ponds and have a direct impact on productivity, even where intensive, artificial feeding is practiced. Therefore, the probiotics prepared with microorganisms have important roles in pond culture, particularly with respect to productivity, the nutrition of the cultured animals, disease control, water quality, and environmental impact of the effluent.

### 6.4.3. Probiotic preparation

The retention of high viability during preparation and storage presents particular challenges and can be regarded as a major bottleneck in commercial probiotic production. This is particularly the case for ‘‘technologically sensitive” **strain** s (for example some LAB species) with the result that most successfully marketed probiotics are usually robust only in nature. Most liquid/frozen probiotic cultures require refrigeration for storage and distribution, thereby adding expense and inconvenience to their widespread use in aquaculture. The survival and viable cell count of probiotic bacteria vary depending on the strains and manufacturers. To maintain confidence in probiotic products used in aquaculture, it is important to demonstrate good survival of the bacteria in products during their shelf life.

Interest in LAB stems from the fact that they are natural residents of the human GIT with the ability to tolerate the acidic and bile environment of the intestinal tract. LAB also function to convert lactose into lactic acid, thereby reducing the pH in the GIT and naturally preventing the colonization by many bacteria. The most widely studied and used LAB are the lactobacilli and bifidobacteria. Other commonly studied probiotics include the spore forming *Bacillus* spp. and yeasts.

### 6.4.3.1 *Bacillus* spp.

The genus *Bacillus* is Gram-positive rods that form a single endospore (spore) and represent a peculiar case among the bacteria used as probiotics.

The species *B. subtilis, B. cereus, B. coagulans, B. clausii, B. megaterium and B. licheniformis*, are used as probiotics.

Due to the physical and biological characteristics of the spore, these preparations (powders or spore suspensions in distilled water) are extremely resistant to the environment and have a prolonged shelf life. Also the cost of production of spores for aquaculture is low with respect to production of purified components. The option to use these organisms as probiotics is made even more feasible by the well-described systems including the specific **plasmid** s available for **genetic engineering** of *B. subtilis*. *Bacillus* spp. possess adhesion abilities, produce bacteriocins (antimicrobial **peptide** s) and provide immunostimulation and can be kept in the spore form and therefore stored indefinitely on the shelf.

### *6.4.3.2. Saccharomyces cerevisiae*

The yeast, *Saccharomyces cerevisiae*, also has been commonly studied whereby immunostimulatory activity was demonstrated and production of inhibitory substances shown.
The stability is also critical to guarantee the efficacy of probiotics and its ability to induce in the host the beneficial effects in the final product formulation.

Therefore, to be effective and confer these health benefits, probiotic cultures must be able to retain their probiotic properties after processing, and with sufficient numbers survived during shel flife/storage.

It is well known that the stability of probiotics is influenced by various factors, including the species, **strain** biotype, water activity, temperature, pH, osmotic pressure, mechanical friction and oxygen. Consequently, special attention and techniques are needed during the resistance of these probiotics against adverse conditions have been proposed, including use of oxygen-impermeable containers, two-step fermentation, stress adaptation, incorporation of micronutrients such as peptides and **amino acids** , and microencapsulation.

### 6.4.4 Safety and evaluation of probiotics

The **gene** ral concept that the use of probiotics in aquaculture may produce various beneficial effects has been proven beyond doubt. The scientific application of this concept to hydrobionts health and disease, although still in its infancy, has already produced some positive results. However, safety considerations neglected for a long time are now taken into account for the development and marketing of probiotics. Safety is the state of being certain that adverse effects will not be caused by an agent under defined conditions.

New species and more specific **strain** s of probiotic bacteria are constantly identified. It cannot be assumed that these novel probiotic organisms share the historical safety of tested or traditional strains. Prior to incorporating them into products, new strains should be carefully assessed and evaluated for both safety and efficacy. In addition, Probiotic manufacturers should apply modern molecular techniques to ensure that the species of bacteria used in their products are correctly identified, for quality assurance as well as safety.
Lactic acid bacteria treated as probiotics are generally considered to be non-pathogenic. However, a growing number of diseases that appeared with the worldwide development of aquaculture may be assigned to distinct bacteria belonging to the genera *Streptococcus, Lactococcus, Vagococcus* and *Cornybacterium*.

Therefore, the safety profile of a potential probiotic strain is of critical importance in the selection process. This testing should include the determination of strain resistance to a wide variety of common classes of **antibiotic** s such as tetracyclines, quinolones and macrolides and subsequent confirmation of non-transmission of drug resistance genes or virulence **plasmid** s. Evaluation should also take the end-product formulation into consideration because this can induce adverse effects in some subjects or negate the positive effects altogether.

### 6.4.5. Prebiotics

The concept of prebiotics in feed is fairly recent. Prebiotics are basically feed for probiotics where they are resistant to attack by **endogenous enzymes** and hence reach the site for proliferation of gut microflora.

Some of the prebiotics, which are currently used in animal feed, are

- Mannan-oligosaccharides (MOS),
- fructo-oligosaccharide and
- mixed oligo-dextran.

Mannan-oligosaccharides are mainly obtained from cell walls of yeasts. Other sources of MOS are copra or palm kernel meal. MOS interferes with the

colonization of the pathogens. Cell surface carbohydrates are primarily responsible for cell recognition. Bacteria have lectins (glyco **protein** ) on the cell surface that recognize specific sugars and allow the cell to attach to that sugar. Binding of *Salmonella, E.coli and Vibrio*sp. is shown to be mediated by a mannose specific lectin like substance present on the bacterial cell surface.

Similarly fructo-oligosaccharides from chicory have been used as prebiotics to competitively exclude pathogenic bacteria. The pH of the lumen gets reduced thus preventing the entry of pathogenic bacteria.

The concept of using prebiotics has not yet been accepted but the advantages of prebiotics are that it can stand high pelletizing temperatures in the feed and also have a long shelf life.

## Chapter 5: Biosensor

### 6.5.1. Introduction

**A biosensor is an analytical device incorporating a bio recognition element intimately associated with or integrated within a transducer that converts the biological response into an electrical signal.**

A great variety of detection techniques can be included in this definition.

- The biological response could be anything from enzyme activity or **antibody** /receptor binding to cell responses.
- The transduction to an electrical signal could also be diverse.

These techniques have been adapted to detect analytes of interest based on the interaction with or functionality modification of a biological target, which could be nucleic acids, **enzymes** , antibodies, receptors, cell organelles or whole cells. The specificity of the detection is determined by the biological component of the method.

For example, a method based on binding to a specific antibody would be very specific, however whole cell-based biosensors usually lack that degree of specificity and that characteristic could be used as an advantage in a broad-spectrum detection/monitoring technique. The sensitivity, on the other hand, as well as the portability of the device, depends on the signal transducer.

Biosensor assays may have mainly two designs, a direct or an indirect format.

- The direct format is based on the detection of analyte binding to a target or being cleaved by an enzyme, for example.

- In the indirect format an additional reaction has to occur in order to detect the analyte, for example the analyte may inhibit the interaction of the biological target with a “reporting element”.

Indirect assays are often used in food analysis because they usually display lower interferences with complex matrixes. Actually, sample preparation is commonly a critical step in method development when working with food samples due to their complexity, and seafood is not an exception. Therefore, attention should be paid not only to the efficiency and sensitivity of the biosensor assay but also to the sample preparation procedure.

Biosensors refer to intact, living cells that have been genetically engineered to produce a measurable signal in response to a specific chemical or physical agent in their environment. Biosensors contain two essential genetic elements, a promoter and a reporter gene. The promoter is turned on when the target agent is present in the cell’s environment. The promoter in a normal cell is linked to other genes that are then likewise transcribed and then translated into proteins that help the cell in either combating or adapting to the agent to which it has been exposed. In the case of a biosensor, these genes, or portions thereof, have been removed and replaced with a reporter gene. Consequently, turning on the promoter now causes the reporter gene to be turned on. Activation of the reporter gene leads to production of reporter proteins that ultimately generate some type of a detectable signal.

Therefore, the presence of a signal indicates that the biosensor has sensed toxic levels of a particular target agent in its environment. Biosensors can be designed to signal levels of pollutants toxic to prokaryotic or eukaryotic systems in general by selecting appropriate host organisms. Bacterial and zebrafish biosensors have been designed and are being used currently as prokaryotic and eukaryotic biosensors, respectively.

### 6.5.2. Advantages (bioavailability, etc) and concept

Biosensors measure the bio-available concentration of the contaminant they are designed to detect. In a biosensor, the expression of a reporter gene is controlled by a genetic regulatory unit (receptor), which responds to the given analyte (heavy metals, genotoxic elements etc), using a receptor–reporter concept. Bioavailability is a critical issue in determining metal toxicity the potential for metal bioaccumulation, and the efficiency of bioremediation of metal-contaminated environments. Regulatory genes responsible for induction of specialized microbial systems can be considered sensitive receptors specifically responding to a target compound at physiologically significant concentrations. Bioluminescence genes (*lux*) have been recognized as a convenient reporter system. Several *lux* operons have been cloned from a number of luminescent bacteria and studied in detail, and their utility as the reporter part of biosensors has been demonstrated for hydrocarbons and mercurial compounds.

The greatest advantage of biosensors is their ability to detect the bio-available fraction of the contaminant, as opposed to the total concentration, which allows for a more accurate assessment of the site and the potential risks involved. Biosensors are also fast, less expensive and less labor intensive than other traditional methods such as atomic absorption spectrometry, inductively coupled atomic electron spectrometry, and sequential extraction procedure. The results obtained from biosensors are compatible with and comparable to chemical analysis, while being free of chemical extractions and analytical procedures. Biosensors can also be more sensitive than chemical methods. They are particularly appealing for field work or *in situ* analysis, since the procedure for using biosensors does not involve the bulky, fragile equipment or specialized training that most analytical methods require.

#### 6.5.2.1. Prokaryotic biosensors

Bacteria can be used as biosensors to demonstrate the toxicity of a variety of environmental media including soil, sediment, and water by coupling bacteria to transducers that convert a cellular response into detectable signals. These bacterial biosensors are engineered by pairing a reporter gene that generates a signal with a contaminant-sensing component that responds to chemical or physical change, such as exposure to a specific analyte. When the biosensor is exposed to such a change, the sensing component stimulates the reporter gene through a biochemical pathway in the cell. The reporter gene then produces a measurable response, such as emitting visible light, which is indicative of the degree of chemical or physical change.

#### 6.5.2.2. Eukaryotic biosensors

It has been shown that transgenic zebrafish can be used as pollution indicators. Using estrogen-inducible and stress–responsive promoters linked to fluorescent reporter genes, Gong Zhiyuan and his team from National University of Singapore have generated transgenic zebrafish that display color when exposed to estrogens and other stressful conditions ( **www.nus.edu** ).

### 6.5.3. Components of a biosensor

#### 6.5.3.1. Receptors

A large number of DNA motifs called response elements located in the regulatory regions of all inducible genes that respond to specific intra- or extra-cellular stimuli have been documented. A signal transduction cascade results in activation of transcription factors that bind to these response elements causing up- or down-regulation of gene expression.

#### 6.5.3.2. Reporters

A reporter gene encodes for a mechanism that produces a detectable cellular response. It determines the sensitivity and detection limits of the biosensor. Specific characteristics are needed for the reporter gene to be used in a biosensor. The gene must have an expression or activity that can be measured using a simple assay and reflects the amount of chemical or physical change. Firefly luciferase is by far the most commonly used of the bioluminescent reporters from the firefly *Photinus pyralis*.

### 6.5.4. Applications

There are many potential applications of **biosensor** s of various types. The main requirements for a biosensor approach to be valuable in terms of research and commercial applications are the identification of a target molecule, availability of a suitable biological recognition element, and the potential for disposable portable detection systems to be preferred to sensitive laboratory-based techniques in some situations. Some examples are given below:

- Glucose monitoring in diabetes patients
- Environmental applications (the detection of **pesticides** and river water contaminants)
- Detection of pathogens
- Determining levels of toxic substances before and after **bioremediation**
- Detection and determining of **organophosphate**
- Routine analytical measurement of **folic acid** , **biotin** , **vitamin B12** and **pantothenic acid** as an alternative to **microbiological assay**
- Determination of drug residues in food, such as **antibiotics** and **growth promoters** , particularly meat and honey.
- Drug discovery and evaluation of biological activity of new compounds.
- Detection of toxic metabolites such as **mycotoxins.**

#### 6.5.4.1. Biosensors in food analysis

- There are several applications of biosensors in food analysis.
- In food industry optic coated with antibodies are commonly used to detect pathogens and food toxins.
- The light system in these biosensors has been fluorescence, since this type of optical measurement can greatly amplify the signal.
- A range of immuno- and ligand-binding assays for the detection and measurement of small molecules such as **water-soluble vitamins** and chemical contaminants ( **drug residues** ) such as **sulfonamides** and **Beta-agonists** have been developed, often adapted from existing **ELISA** or other immunological assay. These are in widespread use across the food industry.

## 8.5.8 Conclusion

- There have been great advances in the use of biosensors for marine toxin detection and the future is still more promising.
- i) In general, biosensor technologies have some advantages versus analytical methods and animal bioassays that include low cost, ease-of-use,speed, no need of highly trained lab personnel and automation, most of them with very good reproducibility and robustness.
- ii) These methods do not entail legal or ethical issues related to the use of laboratory animals.
- iii) The evaluation of the toxicity of a sample with biosensor-based techniques does not require the use of a toxin standard of every compound of a toxin group, just are presentative member would suffice, which is one of the more important drawbacks of analytical methods, since certified standards for many marine toxin analogues are not available or easy to produce.
- iv) Biosensors are characterised by the simplicity of use, even for non-skilled personnel, and the low cost.
- v) At present, biosensors should be seen as bioanalytical tools for preliminary screening the toxicity of a sample. If the sample is considered suspicious, complementary analytical techniques should be used in shellfish may interfere in the measurements, a glutamate decarboxylase has been incorporated to a pre-treatment step, improving the limit of detection and the selectivity of the assay.
- In summary, the existing biosensors seem to be highly promising as biotools for seafood toxicity screening, since they allow detection of phycotoxins with appropriate sensitivities. However, these devices still suffer from limitations that compromise their applicability and further work is required for their commercial exploitation.

**Chapter 6: Bio-processing**

The engineering component of the commercial exploitation of biological materials, living organisms, and their activities is known as **bioprocessing.** Four aspects of bioprocessing include: **enzyme engineering, whole–cell bioreactors, transport and adhesion of cells and bioseparation.**

## 9.1.1 Enzyme Engineering

Enzymes are an alternative to live cells for synthesis of biological products. The applications vary from alternate fuel synthesis to environmental remediation to biosensors to food processing. Cellulase is an example of an enzyme used in an immobilized configuration for the conversion of biomass to feed for ethanol in the production of alternate fuels. In addition, these systems are beginning of prove

useful in the environmental area for the selective removal of heavy metals or the transformation of hazardous substances, such as organics, into nontoxic compounds.

Enzyme technology is exploited in the area of biosensors.

- Enzyme-coated electrodes provide a highly selective and sensitive method for determining the amount of a given substrate.
- E.g., sensors for common substrates like glucose, urea, and nitrate as well as sensors for fermentation products and intermediates like amino acids, lactic acid, penicillin, and alcohols.
- The future use of these specific electrodes will facilitate control of bioprocess, and thus, higher productivity in all aspects of bioprocessing.

At the level of everyday use, enzymes and other bioproducts are among employed in the food industry as preservatives, thickeners, coloring and flavoring agents, and emulsifiers. Enzymes are able to replace or minimize the number of additions used in the food processing industry. Enzymes are also used to increase the self life of many fruits and vegetables including fishes.

## 9.1.3 Whole – cell Bioreactors

Living cells can be viewed as small biochemical reactors of great complexity. Enzymes came from cells. The earliest bioreactors or fermenters were used in the brewing and wine making industries. During the last 65 years, fungal fermentations have been used to make antibiotics, beginning with penicillin. Now, fermentation's is also extensively used in the developing biotechnological/pharmaceutical industry. The first major products made by recombinant organisms were:

insulin (Elililly), tissue plasminogen activator (Genetech), and erythropoietin (Amogen).

- The most common organism used in recombinant fermentations is *Escherichia coli*, a genetically well-characterized bacterium.
- More current areas of research include limitation of protein synthesis by the amount of mRNA in the cells,
- decreasing protease activity in cells to reduce cellular protein degradation,
- manipulation of cells to inhibit inclusion body formation, and
- development of new vector-host system for the over exploitation of proteins.

Plant, mammalian, and insect cells hold greater potential for the biotechnology industry. However, their complexity has slowed development of industrial processes.

- As many as 25% of today's pharmaceuticals are extracted from plants naturally grown.
- A most promising area for plant reactors is the production of Taxol, an anticancer drug, recently approved by the US. Food and Drug administration.
- Currently, Taxol is extracted from yew tree bark, but the demand is greater than the amount usually available from the natural source.

Cell cultures derived from insects:

- a relative new comer to biotechnology, are more complex than plant cells but easier to establish than mammalian derived cell lines.
- A successful experimental system is the baculovirus system. It consists of a strong promotor that allows a cell to make up to 40% of its protein as the target or product protein with practically no cell multiplication.

Many proteins of mammalian origin are not simply products of gene expression they require glycosylation and sometimes other secondary enzymatic processes for biological activity. Since bacteria cannot carry out these conversions, production of such proteins requires mammalian or at least eukaryotic cell lines.

Mammalian cell grow much more slowly than bacteria, are more demanding in terms of media composition, and may require anchorage to solid supports. They are also very sear sensitive and may be suppressed by their own metabolites. It is relatively easier to establish long-lasting or nearly permanent mammalian culture cells using hybridoma cells for the production of monoclonal antibodies. Hybridoma cells do not require anchorage and have as a rule, the longevity of their parent cancer cell lines.

A technology that is still with infancy is the use of transgenic plant and animals to produce proteins.

- The living plant or animal became the "bioreacter". This technology involves inserting new genetic information in the embryo and having the nontoxic protein expressed by the mature animal.
- One strategy is to have a protein secreted into an animal's milk. Then the product is simply separated form the rest of the milk constituents.

To optimize production, fermentation processes are usually performed under conditions of controlled pH, dissolved oxygen, agitation, and temperature. More complicated control strategies involve regulating protein secretion or substrate addition. New biosensors are being developed to aid in determining the amount of a substrate or product. These sensors include enzyme (used to detect specific products or substrates), bacteria (to determine BOD), *Desulfovibrio desulfuricus* (to determine sulfur content).

### 9.1.4 Transport and adhesion of cells

One of the important parameters involved in sensing and controlling reactors is understanding the transport of cells and chemicals.

Cellular and viral transport and adhesion are of importance in

- immobilized reactors,
- development of new drugs, and
- *in situ* bioremediation efforts.

Undertaking the mechanism of intra and intercellular transport is key to the development of site–directed or target-specific drugs.

- monoclonal antibodies may be used to deliver drugs to cancer cells only, through specific ligand– receptor binding.

Understanding cellular transport can also aid in environmental remediation.

- Once microorganism has been injected into the environment, they must move from the point of injection to the contamination site. When cells move slowly toward a chemical contaminant as they degrade it, chemotaxis is useful in bioremediation efforts.

## 9.1.5 Bioseparation

Although a large quantity of work has been done on fermentation and other upstream processing, the main cost of making a biotechnological product is the downstream processing or separation steps.

After a product has been made by a cell, it must be separated from the rest of the by-products.

- The standard industrial techniques for separating cells from spent medium are **centrifugation** and **cross-flow filtration**.
- If the cell product is an intracellular product, the cells have to be lysed through homogenization or osmotic shock.
- Bacterial cells like *E. coli* typically do not secrete protein products, whether mammalian cell typically will. The expensive part is that the further purification of the product, nor the initial separation of cells from product.
- Precipitation of all cellular protein is a common first step.
- A number of technologies exist for further product separation, including differential precipitation, affinity and other types of chromatography, electrophoresis, liquid-liquid extraction and use of specialized membranes.

As essential step in downstream processing is

- quality control,
- the assessment not only of sterility,
- absence of genetic materials such as viruses or nucleic acids, and
- absence of pyrogens,
- but also of the purity of the final product, such as the desired protein.

## Unit 10. Bioinformatics

### 10.1.1 Bioinformatics

The term *bioinformatics* was coined by Paulien Hogeweg in 1979 for the study of informatic processes in biotic systems. **Bioinformatics** is the application of statistics and computer science to the field of molecular biology. Bioinformatics can also be defined as a "science of solving biological problem using a mathematical and computational approach". Its primary use since the late 1980s has been in genomics and genetics, particularly in those areas of genomics involving large-scale DNA sequencing. Bioinformatics now entails the creation and advancement of databases, algorithms, computational and statistical techniques and theory to solve formal and practical problems arising from the management and analysis of biological data. Over the past few decades rapid developments in genomic and other molecular research technologies and developments in information technologies have combined to produce a tremendous amount of information related to molecular biology. It is the name given to these mathematical and computing approaches used to glean understanding of biological processes.

Common activities in bioinformatics include:

Mapping and analyzing DNA and protein sequences, aligning different DNA and protein sequences to compare them and creating and viewing 3-D models of protein structures.

The primary goal of bioinformatics is to:

Uncover the wealth of biological information hidden in the mass of data and obtains a clever insight into the fundamental biology of organism. It focus on developing and applying computationally intensive techniques (e.g., pattern recognition , data mining , machine learning algorithms, and visualization ).

Major research efforts in the field include:

sequence alignment , gene finding , genome assembly , drug design , drug discovery , protein structure alignment , protein structure prediction , prediction of

gene expression and protein-protein interactions , genome-wide association studies and the modeling of evolution .

## 10.1.2 NCBI

The **National Center for Biotechnology Information** (**NCBI**) is part of the United States National Library of Medicine (NLM), a branch of the National Institutes of Health. The NCBI is located in Bethesda, Maryland. The NCBI houses genome sequencing data in GenBank and an index of biomedical research articles in PubMed Central and PubMed, as well as other information relevant to biotechnology. All these databases are available online through the Entrez search engine. NCBI is directed by David Lipman, one of the original authors of the BLAST sequence alignment program and a widely respected figure in Bioinformatics. The NCBI has had responsibility for making available the GenBank DNA sequence database since 1992.

GenBank coordinates with individual laboratories and other sequence databases such as those of the European Molecular Biology Laboratory (EMBL) and the DNA Data Bank of Japan (DDBJ). Since 1992, NCBI has grown to provide other databases in addition to GenBank. NCBI provides Online Mendelian Inheritance in Man, the Molecular Modeling Database (3D protein structures), dbSNP a database of single-nucleotide polymorphisms, the Unique Human Gene Sequence Collection, a Gene Map of the human genome, a Taxonomy Browser, and coordinates with the National Cancer Institute to provide the Cancer Genome Anatomy Project. The NCBI assigns a unique identifier (Taxonomy ID number) to each species of organism.

The NCBI has software tools that are available by WWW browsing or by FTP. For example, BLAST is a sequence similarity searching program. BLAST can do sequence comparisons against the GenBank DNA database in less than 15 seconds.

## 10.1.3 GenBank sequence database

The **GenBank** sequence database is an open access , annotated collection of all publicly available nucleotide sequences and their protein translations.

This database is produced at National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration , or INSDC .

GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms.

GenBank continues to grow at an exponential rate , doubling every 18 months. GenBank is built by direct submissions from individual laboratories, as well as from bulk submissions from large-scale sequencing centers.

## Submissions

- Direct submissions are made to GenBank using **BankIt** , which is a Web-based form, or the stand-alone submission program, **Sequin** .
- Upon receipt of a **sequence** submission, the GenBank staff assigns an **accession number** to the sequence and performs quality assurance checks.
- The submissions are then released to the public database, where the entries are retrievable by **Entrez** or downloadable by **FTP** .
- Bulk submissions of **Expressed Sequence Tag** (EST), **Sequence-tagged site** (STS), **Genome Survey Sequence** (GSS), and **High-Throughput Genome Sequence** (HTGS) data are most often submitted by large-scale sequencing centers.
- The GenBank direct submissions group also processes complete microbial **genome** sequences.

# 10.1.4 Databases

There are two types of databases i.e. Primary databases and Secondary databases.

**Primary Database**

Primary databases contain information and annotations of DNA and protein sequences, DNA and protein structure and DNA and Protein expresses profiles.

1. Genome Database

Sequence Database, Structural Database

2. Protein Database

Structural Database

3. Complex Database

Protein Nucleic acid Complex Database

**Secondary Database**

Those data that are derived from the analysis or treatment of primary data such as secondary structures, hydrophobicity plots, and domain are stored in secondary databases. They also contain results of analysis of a primary resources including information as sequences patterns of methods, variants, mutation and evolutionary relationships.

1. Protein Database
    1. Sequence Database
2. Complex Database
    1. Protein Nucleic Acid Interaction Database

Primary sequence database for nucleic acid

GenBank

GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences. There are approximately 85,759,586,764 bases in 82,853,685 sequence records in the traditional GenBank divisions and 108,635,736,141 bases in 27,439,206 sequence records in the WGS division as of February 2008.

It maintains the following databases and tools:

1. Uterative Databases e.g. PUBMED, OMIMO

2. Entrey Databases e.g. Connoted Domain Databases, 3D Domains

3. Nueleotide Databases e.g. Expressed sequence Tags(EST)

4. Tools for Sequence analysis e.g. BLAST

5. Tools for 3-D structure display and similarity searching e.g. Cn3D

Databases consisting of data derived experimentally such as nucleotide sequences and three dimensional structures are known as primary databases.

**EMBL**

The European Molecular Biology Laboratory (EMBL) is a molecular biology research institution supported by 20 European countries and Australia as associate member state. In 1974, EMBL was created and it is a non-profit organisation

funded by public research money from its member states. Research at EMBL is conducted by approximately 85 independent groups covering the spectrum of molecular biology. The cornerstones of EMBL's mission are:

- to perform basic research in molecular biology and molecular medicine,
- to train scientists, students and visitors at all levels, to offer vital services to scientists in the member states,
- to develop new instruments and methods in the life sciences, and to actively engage in technology transfer.

Website: WWW.ebi.ac.UK

**DDBJ**

The DNA Data Bank of Japan is a DNA data bank. It is located at the National Institute of Genetics of Japan. It shares its data with European Molecular Biology Laboratory at the European Bioinformatics Institute and with GenBank at the National Center for Biotechnology Information. The DNA Data Bank of Japan (DDBJ, has made an effort to collect as much data as possible mainly from Japanese researchers. The increase rates of the data collected, annotated and released to the public in the past year are 43% for the number of entries and 52% for the number of bases.

The increase rates are accelerated even after the human genome was sequenced, because sequencing technology has been remarkably advanced and simplified, and research in life science has been shifted from the gene scale to the genome scale.

Search is done by FASTA and BLAST. Analysis is done by clustalW.

## 10.1.5 Other nucleotide databases

***UniGene***

UniGene is an NCBI database of the transcriptome and thus, despite the name, not primarily a database for genes. Each entry is a set of transcripts that appear to stem from the same transcription locus (i.e. gene or expressed pseudogene) together with the information on protein similarities, gene expression, cDNA clones, and genomic location is included with each entry.

***SGD***

SGD is a scientific database of the molecular biology and genetics of the yeast *Saccharomyces cerevisiae*, which is commonly known as baker's or budding yeast.

***EBI Genomes***

It provides the access to the information of the completed genomes. The first completed genomes from viruses, phages, and organelles were deposited into the EMBL Database in the early 1980's. Since then, molecular biology's shift to obtain the complete sequences of as many genomes as possible combined with major developments in sequencing technology resulted in hundreds of complete genome sequences being added to the database, including Archaea, Bacteria and Eukaryota.

***Genome Biology***

NCBI provides several genomic biology tools and resources, including organism-specific pages that include links to many web sites and databases relevant to that species.

***Ensembl***

Ensembl is a joint project between (EMBL) - (EBI) and the Welcome Trust Sanger Institute (WTSI) to develop a software system which produces and maintains automatic annotation on selected eukaryotic genomes. The project consists of: a database scheme and associated API to store genomic information of about 40 genomes and many extension databases to represent functional, comparative and variational genomics.

## 10.1.6 Tools for Sequence Analysis

**1. BLAST (Basic Local Alignment Search Tool)**

It performs “local” alignment. It finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches.

Types of BLAST

I. Blastn: searches a nucleotide database using a nucleotide query.

II. Blastp: searches a protein database using a protein query.

III. Blastx: searches a protein database using a translated nucleotide query.

IV. Tblastx: searches a translated nucleotide database using a translated nucleotide query.

**2. CLUSTALW**

Clustalw is a multiple sequence alignment program for proteins or nucleotides which is available at <ebi.ac.uk/clustalw>.

Multiple sequence alignment means an extension of pairwise alignment to incorporate more than two sequences at a time.

It is often used in identifying conserved sequence regions across a group of sequences hypothesized to be evolutionarily related.

It calculates the best match for the selected sequences so that differences, similarities and identities can be seen.

Evolutionary relationship can be seen using **cladograms** and **phylograms**.

**Phylogram** is a branching diagram (tree) assumed to be an estimate of a phylogeny, branch lengths are proportional to the amount of inferred evolutionary change.

**A Cladogram** is a branching diagram (tree) assumed to be of a phylogeny where the branches are of equal length, thus cladograms show common ancestry, but do not indicate the amount of evolutionary "time" separating taxa.

It can align either nucleotide or protein sequences. In the case of nucleotide sequences, it will align them as they are input – the program does not provide the option of specifying DNA strands.

The program accepts sequences in the formats like:- NBRF/PIR,EMBL/UniProt, Pearson (Fasta), GDE, ALN/ClustalW. The sequences can either be pasted into the web form or uploaded to the web form in a file.

## 3. FASTA

Fasta is a Protein similarity search. It provides sequence similarity searching of query sequence against nucleotide & protein databases using fasta programs. It can be used for fast protein comparison or fast nucleotide comparison. This format contains a one line header followed by lines of sequence data.

The sequence in fasta formatted files are preceded by a line starting with a ">" symbol. The first word on this line is the name of the sequence. The rest of the line is a description of the sequence. The remaining lines contain the sequence itself. Fasta files containing multiple sequences are just the same, with one sequence listed right after another. This format is accepted for many multiple sequence alignment programs.

# 10.1.7 Primary sequence databases for protein

**Protein Databases**

Protein databases are the most comprehensive source of information on protein. It is necessary to distinguish between universal databases covering protein from all species and specialized data collections storing information about specific families or protein or a group or an organism.

Types

Ø Primary Protein Databases like UnitProt/Swiss-Prot.

Ø Secondary Protein Databases like Interpro.

Ø Specialised Protein Databases like GOA, ENZYME.

Ø Structure Databases like PDB.

**UniProt:**

- It stands for Universal Protein resources.
- It is a central repository or protein sequences and function, created by joining the information contained in UniProt/SwissProt.

**PDB:**

- PDB stands for protein Data Bank.
- The RCSB PDB provides a variety of tools and resources for studying the structures of biological macromolecules and their relationships to sequence, function, and disease.

**PDB viewer:**

- It provides an introduction to macromolecular modeling with Deep View, including review of many basic concepts in protein structure.
- Structure files are used for viewing, and then carry out exercises in manipulating, analyzing, and comparing protein structures.

**SWISS PDB VIEWER:**

- Deep View (formerly called Swiss-Pdb Viewer) is a friendly but powerful molecular graphics program.
- It is designed for use with computing tools available from the Expert Protein Analysis System, or ExPASy Molecular Biology Server in Geneva, Switzerland.

- It allows us to build models, by giving an amino-acid sequence.
- It can find hydrogen bonds within proteins and between proteins and ligends.
- It allows us to view several proteins simultaneously and superimpose them to compare their structures and sequences.
- It computes electrostatic potentials and molecular surfaces, and carries out energy minimization.

Getting started:- website WWW.rcsb.org

Enter the name of protein name or PDB ID (contng. Four characters) into the search box. Then download Pdb file.

# RASMOL

RasMol is a computer program written for molecular graphics visualization intended and used primarily for the depiction and exploration of biological macromolecule structures, such as those found in the Protein Data Bank. It is available for Windows, Macintosh and UNIX platforms.

# Cn3D

- Cn3D is a helper application that allows us to view 3-dimensional structures from NCBI's entrez retrieval service.
- Cn3D runs on Windows, Macintosh, and Unix.
- Cn3D is a visualization tool for biomolecular structures, sequences, and sequence alignments and has powerful annotation and alignment editing features.
- Cn3D displays structure-structure alignments along with their structure-based sequence alignments, to emphasize what regions of a group of related proteins are most conserved in structure and sequence.

While working on it two windows appear: the main Cn3D structure window where the protein is displayed, and a sequence window that shows the chain's amino acid sequence.

When a single structure is a loaded into Cn3D, the sequence viewer shows the sequences of all protein and nucleic acid chains in the structure. The color of each residue is coordinated between the structure and sequence windows: each letter of the sequence represents a residue in the structure, and always adopts the color of the backbone's alpha carbon (or phosphorus, for nucleotides), even if side chains are colored differently from backbone in the structure window Cn3D's sequence window also functions as an alignment viewer when displaying more than one structure or a structure to which multiple sequences have been aligned.

## PIR

The Protein Information Resource (PIR), located at Georgetown University Medical Center (GUMC) is an integrated public bioinformatics resource to support genomic and proteomic research, and scientific studies.PIR was established in 1984 by the National Biomedical Research Foundation (NBRF) as a resource to assist researchers in the identification and interpretation of protein sequence information.

## MIPS

The MIPS group [Munich Information Center for Protein Sequences of the German National Center for Environment and Health (GSF)] at the Max-Planck- Institution for Biochemistry, Martinsried near Munich, Germany, is involved in a number of data collection activities, including a comprehensive database of the yeast genome, a database reflecting the progress in sequencing the *Arabidopsis thaliana genome*, the systematic analysis of other small genomes and the collection of protein sequence data within the framework of the PIR-International Protein Sequence Database.

## SWISS PROT

Swiss-Prot is a protein sequence database created in 1986 by Amos Bairoch and developed by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute. It is a manually curated biological database of protein sequences. Swiss-Prot was  Swiss-Prot strives to provide reliable protein sequences associated with a high level of annotation (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases. In 1996, a computer annotated supplement to SWISS-PROT was created, termed TrEMBL.

## TrEMBL

It was created in 1996 as a computer annotated supplement to SWISS PROT. The database helps the SWISS PROT format and contains translations of all coding sequences (CDS) in EMBL. It has two main sections:

**i) SP-TrEMBL** : (SWISS PROT -TrEMBL): It contains the entries that eventually be incorporated into SWISS PROT; that have not yet been manually annotated.

**ii) REM-TrEMBL** : it contains sequences that are not destined to be included in SWISS PROT. These include: immunoglobulins, T-cell receptors, fragments of fewer than eight amino acids, synthetic sequences, patented sequences, codon translations.

## 10.1.8 Other database

***Ens EMBL***

It is a collaborative project of EMBL, EBI and the Sanger Center to automatically track sequence fragment of human genome and assemble them into longer structures.

Automated analysis methods such as: gene finding feature finding tools, sequence comparison tools, are then applied to the assembled sequences.

These are made available to users through a web interface.

Resources

1. EBI: www.ebi.ac.uk

2. NCBI: www.ncbi.nlm.nih.gov/

3. NCBI-BLAST: http://blast.ncbi.nlm.nih.gov/Blast.egi/

4. DDBJ: www.ddbj.nig.ac.jp/

5. Swiss-Prot: http://expasy.org/sprot/

6. InterPro: www.ebi.ac.uk/interpro/

7. PDB: www.pdb.org

8. CLUSTALW program:

http:// www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalw2

http://align.genome.jp/clustalw/

FASTA: http:// www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html

**PRACTICALS**

# 1. Isolation of genomic DNA from fish tissues

In principle the isolation of nucleic acids from any prokaryotic or eukaryotic cells (DNA,RNA and plasmids) two major steps are followed *viz*, cell disruption and separation of nucleic acids from the associated proteins. In step one, the cells are broken and the nucleic acids are dissociated from other cellular components. This can be done by osmotic shock, freezing-thawing and ultrasonic sound treatment. Quite often surface-active agents such as Sodium Dodecyl sulfate (SDS) and lysozyme are used to disrupt cells. In the second step the cellular debris are removed by centrifugation. The associated proteins are removed from the nucleic acids either by high salt or phenol extraction.

The phenol extraction gives fairly undegraded DNA. In this method, chromatin proteins are dissociated by treatment with SDS. The proteins are denatured by phenol-chloroform treatment. The denatured proteins become insoluble and can be separated by centrifugation.

Isoamyl alcohol reduces foaming during the extraction and facilitates the separation of the aqueous and organic phases. The aqueous phase will contain DNA which can be precipitated with ethanol. Since DNA is a high molecular weight polymer, care should be taken not to break or shear the DNA. Pipetting through narrow bore pipettes should be avoided. Tissues chosen for DNA extraction can be frozen in 100% ethanol and kept at -20 ° C. The tissues can be stored even in room temperature but the DNA may get degraded.

# Procedure

1. Take the sample tissue (fins or muscles) and cut into small pieces with help of a pair of fine scissors. Every time wash in ethanol and cleaned with tissue paper. The fresh tissues should be homogenized.

2. Add 250 m l TEN Buffer + 20 m l 20% SDS and 10 m l Proteinase K.

TEN Buffer

100 mM Tris

10 mM EDTA pH 8.0

250 mM NaCl

Filtered 20% SDS. (add SDS to filtered water)

Proteinase K – 10 mg/ml

3. Keep the eppendorf tube containing extraction buffer/proteinase K and sample for digestion in a water bath at 55°C. The period depends upon the completion of digestion. It can be extended up to 3 days for frozen old samples. If tissue sample was more and incomplete digestion was observed some amount of Proteinase K can be (10 m l) added. For fresh tissue 3-6 hr of proteinase K digestion is enough.

4. After Proteinase K digestion take the eppendorfs from the water bath to fume hood chamber and add 300 m l phenol. Shake the tube vigorously for 20s by hand; followed by gentle mixing. Invert the tubes repeatedly. Phenol removes proteins.

5. Spin at 12000 rpm for 5 min. If rpm is less then spin for more time.

6. Remove the aqueous phase (top layer) and transfer to another eppendorf tube (when spinning, aqueous phase and organic phase are formed. Nucleic acid gets into the aqueous phase at right pH). Do not disturb tissue debris at the interface.

7. Repeat the above step once again by adding 300 m l of phenol.

8. Add 300 m l of 24:1 chloroform : isoamyl alcohol to aqueous phase. Chloroform removes phenol. Shake the tube vigorously for 20s by hand; followed by gentle mixing. The DNA and chloroform mixture should be mixed gently.

9. Spin at 12,000 rpm for 3 min.

10. Remove the bottom layer containing chloroform and discard it. Come out from the fume hood chamber.

11. Add 200 m l of isopropanol (when the eppendorf tube was shaken, clumping may be noticed as an indication for the presence of high molecular weight DNA. If the solution is clear, the DNA got degraded and broken to small fragments. Isopropanol precipitates the DNA). Mix by rapid and abrupt invertion of the tubes 5 or 6 times.

12. After adding isopropanol keep the eppendorf tube in ice for 1 hr or overnight for precipitation.

13. Spin at 12,000 rpm for 20 min. (If no pellet was observed, continue spinning for another 10 min).

14. Pour or discard the isopropanol with help of a pipette. Collect upper aqueous phase using a wide bore pipette.

15. Wash the pellet with 500 m l of 70% ethanol by gentle mixing and allow DNA to precipitate at – 20 ° C for at least 1 hr.

16. Spin at 12,000 rpm for 10 min.

17. Decant off the 70% ethanol. Remove any ethanol if present with a micropipette

18. allow the DNA to partially dry at room temperature (5-10 min) Do not over dry.

19. Add 30 m l of autoclaved nanopure water and resuspend in water bath at 55 ° C for 1 hr. DNA can be resuspended in TE buffer (10mm Tris, 1mM EDTA, pH 8.0). Allow the DNA at least 24 hrs to dissolve fully.

20. Use 1 m l aliquots for PCR.

# Caution

Phenol is highly corrosive and can cause severe burns. Safety glasses and gloves should be worn. Any areas of skin that come into contact with phenol should be rinsed with a large volume of water and washed with soap and water. Do not use ethanol.

# DNA storage

Most protocols suggest using a Tris-EDTA buffer for DNA storage. However, the EDTA in such buffers will chelate the $Mg^{2+}$ in the PCR buffer, the concentration of which is vital, and so may cause problems with the sensitivity and specificity of the reaction. For this reason DNA can be simply stored in distilled water.

# Note

1. For extracting DNA from fish blood insert 2 ml syringe containing anticoagulant near the caudal region below the lateral line and with draw 0.5 ml blood from the caudal vein and transfer to eppendorf tube. After adding lysis buffer keep in a water bath at 60 ° C for 1-2 hr. Proceed with phenol–chloroform extraction.

2. SDS- Don’t keep it in refrigerator, it will get precipitate. If stored in refrigerator before use warm it. Cap it airtight and store on the work bench.

**P2: EXTRACTION OF DNA FROM FISH BLOOD (RBC)**

For extracting DNA from fish blood insert 2 ml syringe containing anticoagulant near the caudal region below the lateral line and with draw 0.5 ml blood from the caudal vein and transfer to eppendorf tube. After adding lysis buffer keep in a water bath at 60 ° C for 1-2 h. Proceed with phenol–chloroform extraction.

# Procedure

1. Collect the blood in ethanol, store in -20°C.

2. Mix well, take 100µl and add 100 µl of TE (Tris EDTA solution, pH 8.0).

3. Centrifuge at 3000rpm for 5 min.

4. Remove the supernatant and add 200 µl of TKEM buffer (10mM Tris-HCl,

pH 7.6, 10mM KCl, 2mM EDTA containing 4mM $MgCl_2$ )

5. Centrifuge at 3000rpm for 5 min. Remove the supernatant and add 200 µl TKEM and again centrifuge and remove the supernatant.

6. Add 200 µl of TKEM buffer and add 15 µl of 10% SDS. Mix well, keep at 55°C for 5 min.

7. Add 75 µl of 6M NaCl. Mix well, centrifuge at 10,000 rpm for 5 min.

8. Collect the supernatant and add ethanol to precipitate the DNA. The DNA will appear as threads. Keep overnight at -20°C.

**P3: DNA Quality and quantification**

**1. Solutions required**

50X TAE (generally diluted to 1X for use) :

242 g Tris; 700 ml ddH2O; 57.1ml glacial acetic acid; 100 ml 0.5 M EDTA pH 8.0. Adjust volume to 1L using dd H2O. Autoclave and store at room temperature.

**1X TAE agarose gel (1%)** :

2 g agarose; 200 ml 1 X TAE; heat in microwave oven until agarose dissolved (do not boil). Store in 65°C oven.

**Load dye** : 50 mM EDTA; 30% Glycerol; 0.25% bromophenol blue; 0.25% xylene cyanol

**Marker** : 50 mM EDTA; 20 µl load dye; 175 µl 1 X TAE; 5 µl DNA marker (HindIII digest of λ phage DNA for example)

**Ethidium bromide** : HAZARD!!! Wear double gloves when handling.

# Methods

There are two methods to quantify DNA sample. The conventional method of electrophoresis of DNA sample of unknown concentration with a known standard is applied in most labs where a spectrophotometer is not available.

Another method is using a spectrophotometer that directly quantifies the DNA concentration in the resuspended DNA extraction.

# A. Electrophoresis of a DNA sample of unknown concentration with a known standard

1. Place the gel plate into gel mould, position the comb and ensure that the gel is horizontal – check with a spirit level is necessary (different supplier have different designs, do follow the instructions from manufacturers).

2. Prepare a 1% agarose gel: dissolve 1g agarose in 100 ml 0.5x TBE or 1x TAE. Heat the mixture in a microwave oven until completely dissolved.

3. Cool to 60°C.

4. Pour agarose onto the gel tray and allow it to set for at least 30 min.

5. Remove the comb. Place the gel into the electrophoresis tank and pour 0.5xTBE or 1xTAE (same as the buffer that was used to make gel) until the gel is completely covered.

6. Mix 1 µl loading dye and 2 µl DNA and load into the well.

7. Load 2 of DNA marker (Hind III digested λ DNA for example) into one of the wells.

8. Run the gel at 70-100V until the dye is about 2.5 cm from the origin.

9. Move the gel to a tray with ethidium bromide (1µl ethidium bromide in 100 µl dd$H_20$) (HAZARD!!!).

10. Let the gel stain for 5- 10 min, and then de-stain for about 2 min in another container with dd$H_2O$ only.

Illuminate the gel with UV light (CAUTION – UV LIGHT IS HAZARDOUS!!! – WEAR MASK OR UV PROTECTION GLASSES IF EXPOSED TO UV LIGHT).

11. Photograph the gel under the UV.

12. Compare the intensity of the DNA bands of the samples with the intensity of the λ bands. As the amount of DNA present in each λ band is known (information is often provided by the supplier), the amount of DNA of each sample can be estimated by comparing the fluorescent yield of the sample with those of the λ bands.

13. Quality of extracted DNA can also be assessed by looking at the gel. Good quality DNA will show as a sharp intense band. Degraded DNA extracts will show various degree of smearing.

# B. Spectrophometric determination of DNA concentration

Dilute 1.5 µl of DNA to 1500 with deionised water and read at $A_{230}$, $A_{260}$ and $A_{280}$.

The $A_{260}/A_{280}$ ratio provides an estimate of the purity of the DNA. In a pure sample, this ratio is approximately 1.8. Lower values indicate protein or phenol contamination. $A_{230}$ should be less than $A_{260}$ and may be the same as $A_{280}$. High $A_{230}$ reading indicates that residual phenol remains in the preparation. An $A_{260}$ of 1 corresponds to approximately 50 µl/ml of double-stranded DNA in a 1 cm quartz cuvette. Nucleic acid concentration is calculated as follows:

$A_{260}$ * 50 mg/µl * 0.001 µl/ml * dilution factor (1500 µl/1.5 µl) (µg/µl)

# Trouble Shooting

Common problems and solutions are summarised in Table1.

**Table 1. Common problems and appropriate solutions**

| Sl. No. | Problem | Possible causes / solutions |
|---|---|---|
| 1. | Lysis of the cell is incomplete | Incubation time is not long enough. At least one of the ingredients is lacking in cell lysis solution, especially SDS. Check by shaking the tube, if no foam formation is seen, then add 100 µL of 10% SDS. If there is foam formation, add 5 µL of Proteinase K (20mg/ml) |

| | | |
|---|---|---|
| | | and continue to incubate for One hour. |
| 2. | No DNA precipitation | Cell lysis is not complete. Short centrifuge time and/or low speed Wrong precipitation solution (ethanol/ isopropanol) |
| 3. | No DNA on gel | DNA pellet is lost during wash step. The gel was not stained with ethidium bromide. |
| 4. | DNA extraction fail from preserved samples | .The two methods described in this manual are often used to produce reasonably good DNA quality. If these two methods fail, especially when dealing with preserved samples, the best solution is probably to use commercial DNA extraction kits. |

**P4: SDS – Poly acrylamide gel electrophoresis**

Electrophoresis is a technique used for the separation of proteins based on the migration of charged proteins in an electric field. This technique is used as an analytical method. In this technique proteins can be visualized and also separated. The number of different proteins in a mixture or the degree of purity of a particular protein preparation can be estimated quickly. Also electrophoresis allows determination of crucial properties of a protein such as its iso-electro point and approximate molecular weight.

Electrophoresis of proteins is generally carried out in gels made up of the cross linked polymer polyacrylamide. The polyacrylamide gel acts as a molecular sieve, slowing the migration of proteins approximately in proportion to their charge-to-mass ratio. Migration may also be affected by protein shape. In electrophoresis, the force moving the macromolecule is the electrical potential. The electrophoresis mobility of the molecule, $\mu$ is the ratio of the particle, V, to the electrical potential (E).

$\mu = V/E$

The migration of a protein in a gel during electrophoresis is therefore a function of its shape and its size. Normally SDS (Sodium dodecyl sulphate), a detergent is used for the estimation of purity and molecular weight. SDS binds to most proteins (probably by electrophoretic interactions) in amounts roughly proportional to the molecular weight of the protein *i.e.* 1 molecule of SDS for every two amino acid

residues. The bound SDS contributes a large net negative charge, rendering the intrinsic charge of the protein insignificantly. The native conformation of a protein is altered when SDS is bound and most proteins assume a similar shape. Electrophoresis in the presence of SDS therefore separate proteins almost exclusively on the basis of mass (molecular weight) with smaller polypeptides migration more rapidly. After electrophoresis the proteins are visualized by adding a dye such as coomassie blue, which binds to proteins, but not to the gel itself. By comparing with the position of the known protein, the molecular weight of the unidentified protein can be measured.

Polyacrylamide gels are formed by the linking co-monomer bisacrylamide. The gel consists of lower 'separating gel' and upper 'stacking gel'. The pH of the buffers is adjusted such that the protein sample is concentrated in the stacking gel(pH 6.8) and starts moving to the separating gel (pH 8.8). The proteins unstuck and begin to separate according to their molecular weights. Treatment of proteins with the detergent SDS (Sodium dodecyl sulphate) and a reducing agent (μ-mercaptoethanol or ditiothreitol) changes their three dimensional shape into simple rod-like structures.

# Stock solutions

1. Acrylamide (30%): Dissolve 30g acrylamide and 0.8 g bisacrylamide in 70 ml of distilled water and make up to 100 ml with the same.

2. Separating buffer: Dissolve 36.34g Tris in 150 ml distilled water and adjust the pH to 8.8 with con. HCl. Add 0.8g SDS and make up the volume to 200ml.

3. Stacking buffer: Dissolve 12.1g Tris in 150ml of distilled water and adjust the pH to 6.8 with con. HCl. Add 0.8g SDS and make up the volume to 200 ml with distilled water.

4. Running buffer: Dissolve 28.8 g glycerine, 6.1g tris and 1g SDS in 800 ml distilled water and make up the volume to 1 litre.

5. Sample buffer: Mix 3 ml of glycerol, 1.2 ml of 1M Tris (pH 6.9), 200 μl mercaptoethanol and 600μl of 0.02% bromophenol blue and dissolve 1g of SDS in the mixture. The buffer should be prepared fresh and stored at 4°C.

6. Ammonium persulphate (APS): Dissolve 50 mg of APS in 0.5 ml distilled water. This solution should be prepared fresh and stored at 20°C in aliquots.

*Gel recipes (Separating gel)- TABLE 2.*

| Chemicals | 7% | 10% |
|---|---|---|
| Acrylamide (30% Stock) | 7ml | 10ml |
| Double distilled water | 15.4 ml | 2.4 ml |
| Separating gel buffer | 7.5 ml | 7.5 ml |
| TEMED Ammonium | 30 µl | 30 µl |
| persulphate | 70 µl | 70 µl |

**CAUTION**

Acrylamide and bis-acrylamide are neurotoxins and should be handled with care

# Procedure

1. Wash the plates neatly with distilled water and dry

2. Wipe the plates with 0.1% SDS solution and clean them properly

3. Fix the strips with vacuum grease and seal them with 1% agar solution(Check for leakage with distilled water)

4. Prepare the solutions as described above and pour the gel. Overlay the gel with 0.15 SDS solution, care should be taken to note that the upper layer is even and gel is 2 cm below the notch.

5. Allow the gel to polymerise uniformly

6. After proper polymerization remove the overlay solution and wipe it with filter paper

7. Then place the comb and pour stacking gel

8. Allow stacking gel to polymerise. Polymerization must be even and must extended uniformly up between fingers.

9. Sample preparation:

Prepare the samples as per the respective procedure and estimate the amount of protein present. For coomassie blue staining, load 150µg protein per lot. Take care that the volume should be minimum (20-40µl)

10. Take the precast gel and remove the base strip and wipe the extra grease with the help of cotton

11. Set up tank, connect leads and fix the gel. Pour running buffer in the lower chamber

12. Boil the sample with equal volume of sample buffer in a boiling water bath for 2 minutes

13. Load the sample with the help of a micro pipette or micro syringe. Start loading from one end of the gel. Don't load the outermost wells because the tracks may be distorted. Fill the unused wells with sample buffer

14. Fill the upper reservoir with running buffer and overlay the samples with the same

15. With the help of a syringe and bent needle remove the bubbles under the gel between the glass plates

16. Run the gel at 20mA constant current until bromophenol blue reaches the bottom (6-8hrs)

17. Care should be taken to check that the plates do not get heated

18. After completion, open the plates and remove the gel

19. Wash the gel to remove SDS and stain it for 3h

20. Then destain till the background becomes clear

**CAUTION**

1. Pour the gel, preferably 24h prior to running

2. Take care that the sample volume and the amount of protein in the sample should be equal in all slots

3. While loading the sample, work as quickly as possible. If a well is empty and the adjacent well is filled, sample will leak through the finger into the empty well and cross contaminate

4. Don't overfill the wells. Overfilling leads to cross lane contamination

5. When filling the wells fill it slowly with the upper reservoir buffer without disturbing the sample

# Molecular weight determination

Determine the molecular weight of the proteins in the samples as described by Weber and Osborn (1969). Plot the molecular weight of marker proteins in log scale against the electrophoresis mobility of the proteins. Note the mobilities of the proteins of interest and plot on the same group.

# Trouble shooting

1. Poor polymerization: Polymerization is inhibited by contamination with impurities in the water or reagent and contaminants from the glass, comb, spacers or tubing. Ammonium per sulphate is highly unstable. So store the crystals in a desiccator. Every time prepare freshly or store the solution at 1- 20°C in small aliquots

2. Protein overload: This will result in distorted bands

3. Wavy bands: This is because of excessive heating of the sample. To avoid heating run the gel at low current and low temperature. Inadequate polymerization will also cause this.

**P5: Agarose gel electrophoresis**

Agarose forms a gel by hydrogen bonding and the gel pore size depends on the agarose concentration. The DNA molecules are separated by electrophoresis on the basis of their size, shape and the magnitude of net charge on the molecules. When an electrical field is applied across the gel, DNA which is negatively charged at neutral pH, migrates towards, the anode. Larger molecules migrate more slowly through the pores of the gel less efficiently than smaller molecules. A linear DNA fragment of a given size migrates at different rates though gel containing different concentration of agarose. There is a linear relationship between the electrophoretic mobility of DNA and the gel concentration. Thus, by using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules.

Agarose gel has lower resolving power than polyacrylamide gel but have greater range of separation of DNAs from 200 bp to 50 kb in targets can be separated. Agarose gel is run horizontally and can be fragile and difficult to handle safely. When preparing very concentrated (4% or greater) gels the agarose should gradually be added to a vigorously stirred buffer to avoid clumping. Should finer resolution be necessary, polyacrylamide or sequencing gels can be used. Approximately a quarter of the PCR product should be sufficient to be seen directly on the ethidium bromide stained gel.

Ethidium bromide can be added to the gel to a concentration of 1 m g/ml, or gels can be stained after electrophoresis. The dye ethidium bromide intercalates between the bases of RNA and DNA and fluoresces orange when irradiated with UV light. Polyacrylamide gels are effective for separating small fragments of DNA (5-500 bp), their resolving power is extremely high, and fragments of DNA that differ in size by little as 1 bp can be separated from one another. The disadvantage is that more difficult to prepare and handle.

# Procedure

1. Prepare 1% gel using ultrapure agarose

(0.5 g of agarose in 25 ml of TBE /TAE buffer)

TBE Buffer (10x - 1 lit)

Tris - 108 g

Boric acid - 55 g

EDTA - 9.3 g

pH - 8.3

To dissolve the agarose boil for few min in microwave oven.

2. After cooling add 1 m l of Ethidium Bromide and swirl gently without bubbling.

(EtBr stock - 10 mg/ml, 5 m l /100 ml of buffer or agarose mix)

3. Pour the gel gently in the gel tray after placing comb and leave for 30 min.

4. Fill the gel tank with TBE buffer.

5. Place the gel tray in the tank after removing the combs from the gel gently. Lift the combs without shaking from both sides.

6. Mix 1 m l of loading buffer with 2 m l of sample DNA and loaded in the wells. Use separate tips for each sample to avoid cross-contamination.

7. Load marker DNA also.

8. Run the gel at 160 V (80 milli amperes) for a period of 45 min. (Before loading the samples the apparatus can be checked by connecting to electric circuit. On both sides from the electrodes bubbles will come. This ensures the powerpack is in good condition)

9. Stop the gel run when the tracking dye migrated the other end.

10. Observe the gel in the UV Transilluminator using goggles.

Note : Take care while handling the gel because it contains ethidium bromide a carcinogen. Always use gloves. Discard the TBE buffer.

**P6: The Polymerase Chain Reaction ( PCR)**

The **polymerase chain reaction** (**PCR**) is a technique widely used in molecular biology . With PCR it is possible to amplify a single or few copies of a piece of DNA generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling , consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations . Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase , an enzyme originally isolated from the bacterium *Thermus aquaticus* . This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides , by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers ), which are required for initiation of DNA synthesis.

Developed in 1984 by Kary Mullis , PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing , DNA-based phylogeny , or functional analysis of genes ; the diagnosis of hereditary diseases ; the identification of genetic fingerprints (used in forensic sciences and paternity testing ); and the detection and diagnosis of infectious diseases . In 1993 Mullis was awarded the Nobel Prize in Chemistry for his work on PCR

# PCR principles and procedure

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. A basic PCR set up requires several components and reagents. These components include:

1. *DNA template* that contains the DNA region (target) to be amplified.

2. Two *primers* , which are complementary to the DNA regions at the 5' (five prime) or 3' (three prime) ends of the DNA region.

3. Taq polymerase

4. Deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerases synthesizes a new DNA strand.

5. Buffer solution , providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

6. Divalent cations , magnesium or manganese ions; generally $Mg^{2+}$ is used, but $Mn^{2+}$ can be utilized for PCR-mediated DNA mutagenesis, as higher $Mn^{2+}$ concentration increases the error rate during DNA synthesis.

7. Monovalent cation potassium ions.

The PCR is commonly carried out in a reaction volume of 10-200 µl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler . The therm al cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps. The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

1. **Initialization step** : This step consists of heating the reaction to a temperature of 94-96°C which is held for 1-9 min. It is only required for DNA polymerases that require heat activation by hot-start PCR .

2. **Denaturation step :** This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 secs. It causes melting of DNA template

and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

3. **Annealing step :** The reaction temperature is lowered to 50-65°C for 20-40 secs allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5ºC below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

4. **Extension/elongation step** : The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'- phosphate group of the dNTPs with the 3'- hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

5. **Final elongation :** This single step is occasionally performed at a temperature of 70-74°C for 5-15 min after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

6. **Final hold :** This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

**P7: Enzyme Linked Immunosorbent Assay (ELISA)**

ELISA is an assay for detection or quantitation of an antibody or antigen using a ligand (e.g. an anti- immuunoglobin) conjugate to an enzyme which changes the colour of a substrate . The most common assay involves antibody or antigen coupling to enzymes which gives a colored soluble reaction product. Enzyme such as horse radish peroxidase (HRP) and alkaline phosphatase (AP) which are readily available, stable and have a high turnover number, have been widely employed. One clever play for amplifying the phosphatase reaction is to use NADP as a substrate to generate NAD which now acts as a coenzyme for a second enzyme system. Pyrophosphatase from *E. coli* provides a good conjugate because the enzyme is not present in tissue, is stable and gives a good reaction color. Chemiluminescent systems based on enzymes such as luciferase can also be applied. A genetically engineered metapyrocatechase-protein A fusion molecule is a recent innovation for detection of Ig G antibodies.

# Microwell assays

Assays for antigens or antibodies are routinely performed in plastic (PVC or polystyrene) microwell plates. The basis for these assays is the fact that proteins adsorb to the surfaces of many plastics and will remain adsorbed throughout subsequent incubation steps. A micro well assay in which an antiserum is bound to the well via protein A or protein G and the antibody of interest is detected using an enzyme conjugate.

Assays for antigens or antibodies are routinely performed in plastic (PVC or polystyrene) microwell plates. The basis for these assays is the fact that proteins adsorb to the surfaces of many plastics and will remain adsorbed throughout subsequent incubation steps. A micro well assay in which an antiserum is bound to the well via protein A or protein G and the antibody of interest is detected using an enzyme conjugate.

# Advantages

1. It is used to detect antigen (pathogen) and antibody

2. It is used to evaluate the binding of antigens by derivatized antibodies

3. It is used for isotyping of immunoglobulins

4. It provides an extremely efficient means of screening hybridoma cultures (i.e. those secreting antibodies of desired specificity)

5. It provides a quick, qualitative color reaction and it can also be used as a quantitative assay if a plate reader (ELISA reader) is available for reading absorbances.

**P8: Decomposition of organic waste by vermiculture technology**

Vermiculture technology is a process by which all types of biodegradable wastes such as farm wastes, kitchen wastes, market wastes, bio wastes of agro based industries, livestock wastes etc. are converted to nutrient rich vermicompost by using earthworms as biological agents. Earthworms eat cow dung or farm yard manure along with other farm wastes and pass it through their body and in the process convert it into vermicompost. Earthworms not only convert garbage into valuable manure but keep the environment healthy. Conversion of garbage by earthworms into compost and the multiplication of earthworms are simple process and can be easily handled by the farmers. Worm castings contains a high percentage of humus and slow release nutrients that are readily available to plants. Earthworm castings are the best imaginable potting soil for greenhouses or houseplants, as well as gardening and farming.

# Method of preparation of Vermicompost Large/community Scale Vermicomposting

**(Large/community scale vermicomposting)**

A thatched roof shed preferably open from all sides with unpaved (katcha) floor is erected in East-West direction length wise to protect the site from direct sunlight. A shed area of 12'x 12' is sufficient to accommodate three vermibeds of 10'x 3' each having 1' space in between for treatment of 9-12 quintals of waste in a cycle of 40-45 days. The length of shed can be increased/decreased depending upon the quantity of waste to be treated and availability of space. The height of thatched roof is kept at 8 feet from the centre and 6 feet from the sides. The base of the site is raised at least 6 inches above ground to protect it from flooding during the rains. The vermibeds are laid over the raised ground as per the procedure given below. The site marked for vermibeds on the raised ground is watered and a 4"-6" layer of any slowly biodegradable agricultural residue such as dried leaves/straw/sugarcane trash etc. is laid over it after soaking with water. This is followed by 1" layer of vermicompost or farm yard manure.

Earthworms are released on each vermibed at the following rates: For treatment of cowdung/agriwaste: 1.0 kg/ bed. For treatment of household garbage: 1.5 kg/ bed. The loaded waste is finally covered with a Jute Mat to protect earthworms from birds and insects. Water is sprinkled on the vermibeds daily according to requirement and season to keep them moist. The waste is turned upside down fortnightly without disturbing the basal layer (vermibed). The appearance of black granular crumbly powder on top of vermibeds indicate harvest stage of the compost. Watering is stopped for at least 5 days at this stage. The earthworms go down and the compost is collected from the top without disturbing the lower layers (vermibed). The first lot of Vermicompost is ready for harvesting after 2-2 ½ months and the subsequent lots can be harvested after every 6 weeks of loading.

# Multiplication of worms in large scale

Prepare a mixture of cow dung and dried leaves in 1:1 proportion. Release earthworm @ 50 numbers/10 kg. of mixture and mix dried grass/leaves or husk and keep it in shade. Sprinkle water over it time to time to maintain moisture level. By this process, earthworms multiply 300 times within 1to 2 months. These earthworms can be used to prepare vermicompost.

# Advantages of vermicomposting

1. Vermicompost is an ecofriendly natural fertilizer prepared from biodegradable organic wastes and is free from chemical inputs.

2. It does not have any adverse effect on soil, plant and environment.

3. It improves soil aeration, and texture.

4. It improves water retention capacity of soil because of its high organic matter content.

5. It promotes better root growth and nutrient absorption.

6. It improves nutrient status of soil-both macro and micro-nutrients.

# Precautions

1. Vermicompost pit should be protected from direct sun light.

2. To maintain moisture level, spray water on the pit as and when required.

3. Protect the worms from ant, rat and bird.

**P9: Western Blotting**

# Introduction

*The transfer of protein bands from an acrylamide gel onto a more stable and immobilizing support is called as protein blottin or western blotting* . The western blot was described by W. Neal Burnette, of Fred Hutchinson Cancer Research Center in Seattle, Washington(1981). Western blotting is commonly used to positively identify a specific protein in a complex mixture and to obtain qualitative and semi quantitative data about that protein. This method is, however, dependent on the use of a high-quality antibo dy directed against a desired protein. A variety of analysis involving immunoblotting, DNA binding proteins, and glycoproteins could then be performed on the proteins blotted onto the filters. This method is an extension of the “southern blot” used to transfer DNA from gels to nitrocellulose filter and is called as the “western blotting”. The benefits of a protein blot include rapid staining/destaining, detection of proteins at low concentrations and rapid localization of the proteins in preparative gels. The blot can be preserved as a replica of the original gel. The transfer of proteins is carried out either by electrophoresis (electroblotting) or by the capillary action of buffer (capillary blotting).

# Principle

The basic principle be hind Western blotting and immune detection is that of antigen and antibody interaction. An antigen bound to the primary anti body is detected by a secondary antibody labeled with an enzyme, when the substrate is added gives a colored insoluble product, which can be detected visually.

The proteins from bacterial cells[hemolysin], tissue culture or any other source are first separated by SDS -PAGE and are transferred or blotted from the gel to anitro cellulose or nylon membrane that binds proteins non specifically. When this membrane is treated with primary antibody directed against desired protein antigen, if the specific antigens are present , then the anti body will bind to them . T hen th e added Enzyme -labeled secondary antibody recognizes and binds to the primary antibody . The antigen-primary antibody  secondary antibody-enzyme complex is detected when the enzyme converts a soluble, color less substrate into an in soluble, colored product . Thus the colored bands appear on the white membrane wherever a protein antigen interacts with the primary anti body

# Materials

**i) Western Blot apparatus and accessories**

**ii) SDS - PA G E**

Acrylami de stock (30%)

1 .5 M Tris- H CI (pH 8.8)

1 M Tris-HCl (pH 6.8)

10% Ammonium Persulphate 10% SDS

TEMED (N , N , N', N' – Tetramethyl ethylenediamine)

Tris, Glycine , SDS-Running buffer

Sample Buffer 4X

Water saturated n-butanol

**Note:** Polyacrylamide gels are formed from the polymerization of two compounds, acr y lamide and N, N-methylene bisacryl a mide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium per sulfate along with either DMAP or TEMED. The separation of molecules within a gel is determined by the relative size of the pores formed within

the gel . The pore size of a gel is determined by two factors: the total amount of acrylamide present (designated as % T) and the amount of cross-linker (%C) . As the total amount of acrylamide increases, the pore size decreases.

**Rule of thumb:** The smaller the size of the protein of interest, the higher the percentage of polyacrylamide (mono:bisacrylamide). The bigger the size of the protein of interest , the lower the percentage of mono: bis .

**iii)Western blotting**

PBS (10X)

Transfer Buffer

Blocking solution

Primary antibody

Secondary antibody

Developing solution

**II. Preparation of stocks**

**i) A c ryla mide 30% ( 1 0 0 ml)**

A c ry lamide : 2 9 . 2g

N -N bis acrylamid e : 0. 8 g

The above mentioned sub stances are dissolved in 100ml steriled is tilled water and stored in brown bottle at 4 ° C .

**ii) 1.5M Tris-HCI**

18.171 g Tris is dissolved in 100 ml water. The pH is adjusted to 8 .8 using HCl until it stabilizes. Then it is filtered through Whatman No . 1 filter paper and auto claved. (Not e : Tris-HCl can be stored at room temperature )

**iii) 1M Tris- HCI**

6.057g Tris is dissolved in 50 ml water . The pH is adjusted to 6 . 8 using HCI until it stabilizes and then filtered through Whatman No. 1 filter paper and autoclaved . (Note: Tris-HCl can be stored at room temperature)

**iv) 10% SDS**

10 SDS is dissolved in 100 ml of distilled water .

v) **SDS gel running buffer**

Tris: 3 g

Glycine : 14 . 4 g

SDS : 1 g

vi) **Sample loading buffer 4X for 5ml**

1M Tris HCL (pH 6.8) : 2.1ml

100% Glycerol : 1.0 ml

β-Mercaptoethanol : 0.5ml

Bromophenol blue (0.05%): 2.5mg

The above mentioned substances are dissolved and made up to 5 ml with steriled is tilled water.

**vii) Water saturated butanol**

Equal volumes of n-but a nol is added to distilled water and mixed well and allowed it to stand for 10 minutes.

viii) **l0%APS**

0 . 1 g of ammonium persulfate is dissolved in1 ml of water .

Precaution: APS to be freshly prepared before every use

**ix) 5% Stacking gel (2ml)**

| | |
|---|---|
| 30% Acrylamide | 0.33 ml |
| 1 . 0 M Tris (pH ~ 6.8) | 0.25 ml |
| 10% SDS | 0.02 ml |
| 1O%APS | 0.02 ml |
| TEMED | 0.002 ml |

The above mentioned substances are dissolved and made, up to 2ml with sterile distilled water.

x) 10% Separating gel (5ml)

Acrylamide 1.7 ml

1.5 MTris (pH - 8 . 8) 1 . 3 ml

SDS 0 . 05 ml

APS 0.05 ml

TEMED 0.002 ml

The above mentioned substances are dissolved and made upto 5ml with sterie distilled water .

xi**) l0 X PBS (1 L)**

NaCl : 80g

KCI : 2g

$Na_2H$ P0 $_{4:}$ 11.5g

$KH_2P04$ : 2 g

Distilled water : 1000ml

**xii) Transfer Buffer ( 1 L)**

25m M T ris :2 .9 g

190m M G l ycin e : 1 4.5g

20% MethanoI : 200m l

Dissolved in IX PBS. : 800ml

**xiii) Blocking Solution**

5% non-fat dry milk and 0 . 1 % Tween 20 are dissolved in Ix PBS

**xiv) Staining solution**

Coomassie Brilliant Blue (R-250) : 0.001 %

Methanol : 50 %

Acetic acid : 7%

**xv) Destaining Solution**

Methanol : 50 %

Acetic acid : 7%

# Procedure

**1 . SDS-PAGE**

1. A separating gel (10%) was cast in the gel assembly system and a small column of stacking gel was poured over it and an appropriate comb was placed and kept for polymerization. The gel was pre run for 10 min at . 100 V

2. Samples [Extracellular products of hemolysin isolates from *Aeromonas hydrophila*] are prepared by boiling at 100 ' C with an equal Volume of sample buffer for 3-5 min. Electrophoresis is done at 80-100 V till the dye front reached the bottom of the gel .

3. After electrophoresis, the gel was kept in the staining solution (0 . 001 % - Coomassie brilliant blue R-250) for 2h or overnight and the excess stain was removed by destaining till the bands were clear .

# Transferring proteins from the gel to nitrocellulose membrane

1. What man no. 1 filter papers of about six numbers are cut and used as absorbent paper and the nitrocellulose membrane is cut to the exact size of the gel.

2. The membrane and filter papers are soaked in the transfer buffer before stacking for transfer.

3. On a solid plastic platform, two pieces of wet absorbent paper are stacked.

4. Another absorbent paper is placed on the gel in the plate and slowly the gel is transferred on to the stack, placing the gel side up.

5. The wet nitrocellulose membrane is placed on the gel, aligning the edges and the remaining absorbent paper is kept on the stack. (Gel and membrane are sandwiched between stacks of filter paper.)

6. All the air bubbles between the layers are removed by rolling a pipette from the center to the edges.

7. The transfer sandwich is positioned to the electro blotter in such a way that membrane is near the positive electrode. The plates of the apparatus are screwed.

8. The transfer buffer is poured into the transfer apparatus and the electrodes are connected and the power supply is set to ~ 60mA. Transfer is done for one hour.

**Note: More transfer tips:**

i) The gel needs to equilibrate for 15-30 minutes in transfer buffer . Failure to do so will cause shrinking while transferring, and a distorted pattern of transfer .

ii) Avoid touching the membrane with your fingers; use tweezers instead. Oils and proteins on the fingers will block efficient transfer and create dirty blots.

iii) After sandwiching the gel and membrane between paper air bubbles between the gel and membrane can be removed by rolling them out with a pipette or 15ml tube, or by assembling the sandwitch in a dish of transfer .

iv) Add buffer to prevent formation of bubbles in the first place . Wear gloves !

v) Make sure the filter paper and membrane are cut to the same size as the gel .

Large overhangs may prevent a current from passing through the membrane in semi-dry transfers .

# Immunological detection of protein

1. After transfer, the membrane is washed in blocking solution ( 5 % milk powder in 1 X PBS) for 20 min . The solution is discarded and this washing is repeated for 2 -4 times .

( Note: Blocking the membrane prevents non-specific background binding of the primary and / or secondary antibodies to the membrane (which has a high capacity at binding proteins and therefore antibodies).

2. Then primary antibody [Anti hemolysin rabbit serum] (1 : 500) diluted in 30 ml of blockig solution is added . The membrane and antibody mixture are rocked gently for 45 min- Ihr.

3. After incubation the solution is discarded and the membrane is washed with IXPBS for three times.

4. Then secondary antibody (Alkaline phosphatase conjugated goat anti-rabbit antibodies) diluted (l :1000) in 30 ml of the blocking solution is added . The membrane and antibody mixture are rocked gently for Ihr .

5. After incubation the solution is discarded and the membrane is washed with IX PBS for three times.

6.The membrane is developed in dark by adding about 20 ml of developing solution (BCIPINBT solution- Generally, BCIPINBT Phosphatase Substrate deposits a permanent, dark purple stain on membrane sites bearing phosphatase)

**P10: Digestion of DNA with Restriction Enzymes**

Restriction Enzymes (REs) recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequences. RE cleavage of DNA into discrete fragments is one of the most basic procedures in molecular biology. A number of factors affect RE reactions. Contaminants found in some DNA preparations (e.g., protein, phenol, chloroform, ethanol, EDTA, SDS, high salt concentration) may inhibit RE activity. The effects of contaminants may be overcome by increasing the number of enzyme units added to the reaction mixture (10 to 20U per microgram DNA), increasing the reaction volume to dilute potential inhibitors, or increasing the duration of incubation. Digestion of genomic DNA can be facilitated by addition of the polycation spermidine (final conc 1 to 2.5 mM) which acts by binding negatively charged contaminants. Larger amounts (up to 20-fold more) of some enzymes are necessary to cleave supercoiled plasmid or viral DNA as compared to the amount needed to cleave linear DNA. In addition, some enzymes cleave their defined sites with different efficiencies, presumably due to differences in flanking nucleotides. Some REs are inhibited by methylation of nucleotides within their recognition sequences.

The typical RE buffer contains $MgCl_2$, NaCl or KCL, Tris-Cl, b -ME or DTT, and BSA. A divalent cation, usually $Mg^{2+}$, is an absolute requirement for enzyme activity. Some are very sensitive to the concentration of $Na^+$ or $K^+$ ion, while others are active over a wide range of ionic strength.

# Digesting DNA sample with a single RE

RE cleavage is accomplished simply by incubating the enzymes(s) with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentration, and the temperature and duration of reaction may vary depending upon the specific application and source of the RE.

# Materials

1 mg/ m l DNA sample in $H_2O$ or TE buffer

10 ´ RE buffers

REs

0.5 MEDTA, pH 8.0

3M Sod. Acetate (pH 5.2)

Phenol saturated with Tris-Cl (pH 8.0)

24:1 Chloroform-isoamyl alcohol mix

Absolute alcohol, 70% ethanol

# Method

1. Pipette the following in 1.5 ml microcentrifuge tube:

50 m l DNA (50 m g DNA in $H_2O$ or TE buffer)

50 m l 10X restriction buffer

140 m l deionized $H_2O$

10 m l RE (10 U/ m l)

2. Incubate the reaction mixture overnight or 4 hrs at the recommended temp (in general, $37^0$C)

3. Stop the reaction by adding 0.5M EDTA (pH 8.0) to a final concentration of 10mM.

4. Extract once with phenol: chloroform isoamyl alcohol and once with chloroform isoamyl alcohol and precipitate the DNA with ethanol for 2-4 hrs at $-20^0$C.

5. Collect the DNA by centrifuging for 15 min, wash once with 70% ethanol, air dry the pellet and dissolve in 25 m l 1 X TE

6. If digestion is incomplete add second shot of the enzyme and repeat the above steps.

# Resolution of restriction fragments on agarose gel

**Materials**

Electrophoresis buffer -50 X TAE

EtBr solution (10 mg/ml)

Agarose, electrophoresis-grade

6X loading buffer

DNA m.w markers

**Method**

1. Prepare the 1% gel, using electrophoresis buffer (final conc 1X) and electrophoresis-grade agarose by melting in a microwave oven, cooling to $55^0$C, add EtBr to a final conc of 0.5 m g/ml, pouring into a sealed gel casting platform, and inserting the gel comb.

2. After the gel has hardened, remove the seal from the gel casting platform and withdraw the gel comb. Place into an electrophoresis tank containing sufficient electrophoresis buffer (1X) to cover the gel ~1mm.

3. Take OD of the DNA samples at 260nm.

4. Prepare DNA samples (15-20 m g) with water and an appropriate amount of 6X loading buffer and load samples into wells with a pipette. Load appropriate DNA m.w. markers.

5. Attach the leads so that the DNA migrates to the anode or positive lead and electrophorese at 5 V/cm of gel.

6. Turn off the power supply when the bromophenol blue and xylene cyanol dye from the loading buffer have migrated a distance judged sufficient for separation of the DNA fragments.

7. Photograph a stained gel directly on a UV transilluminator.

8. Put a transparency on the gel and under UV-illumination , mark the m.w markers with the help of a transparency marking pen. Keep the transparency for alignment with the X-ray film of Southern blot.

**P11: Southern blotting**

# Southern blotting

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane support resulting in immobilization of the DNA fragments. So the membrane carries a semi permanent reproduction of the banding pattern of the gel. Southern hybridization technique was developed by E.M. Southern (1975)

There are three methods of transfer of DNA fragments from agarose gels to solid support

1. Capillary transfer

2. Electrophoretic transfer

3. Vacuum transfer

Vacuum transfer is the most efficient and rapid method. Depurinated and denatured DNA is transferred in approximately 30-45 mins from gels of normal thickness (4-5 mm) and normal agarose concentration (0.7-1.0%)

# Materials

Depurination solution: 0.25M HCL

Denaturation solution: 1.5M NaCl, 0.5 M NaOH (store at Room temperatue)

Neutralization solution: 1.5M NaCl, 1M Tris Cl, pH 7.5 (store at Room temperatue)20X and 2X SSC

Nylon or nitrocellulose membrane

# Method

1. Place the gel in a clean, baked glass baking tray.

2. Depurinate the DNA in the gel with 0.25M HCL for 8 min (until the Bromophenol blue turns yellow), with occasional shaking. Rinse 2-3 times with distilled water.

3. Denature the DNA twice by soaking the gel for 15 min in several vol. of denaturation solution, with constant gentle shaking. Again rinse the gel 2-3 times with distilled water.

4. Neutralize the DNA twice by soaking the gel for 15 min in several vol. of neutralization solution. Then finally soak the treated gel in transfer solution (20X SSC)

5. Connect the pump inlet on the front panel to the liquid trap and connect the trap to the base of the VacuGene XL blotting unit.

6. Pre-wet the nylon membrane as recommended (either in $H_2O$ or 2X SSC) and place it on the porous screen (nitrocellulose membranes can be sensitive to the NaOH solution). Place the plastic mask on the membrane in such a way that it overlaps on each side of the membrane by approximately 5mm.

7. Fit the top frame and secure it by tightening the four locking clamps.

8. Starting with one of the gel edges, let it gradually slide onto the membrane. Avoid trapping air bubbles. Make sure that gel and mask overlap by atleast 2mm. Small cracks and the walls in the gel must be filled with melted agarose to get a good seal. (All leakages can be sealed with agarose preferably low melting point agarose).

9. Switch on the VacuGene XL pump. Stabilize the vacuum between 50 and 55 mbar. The gel must always be covered with transfer solution when the vacuum is on.

10. Carry–on the transfer for approximately 90mins. Ensure that the gel remains immersed during this time. Remove the transfer solution.

11. Turn off the pump. Mark the wells. Remove the gel.

12. Wash the filter in 6X SSC, to eliminate any bit of adhering agarose. Air dry the filter for 30 min. Bake the filter at $80^0$C for 2 hours under vacuum (200 mbar).

**P12: Random primer labeling of DNA**

# Random primer labeling of DNA

The Klenow fragment of *E. coli* DNA polymerase I carries the polymerase and 3'→5' exonuclease activities but lacks the 5'→ 3' exonuclease activity. This method originally developed by Feinberg and Vogelstein (1983) employs its 5'→ 3' polymerase activity. Synthetic random primers of 6-10 bases are used as primers which form hybrids at many positions along the template. The template for labeling must be denatured prior to the reaction.

# Materials

10X labeling buffer

0.5M Tris-Cl, pH 7.5, 0.1M $MgCl_2$, 10mM DTT, 0.5mg/ml BSA

dCTP, dGTP and dTTP (0.5mM in Tris-Cl pH 7.0)

a $^{32}$P dATP – 3000 Ci/mmole

Klenow polymerase 10units/ml

Sephadex G-50

Silliconized glasswool

TE buffer

**Method**

1. Digest DNA with an appropriate RE. Purify by gel electrophoresis or ethanol precipitation. Resuspend in TE buffer.

2. Dossolve 25ng of template DNA in nuclease free water (1-34 m l)

3. Denature in boiling water for 5 min and chill on ice for 5 min. Centrifuge briefly.

4. Add the following to the DNA

5.0 m l 10X Klenow fragment buffer

6.0 m l dNTP mix (2 m l of 0.5mM dTTP, dCTP, dGTP)

4.0 m l 3000Ci/mmol [ a $^{-32}$ P]dATP (40 m Ci)

1.0 m l Klenow fragment (5U)

5. Incubate for 1hr at $37^0$C.

6. Stop reaction with 1 m l of 0.5M EDTA.

7. Separate labeled DNA from unincorporated radioactive precursors by column chromatography.

8. Remove 1 m l aliquot and determine $^{32}$P labeling by Scintillation counter. Specific activity should be $10^9$ cpm/ m g DNA.

# Removal of unincorporated nucleotides by spin-column chromatography

1. Plug a 1ml syringe with glass wool, and add swollen sephadex G-50 fine resin (Pharmacia) unto top.

2. Place the syringe inside a 40ml centrifuge, spin at 1000g for 5 min.

3. Add the sample at the top of the column. Place a 1.5 ml tube before placing the column inside the centrifuge tube. Confirm that the outlet of the column hangs inside the microfuge tube.

4. Spin at 1000g for 5 min

5. Take out the microfuge tube carefully. The elute in the microfuge tube can be used without further purification.

**P13: Hybridization analysis of DNA blots**

The principle of hybridization analysis is that a single stranded DNA or RNA molecule of defined sequence (the "probe" which is usually labeled) can base-pair to a second DNA or RNA molecule that is immobilized and contains a complementary sequence (the "target"), with the stability of the hybrid depending on the extent of base pairing that occurs. The technique permits detection of single copy genes in complex genomes. This protocol is suitable for hybridization analysis of Southern transfer and dot and slot blots with a radioactively labeled DNA probe 100 to 2000 bp in length.

# Materials

Probe DNA labeled to a specific activity > 1 X $10^8$ dpm/ m g

20 X SSC

50X Denhardts solution

200 m g/ml denatured Salmon sperm DNA

10%(w/v) SDS

Hybridization solution

Prehybridization solution + denatured probe + 0.1mM EDTA pH 8.0

# Method

1. Wet a membrane carrying immobilized DNA in 2X SSC

2. Place the membrane in a polythene bag, and add ~1 ml prephybridization solution per

5$Cm^2$ of membrane. Incubate 3 hr in hybridization incubation oven with occasional

shaking at $60^0$C

3. Just before the end of the prehybridization incubation, denature probe DNA 10min at $100^0$C. Place in ice.

4. Pour out the prehybridization solution from the polythene bag and replace with half the vol. of prewarmed ($60^0$C) hybridization solution. Add denatured probe and incubate with shaking overnight at $60^0$C.

5. Pour out the solution and take out the membrance in a glass tray containing 6X SSc and 0.5% SDS. Keep on shaking the tray for 15min at RT. Change the wash solution after 15min.

1. Replace the wash solution with an equal vol. of 2 X SSC and 0.5% SDS and incubate for 10min at RT. Change the wash solution after 15 min.

7. Carry out 15-min medium stringency wash using $45^0$C 2X SSC and 0.5% SDS.

8. If desired, carry out two 15-min high-stringency washes in 2X SSC and 0.5% SDS at $50^0$C.

9. Pour off the final wash solution and blot excess liquid. Wrap in plastic wrap. Carry out autoradiography by placing an X-ray film over the membrane inside an X-ray film holder with intensifying screen at $-70^0$C overnight.

**P14: Immunofluorescence**

Immunofluorescence is an antigen-antibody reaction where the antibodies are tagged (labelled) with a fluorescent dye and the antigen-antibody complex is visualized using ultra-violet (fluorescent) microscope. Fluorochromes are dyes that absorb ultra-violet rays and emit visible light. This process is called fluorescence. Commonly used fluorochromes are Acridine Orange, Rhodamine, Lissamine and Calcofluor white. However, these fluorochromes are used for general fluorescence. When fluorescein (FITC) is excited by a blue (wavelength 488nm) light, it will emit a green (520nm) colour. Phycoerythrin (PE) emits an orange (570nm) colour. The fluorochromes commonly used in immunofluorescence are fluorescein isothiocyanate (green) and and tetramethyl rhodamine isothiocyanate (red)

# Types of immunofluorescence

· Direct immunofluorescence

· Indirect immunofluorescence

# Direct immunofluorescence

The procedure for the direct immunofluorescence is similar to that of the indirect method except for the primary antibody (eg. Monoclonal antibody to HPV antigen raised in rabbits) is conjugated to the fluorochrome. Hence, the procedure would involve blocking, incubating with the primary antibody, washing and

examining the specimens after covering with antifade. Use of monoclonal antibody is of advantageous because of the specificity.

# Indirect immunofluorescence

### Materials required

10X Phosphate buffered saline (PBS)

Formaldehyde , 16%, methanol free

Blocking Buffer (1X PBS with 5% normal goat serum and 0.3% Triton X-100) Antibody Dilution Buffer (1X PBS with 1% BSA and 0.3% Triton X-100) Fluorochrome-conjugated secondary antibody

Antifade Reagent

Xylene

Ethanol , anhydrous denatured, histological grade, 100% and 95%

Antigen Unmasking: 10 mM Sodium Citrate Buffer pH (6.0) or 1 mM EDTA pH (8.0)

### Specimen preparation

### a. Cultured cells

Aspirate the medium and fix with 4% formaldehyde in PBS for 15 min at room temperature. Remove the fixative and rinse three times in PBS and proceed for Immunostaining

### b. Paraffin sections

# Deparaffinization/Rehydration

1. Incubate sections in three washes of xylene for 5 minutes each.
2. Incubate sections in two washes of 100% ethanol for 10 minutes each.
3. Incubate sections in two washes of 95% ethanol for 10 minutes each.
4. Rinse sections twice in $dH_2O$ for 5 minutes each.
5. **Antigen Unmasking**

6. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0, then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
7. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.
8. **a. Frozen tissue sections**
9. Fix the tissue sections with 2-4% formaldehyde in PBS for 15 min at room temperature. Rinse the slides in PBS three times for 5 min each. Proceed for immunostaining.

# Immunostaining

1. Block specimen in Blocking Buffer for 60 minutes.
2. While blocking, prepare primary antibody by diluting to the appropriate dilution in the antibody dilution buffer
3. Aspirate blocking solution, apply diluted primary antibody (eg. Antibody against HPV antigen raised in rabbits).
4. Incubate overnight at 4°C.
5. Rinse three times in PBS for 5 minutes each.
6. Incubate specimen in fluorochrome-conjugated secondary antibody (mouse anti-rabbit) of appropriate dilution in Antibody Dilution Buffer for 1–2 hours at room temperature in dark.
7. Rinse in PBS as in step 5.
8. Cover the specimen with antifade reagent
9. Examine specimens immediately using appropriate excitation wavelength. For long-term storage, store slides flat at 4°C protected from light.

**P15: Immunohistochemistry**

Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context. While there are multiple approaches and permutations in IHC methodology, all of the steps involved are separated into two groups: sample preparation and labeling.

# Materials required

· *Xylene*

· Ethanol, anhydrous denatured, histological grade (100% and 95%)

· Deionized water ($dH_2O$)

· Hematoxylin (optional)

· Wash Buffer: 1X TBS/0.1% Tween-20 (1X TBST)

· 10X Tris Buffered Saline (TBS) 24.2 g Trizma® base ($C_4H_{11}NO_3$) and 80 g sodium chloride (NaCl) to 1 L $dH_2O$. Adjust pH to 7.6 with concentrated HCl.

· Antibody Diluent: TBST/5% normal goat serum

· Antigen Unmasking- Citrate ( 10 mM Sodium Citrate Buffer) or EDTA ( 1 mM EDTA)

· 3% Hydrogen Peroxide: To prepare, add 10 ml 30% $H_2O_2$ to 90 ml $dH_2O$.

· Blocking Solution

· *Secondary antibody conjugate to enzyme*

· Substrate solution (DAB: 3,3´-diaminobenzidine tetrahydrochloride or NBT-BCIP: 5-bromo-4-chloro-3-indolyl-phosphate/ nitro-blue tetrazolium)

# Methodology

Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

- Incubate sections in three washes of xylene for 5 minutes each.
- Incubate sections in two washes of 100% ethanol for 10 minutes each.
- Incubate sections in two washes of 95% ethanol for 10 minutes each.
- Wash sections twice in $dH_2O$ for 5 minutes each.

# Antigen Unmasking

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.

# Staining

1. Wash sections in $dH_2O$ three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in $dH_2O$ twice for 5 minutes each.

4. Wash sections in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100-400 µl primary antibody diluted in recommended antibody diluent to each section. Incubate overnight at 4°C.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl of the secondary antibody, diluted in TBST to each section. Incubate 30 minutes at room temperature.
9. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
10. Add 100-400 µl of the substrate solution to each section and incubate for 30 minutes at room temperature.
11. Remove the substrate and wash sections three times in water for 5 minutes each.
12. If desired, counterstain sections in hematoxylin
13. Wash sections in $dH_2O$ two times for 5 minutes each.
14. Dehydrate sections:
    1. Incubate sections in 95% ethanol two times for 10 seconds each.
    2. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
    3. Repeat in xylene, incubating sections two times for 10 seconds each.
15. Mount the slides for viewing.

**P16: Immunodot**

ImmunoDOT utilizes an enzyme-linked immunoassay (EIA) dot technique for the detection of antibodies/ antigens. The suspected sample/ test antigen is dispensed as discrete dots onto a solid membrane. After adding specimen to a reaction vessel, an assay strip is inserted, allowing test antibodies (in the case of antibody detection) or primary antibody (in case of antigen detection) reactive with the test antigen/ sample to bind to the strip's solid support membrane. In the second stage, the reaction is enhanced by removal of non-specifically bound materials. During the third stage, an enzyme conjugated secondary antibody is allowed to react. Finally, the strip is transferred to a substrate reagent, which reacts to produce an easily seen, distinct dot.

# Materials required

· Nitrocellulose (NC) or polyvinylidene fluoride ( PVDF ) membrane

· Suspected sample or test antigen

· Test antibody

· Purified primary antibody ( IgG )

· Secondary antibody conjugated to enzyme (HRP or alkaline phosphatase)

· Substrate solution (DAB: 3,3´-diaminobenzidine tetrahydrochloride or NBT-BCIP: 5-bromo-4-chloro-3-indolyl-phosphate/ nitro-blue tetrazolium)

· Phosphate buffered saline (PBS)

· PBST (PBS with 0.5% Tween 20)

· Blocking solution (0.5% skim milk powder solution in PBS)

· Hydrogen peroxide

# Protocol

1. Dot 1-2 ul of the test sample (positive antigen) in case antibody detection or the suspected sample suspension in case antigen detection on to a NC or PVDF membrane and air dry.

2. Incubate the membrane in the blocking solution for 1 hr at $37^{o}$C

3. Rinse the membrane for three times in PBST

4. Incubate the membrane in standardized dilution of test antibody (for antibody detection) or the primary antibody for 1 hr at $37^{o}$C

5. Rinse the membrane for three times in PBST for three times 5 minutes each

6. Incubate the membrane in standardized dilution of secondary antibody enzyme conjugate

7. for 1 hr at $37^{o}$C

8. Rinse the membrane for three times in PBST for three times 5 minutes each

9. Expose the membrane to the substrate solution

10. The positive result are visible as a dot

## P17. Manipulating a DNA Sequence

### A. Reverse complement and other tools

Reverse Complement converts a DNA sequence into its reverse, complement, or reverse-complement counterpart. There are many cases where one might want to work with the reverse-complement of a sequence if it contains an ORF on the reverse strand

- Open **http://www.vivo.colostate.edu/molkit/manip/**

- The tool contains a number of application for nucleic acid sequence analysis.

- In the program enter the DNA sequence into the upper text box and click on the appropriate button.

- The result will be displayed on the lower box

### B. Translating DNA to six reading frames

This tool allows the six frame translation of a nucleotide (DNA/RNA) sequences to a protein sequence in order to locate open reading frame in your sequence

Go to URL **http://www.expasy.ch/tools/dna.html**

Paste your sequence in the box provided and click “Translate Sequence”

You can choose from three options

- Verbose – puts Met & Stop to highlight start and stop codons

- Compact – useful if you want to use output in other programs

- Include nucleotide sequence – nucleotide sequence is above the translation

This returns a six-frame translation of your sequence. You can then choose the correct frame.

### C. Oligo Calculator

**– http://www.pitt.edu/~rsup/OligoCalc.html**

Tool to calculate the length, %GC content, Melting temperature(Tm) and Molecular weight (daltons)

Go to the above URL

Paste your sequence in the box provided and click “Calculate”

**Example:**

## P18: BLAST

### A. Data retrieval from GenBank

1. Access the internet via Internet Explorer or Netscape

2. Type http://www.ncbi.nlm.nih.gov in the location box and press enter

3. From the NCBI All database list click on ‘Nucleotide’

4. Enter the nucleotide name or accession number and click Go

5. IN the results page scroll down to see if any of the exact nucleotide name matches.

6. Download and save the sequence for further analysis

### B. Sequence search using BLAST

Let us take an example of how to carry out a database search. Assuming that we have a nucleotide sequence named Unknown Sequence#1, and we wish to know whether there are any nucleotide sequences in the database similar to Unknown sequence#1, the search can be performed using the following procedures

7. Access the internet via Internet Explorer or Netscape

8. Type http://www.ncbi.nlm.nih.gov in the location box and press enter

9. From the tools list click on BLAST

10. The main BLAST page appears displaying the d ifferent BLAST programs. Choose blastn for aligning nucleotide sequences

You are now in the NCBI BLAST program. From the top to the bottom, there are a number of blank boxes to be filled in or prechosen as defaults.

Click on the **Choose database** link to find out information about the databases that can be used in this nucleotide BLAST search. Once a suitable structure database name has been found close the popup window.

11. In the sequence box, type the name of the sequence in the first line. The > must be included immediately before the first letter, otherwise, the database will not

recognize the name and treat it as an unknown sequence. The BLAST web pages accept input sequences in three formats; **FASTA sequence format**, **NCBI Accession numbers**, or **GIs**. Start from the second line, type a sequence or paste a sequence cut from another file

**BLAST Search Parameters**

## 1. Histogram

Display a histogram of scores for each search; default is yes

## 2. Descriptions

Restricts the number of short descriptions of matching sequences reported to the number specified

## 3. Alignments

Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported.

## 4. Expect

The statistical significance threshold for reporting matches against database sequences; the default value is 10.

## 5. Cutoff

Cutoff score for reporting high–scoring segment pairs (HSP). The default value is calculated from the EXCEPT value HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF values are more stringent, leading to fewer chance matches being reported. Typically, significance thresholds can be more intuitively managed using EXPECT.

**6. Matrix**

Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and TBLASTX. The default matrix is BLOSUM62.The valid alternative choices include : PAM40, PAM120, PAM250

## 7. Strand

Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

**8. Filter**

Mask off segments of the query sequence that have low compositional complexity or segments consisting of short-periodicity internal repeats. Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic, - basic – or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence and the letter "X" in protein sequences Users may turn off filtering by using the "Filter" option on the "Advanced options for the BLAST server" page.

**9. NCBI-gi**

Causes NCBI gi identifiers to be shown in the output, in addition to the accession and /or locus name.

**P19: Multiple sequence alignment**

A **multiple sequence alignment (MSA)** is a sequence alignment of three or more biological sequences, generally protein, DNA, or RNA. In many cases, the input set of query sequences are assumed to have an evolutionary relationship by which they share a lineage and are descended from a common ancestor. From the resulting MSA, sequence homology can be inferred and phylogenetic analysis can be conducted to assess the sequences' shared evolutionary origins. MSA's are used for revealing important conserved residues, for making phylogenies, for secondary structure prediction etc.

In this practical we will make a multiple alignment of a protein sequence family using ClustalW and calculate a tree from the sequences. ClustalW is a general purpose global multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences.

Step 1: ***Getting a set of DNA sequences***

We will consider aligning the several tropomyosin sequences, represented by the accession numbers below.

BF056441, BE8487196, BF022813, BF452255, BG089808, BG147728, BI817778, AF186109, AF186110, AF310722, AF362886, AF362887, AF087679, SSAJ803, SSAJ804

Sequences to be downloaded and entered in the following format.

>Sequence1
ATGAAGGATGAGGAGAAGATGGAGATTCAGGAGATGCAGCTCAAAGA
GGCCAAGCACATTATGAAGGATGAGGAGAAGATGGAGATTCAGGAGA
TGCAGC TCAAAGAGGCCAAGCACATT
>Sequence2
GCAGACGACGCANAGGATCGCGCGCAAGGCCTGCAGCGCGAACTGGA
TGGCGAGCTCTAGGCGGACGAGGCAGAGGATCGCGCGCAGGGCCTGCA
GCGGGAGCTGGACGGCGAGCGCGAG
>Sequence3
GCGGAAGAGGCTGACCGCAAATACGAGGAGGTAGCTCGTAAGCTGGTC
ATCCTGGAGGGTGCGGACGAGGCCGAAGAGCGGGCCGAGATCCTGCA
GAGGGAGGTGGACGCCGAGAGGCAG

**Step 2: Uploading and running the programme**

Consider the following **ClustalW** submission form:

☐ The multiple sequences were uploaded or pasted in the box provided in FASTA format, which consists of a one-line header starting with a ">" symbol, followed by the sequence name/description. The sequence is then entered on new line(s).

**Step 3: Result of a ClustalW alignment**

**Consensus symbols**

**“***" means that the residues or nucleotides in that column are identical in all sequences in the alignment.

":" means that conserved substitutions have been observed.

"." means that semi-conserved substitutions are observed.

☐ Other options are left on "default".

☐ The program is started by clicking on the ‘Run’ button

**Step 4: ClustalW guide tree**

The guide tree is available for download as a .dnd file and contains the information for building the cladogram or phylogram. The ".dnd" file is a file that describes the phylogenetic tree. A Phylogram is a branching diagram (tree) assumed to be an estimate of a phylogeny, branch lengths are proportional to the amount of inferred evolutionary change. A Cladogram is a branching diagram (tree) assumed to be an estimate of a phylogeny where the branches are of equal length, thus cladograms show common ancestry, but do not indicate the amount of evolutionary "time" separating taxa.

These options are now in controlled with new buttons in the output file as well as a pop up menu, that is available by right-clicking on the applet. The buttons on the page include "Show as Phylogram Tree", "Show as Cladogram Tree" and "Show Distances".Tree distances can be shown, just click on the diagram to get a menu of options.