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NET LIFE SCIENCE

APPLIED BIOLOGY

FOR CSIR NET LIFESCIENCE

MODULE 12

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A1. MICROBIAL FERMENTATION

Fermentation has always been an important part of our lives: foods can be spoiled by microbial fermentations, foods can be made by microbial fermentations, and muscle cells use fermentation to provide us with quick responses.

When respiration is not an option, organisms must use an alternative mechanism for the regeneration of the limited supply of coenzymes, reduced during the oxidation of glucose to pyruvate. If NAD(P)H is not oxidized back to NAD(P)⁺, further catabolism will cease. Consequently, a suitable terminal electron acceptor must be found to take the electrons.

Fermentations use an organic molecule, pyruvate or a derivative, as this final electron acceptor, thereby regenerating NAD(P)⁺ and allowing catabolism to continue. This results in the formation of reduced 'waste' products, such as alcohols and acids, that are then excreted from the cell (Figure 1).

However, this is very wasteful in terms of recovery of potential energy from glucose, as little or no further ATP is formed. Conversely, complete oxidation to CO₂, in conjunction with oxidative phosphorylation during respiration, can generate a possible further 36 molecules of ATP per glucose molecule.

Alcoholic fermentation is performed by yeasts and certain filamentous fungi and bacteria. It is a two step process, where pyruvate from the EMP pathway, or via the ED pathway in the case of *Zymomonas*, is first decarboxylated to acetaldehyde; NAD⁺ is then regenerated during reduction of acetaldehyde to ethanol (Figure 1a).

Lactic acid fermentations are carried out by a number of bacteria, including *Streptococcus*, *Lactobacillus*, *Lactococcus* and *Leuconostoc*, along with some fungi, algae and protozoa. Here pyruvate, rather than a derivative of pyruvate, is the electron acceptor and forms lactate. There are two forms of this fermentation:

1. Homolactic fermentation is operated by bacteria such as *Lactobacillus acidophilus* and *Lactobacillus casei*, which reduce virtually all pyruvate generated by glycolysis to lactic acid (Figure 1b); this also occurs in animal muscle deprived of oxygen; and

2. Heterolactic fermentation generates other products along with the lactic acid (Figure 1c). The organisms that perform it include *Leuconostoc mesenteroides* and *Lactobacillus brevis*, which operate the phosphoketolase pathway. They form lactate from pyruvate and ethanol from acetyl phosphate, and in certain cases some acetate may be produced.

Mixed acid fermentation is carried out by *E. coli* and related facultative anaerobes. The products include lactate, acetate, small quantities of ethanol and formate. Some organisms have the ability to further reduce formate to hydrogen and CO₂ (Figure 1d).

2,3-Butanediol fermentation is performed by *Enterobacter*, *Erwinia*, *Klebsiella* and *Serratia*. It is similar to the mixed acid fermentation, but generates butanediol, along with ethanol and acids (Figure 1e).

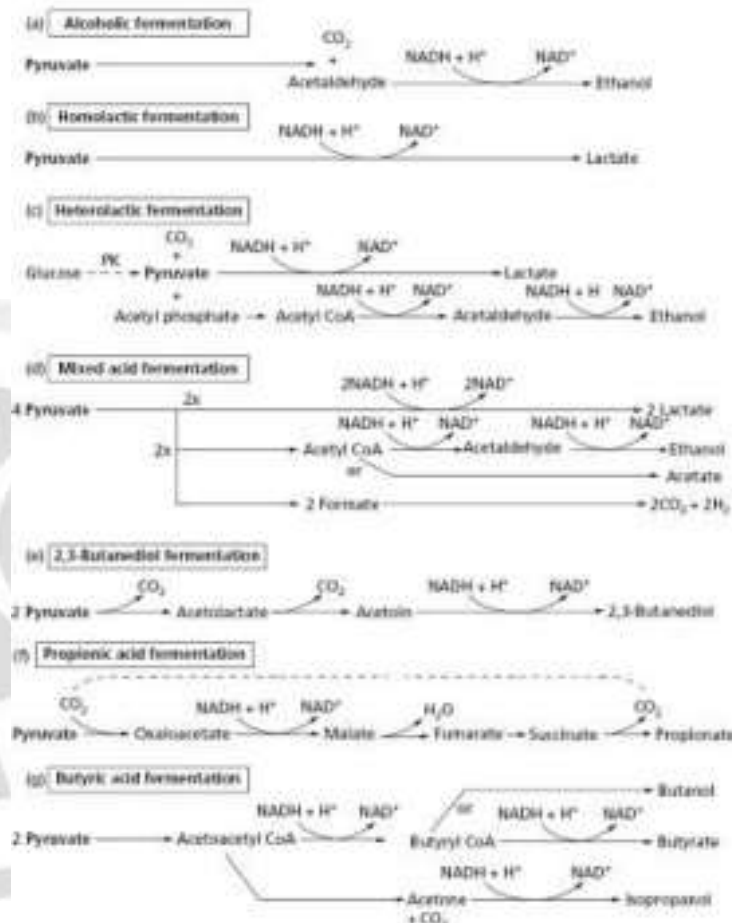


Fig. 1 Common fermentation products derived from pyruvate.

Propionic acid fermentation is conducted by several gut bacteria, such as species *Propionibacterium*, some of which are involved in the commercial production of certain Swiss-type cheeses and vitamin B₁₂ (cobalamin). The propionate is formed from pyruvate via the methyl malonyl CoA pathway, where pyruvate is carboxylated to oxaloacetate, and then reduced to propionate via malate, fumarate and succinate (Figure 1f). This requires the important cofactors, biotin, CoA and CoB₁₂.

Butyric acid fermentation is carried out by species of *Clostridium*. These anaerobic spore formers also produce acetone, butanol, propanol, other alcohols and acids (Figure 1g). They also ferment amino acids and other nitrogenous compounds, as well as carbohydrates.

Stage – 3: Plantlet establishment, elongation and rooting

After repeated subcultures and screening the resulting plantlets are transferred to the final in vitro stage. This stage is designed to arrest rapid multiplication and to induce the establishment of fully developed plantlet. Shoot elongation, root formation, and when required, formation of storage organs (bulb, corms and tubers). This stage also provides the conditions for stimulations of photosynthesis and other physiological changes that are required for autotrophic, ex vitro growth in the acclimatization stage. This is achieved by modifications in culture media (e.g. reduction of cytokinins concentrations, increased levels of auxins, reduced sugar levels etc.) and by modifying environmental conditions.

Stage-4: Acclimatization

The healthy plantlets that come out from the culture vessels are usually incapable of existence in natural field or green house conditions. 4-8 weeks in an acclimatization green house provide a weaning process. The first few days in green house are spent under low light, high temperature and high humidity conditions, often provided by fogging or an incubator for ex vitro plants. As cuticular waxes, stomatal function, and new functional roots develop; photosynthetic activity is increased and plant become ocytotrophic. Over the following few weeks light intensity is raised, and ambient temperature and humidity are regulated to natural environment or growing conditions. In vitro plants that are unable to survive this stage must be considered unsuccessful. Organs initiated in cultures are defined by the culture environment and they have the same characteristics as they did in vitro after transfer to green house. A gradual return to normal characteristics occurs during the acclimatization period in green house.

2 Applications of micropropagation:

Micropropagation offers significant advantages in quality, quantify and economics over conventional methods of vegetative propagation for many species. Some of these are –

- Production of a very large number of clonal propagules within a short time span – the techniques of plant tissue culture given an exponential increase of the propagation. Depending upon the multiplication rate, thousands and millions of plants can be rapidly produced in vitro.
- Production of disease-free plant material with the possibility of eliminating viral, bacterial and fungal contamination.
- Production of large stock of true-to-type clonal propagation material.
- The ability of air ship large quantities of plants material quickly, efficiently and relatively inexpensively.

- The possibility of bringing newly bred plants and selections to market quickly in large quantities.
- Elite selection
- Product uniformity
- Genetically engineered products

C2. Techniques for Micropropagation

2.1 Plant Cell Culture:

It was Haberlandt in 1890s, which put forth the pioneering attempt to culture mechanically isolated mesophyll cells. He succeeded in maintaining these cells alive for 10 days in culture but it did not divide. He failed to achieve division of isolated cells. Since then, the progress in the field of isolating single cell bur and culturing individual cells has been spectacular that it has become possible not only to culture isolated single cells but also induce divisions in such cells in complete isolation and finally , to regenerate whole plant from them. The most important aspect that deserves attention in the isolated cell culture technology is the initiation of division and proliferation of isolated singer cells which starts only when they are placed into conditioned medium. Isolated single plant cell resembles with animal cells in this respect. However, a conditioned medium is prepared by culturing high densities of cells of same or different species on same fresh medium for some days and then the cultured cells are removed by filter sterilization making the medium free of them. This medium now contains essential amino acids such as glutamine and serine as well as growth regulators such as cytokinins. When the isolated single cell are placed in the conditioned media in exactly the same way as microorganisms, they initiate the cell division and instead, of developing a colony as do the microorganism they proliferate to form a callus. The latter is induced to regenerate a plant.

2.2. Applications of cell culture:

- 1. Resistant to diseases:** Tobacco protoplasts selected from resistance to methionine sulphoximine showed enhanced resistance to *Psuedomonas tabaci*.
- 2. Resistant to herbicides:** In the recent past the fertile plants resistant to herbicides such as amitrol, 2,4-D, sodium chlorate, Picloram, Cholorosulfuron and sulfometuron methyl have been selected from cultivated cells of *Nicotiana*.
- 3. Resistant to amino acids and their analogues:** A Potato cell line resistant to 5-methyl tryptophan has been used to demonstrate the presence of two possible Isoenzymes of anthranilate synthase the first regulatory enzyme unique to tryptophan biosynthesis.
- 4. Secondary metabolic production:** A few but well known established examples of secondary metabolite production in high amounts by selected cell lines are- Glutathione and Nicotine from *Nicotiana tobacum*, Anthraquinones from *Merinda citrifolia*, Rosmarinic acid

This approach has been made for developing resistance against at least three herbicides viz. glyphosate, sulphonyl urea and imidazolinones.

a. Resistance against glyphosate: Glyphosate, an active components of round up herbicides, is used as nonselective post-emergence herbicides. It is suggested that glyphosate inhibits aromatic amino acid biosynthesis by competitive inhibitor of enzyme 5-enol-pyruvyl shikimate-3 phosphate synthase [EPSPS]. EPSPS catalyzes the synthesis of phenylalanine, tyrosine and tryptophan. The *aro A* gene which codes EPSPS has been isolated and sequenced from *E. coli*, *Salmonella typhimurium* and *Arabidopsis thaliana*. There are two possible methods for producing glyphosate resistant plants by genetic engineering: (a) transfer of a glyphosate sensitive EPSPS under the control of a powerful promoter causing over expression of the protein' and/or (b) transfer of gene which codes a mutated, glyphosate resistant EPSPS. cDNA of the EPSPS gene with 35 S cauliflower mosaic virus

An alternative method is insertion and expression of mutated bacterial *aro A* gene coding for glyphosate resistant EPSPS. The source of the *aro A* gene was a mutagenized strain of *S. typhimurium* or *E. coli* and was transferred to tomato and/or tobacco. Glyphosate oxidase gene (*gox*) also provide resistance against glyphosate.

b. Resistance against sulfonylurea and imidazolinone herbicides: The herbicidal action of sulfonylureas and imidazolinones is based on their ability to inhibit non-competitively an enzyme of branched chain amino acid biosynthesis, acetolactate synthase (ALS). ALS is the first enzyme in the biosynthetic chain resulting in the synthesis of the branched chain amino acids valine, leucine and isoleucine. Mutated strains resistant to sulfonylureas have been obtained from bacteria, fungi and plant cell cultures. The resistance of all these mutants can be attributed to possession of mutated forms of the ALS. Transgenic tobacco plants expressing a mutant ALS gene from tobacco or *Arabidopsis*, were produced that were tolerant to sulfonylurea herbicides.

c. Resistance against Phosphinothricin: L-Phosphinothricin (PPT) is a naturally occurring amino acid with herbicidal activity and is active ingredient of herbicide 'Basta'. PPT is a potent inhibitor of glutamine synthase and thus causes rapid increase in ammonia concentration in plants. This leads to death of plant cell. 'bar' gene from *Streptomyces hygroscopicus* encodes phosphinothricin acetyl transferase enzyme that acetylates the free-NH₂ groups of phosphinothricin and renders it inactivates the herbicide Basta. This 'bar' gene with CaMV 35S promoter has been transferred to tobacco, tomato and potato plants using *Agrobacterium Ti* plasmid vector system. The transgenic plants have been tested for 'Basta' resistance.

1.2. Engineered Resistance against Pest: For transgenic plant production with pest resistance properties, two major approaches have been developed: a) the introduction of protein delta endotoxins from Bt and b) introduction of protease inhibitors in plants.

a. Bacillus thuringiensis endotoxins: The entomological bacterium *Bacillus thuringiensis* upon sporulation normally produces a parasporal crystalline toxin. When ingested by a susceptible insect, a combination of the high pH and proteinases of the insects mid-gut solubilizes the crystal and yields active toxin. The effects of the toxin occur within minutes of indigestion, leading to disruption of insect's midgut cells. Bt toxin activity has been found against many species of insects within the orders of Lepidoptera, Diptera and Coleoptera. These delta endotoxins are very specific in their action and are safe insecticides, but their use is limited due to high production cost and due to instability of crystal proteins when exposed in the field. The gene for above toxin (*ht2*) from *B. thuringiensis* has been isolated and was used for *Agrobacterium* plasmid mediated transformation of tobacco, cotton, potato and tomato plants. These transgenic plants were resistant to the feeding damage of *Manduca sexta* larvae (tobacco horn worm), a pest of tobacco.

b. Protease inhibitors: Protease inhibitors are proteins with anti-metabolic activity against wide range of insects. Such inhibitors are widely distributed within the plant kingdom and can accumulate to high levels in seeds and storage organs. Other major advantages of protease inhibitors are their inactivation with cooking. In cowpea (*Vigna unguiculata*), trypsin inhibitor (CpTi) level was shown to be responsible for its resistance to attack by the major storage pest of its seeds (i.e. bruchid beetle = *Callosobruchus maculatus*). A variety of insects have shown to be toxic to CpTi. This CpTi gene was joined with a CaMV 35S promoter, and one marker gene with the transgene and was used to infect tobacco leaf discs. *Agrobacterium* was used for transformation and the transgenic tobacco plants express a high level of CpTi. The CpTi gene in transgenic plants is stably inherited and there is no serious 'yield penalty'. Thus like Bt toxin, CpTi can also be used as a protectant against insect attack in transgenic plants. However, extensive field trials are necessary before releasing these transgenic plants to the farmers.

1.3. Engineered Resistance against Viral Infection: Over seven hundred plant viruses are recognised causing various diseases and significant crop losses. Due to the lack of 'viricides', the practical control depends on methods that prevent or restrict virus infection. Various strategies have been applied for protecting viral infection; few are described here like:

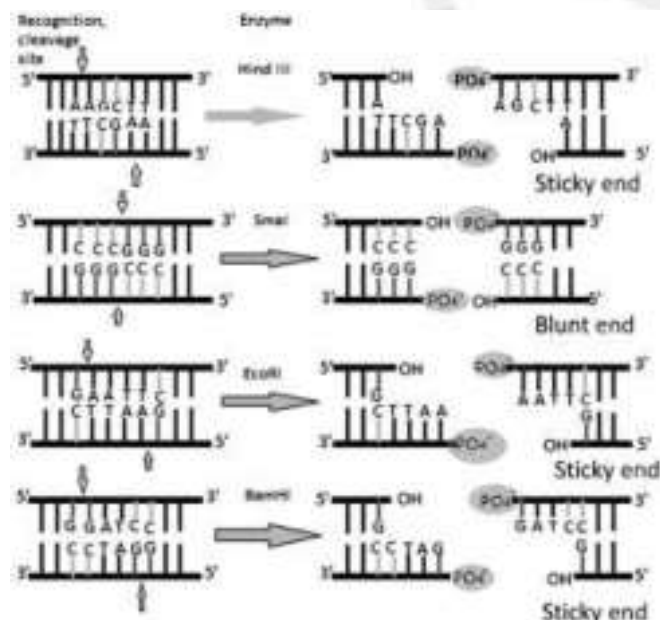
Cross protection: If susceptible strain of a crop is inoculated with a mild strain of a virus, the susceptible strain, as a result, develops resistance against more virulent strains. The phenomenon is known as cross protection. This method has been used to reduce yield

Table 1: Comparative properties of restriction enzymes

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non-specific location > 1000 bp away from recognition site	Cut both strands at a specific, usually palindromic recognition site (4-8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3' recognition site
Restriction and modification	Single multifunctional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg ²⁺ , SAM	Mg ²⁺	Mg ²⁺ (SAM)
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition site	Two recognition sites in a head-to-head orientation
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site

Cleavage Patterns of Some Common Restriction Endonucleases:

The recognition and cleavage sites and cleavage patterns of *HindIII*, *SmaI*, *EcoRI*, and *BamHI* are shown. Cleavage by an endonuclease creates DNA sequence with either a sticky end or blunt end. The blunt ended fragments can be joined to any other DNA fragment with blunt ends using linkers/adapters, making these enzymes useful for certain types of DNA cloning experiments.

**Fig 2: Cleavage patterns of *HindIII*, *SmaI*, *EcoRI* and *BamHI***

Applications:

In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.
- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.

2. Polynucleotide phosphorylase:

- Polynucleotide phosphorylase was first discovered from extracts of *Azotobacter agile* by Grunberg-Manago and Ochoa.
- Polynucleotide phosphorylase (PNPase) catalyzes the synthesis of long chain polyribonucleotides (RNA) in 5' to 3' direction from nucleotide diphosphates as precursors and reversibly catalyzes phosphorolytic cleavage of polyribonucleotides in 3' to 5' direction with a release of orthophosphate in presence of inorganic phosphate.
- PNPase is a bifunctional enzyme and functions in mRNA processing and degradation inside the cell.
- Structural and physiochemical studies in enzymes showed that it is formed of subunits. The arrangements of the subunits may vary from species to species which would alter their properties.
- These enzyme can catalyze not only the synthesis of RNA from the mixtures of naturally occurring ribonucleoside diphosphates, but also that of non-naturally occurring polyribonucleotides

2. In Vivo Gene Therapy

- *In vivo* method of gene transfer involves the transfer of cloned genes directly into the tissues of the patient.
- This is done in case of tissues whose individual cells cannot be cultured *in vitro* in sufficient numbers (like brain cells) and/or where re-implantation of the cultured cells in the patient is not efficient.
- Liposomes and certain viral vectors are employed for this purpose because of lack of any other mode of selection.
- In case of viral vectors such type of cultured cells were often used which have been infected with the recombinant retrovirus *in vitro* to produce modified viral vectors regularly. These cultured cells will be called as vector-producing cells (VPCs). The VPCs transfer the gene to surrounding disease cells.
- The efficiency of gene transfer and expression determines the success of this approach, because of the lack of any way for selection and amplification of cells which take up and express the foreign gene.
- Figure 8 shows various steps of *in vivo* gene transfer.

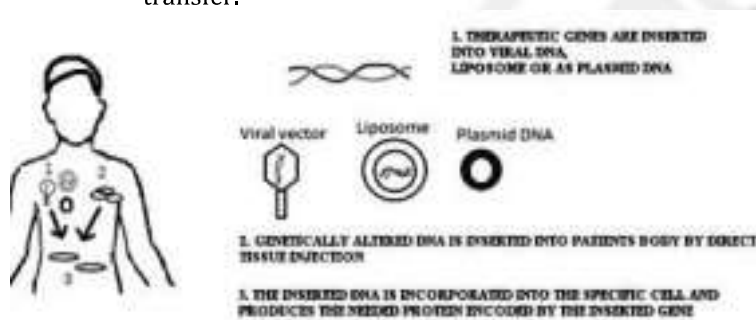


Figure 8: Steps of *in vivo* gene transfer

Target cells for gene transfer	
Disease	Target Cells
Cancer	Tumor cells, antigen presenting cells (APCs), blood progenitor cells, T cells, fibroblasts, muscle cells
Inherited monogenic disease	Lung epithelial cells, macrophages, T cells, blood progenitor cells, hepatocytes, muscle cells
Infectious disease	T cells, blood progenitor cells, antigen presenting cells (APCs), muscle cells
Cardiovascular disease	Endothelial cells, muscle cells
Rheumatoid arthritis	Sinovial lining cells
Cubital tunnel Syndrome	Nerve cells

Vectors for gene therapy

Vectors for gene therapy can be classified into two types:

1. Viral vectors
2. Non-viral

Vectors used in gene therapy	
Viral Vector	Non-viral Vectors
Adenovirus	Lipid complex
Retrovirus	Liposomes
Adeno- Associated Virus	Peptide/protein
Lentivirus	Polymers
Vaccinia virus	
Herpes simplex virus	
• Direct gene transfer methods like mechanical, electroporation, gene gun are also used to transfer genes into target cells	

Viral vectors

Retroviruses, adenoviruses and adeno-associated viruses (AAV) some commonly used viral vectors whereas some less commonly used viral vectors are derived from the Herpes simplex virus (HSV-1), the baculovirus etc.

1. Adenoviral vectors

Adenoviruses are large linear double-stranded DNA viruses that are commonly used for preparing gene transfer vectors. Adenovirus vectors are known to be the second most popular gene delivery vector for gene therapy of various diseases like cystic fibrosis and certain types of cancer. Figure 9 shows how the adenoviruses enter cells by receptor-mediated endocytosis. A primary cellular receptor binds to viral fiber then the virus interacts with secondary receptors which are responsible for its internalization. Coxsackie and Adenovirus Receptor (CAR),

Difference Between *in vivo* and *ex vivo* Gene Delivery Systems

<i>In vivo</i>	<i>Ex vivo</i>
Less invasive	More invasive
Technically simple	Technically complex
Vectors introduced directly	No vectors introduced directly
Safety check not possible	Safety check possible
Decreased control over target cells	Close control possible

Target sites for Gene Therapy

Therapeutic genes have to be delivered to specific target sites for a specific type of disease. This table describes the list of such disease and their target sites for gene therapy.

Amperometric biosensors.

Substrate	Bioreceptor	Product detected
Choline	choline oxidase	H_2O_2
Ethanol	alcohol oxidase	H_2O_2
Methanol	t. dehydrogenase	NADH
Glucose	glucose oxidase	H_2O_2 , O_2
L-Glutamine	Glutaminase and glutamate oxidase	H_2O_2
L-Glutamate	Glutamate oxidase	H_2O_2
Hypoxanthine	Xanthine oxidase	H_2O_2
Lactate	Lactate oxidase	H_2O_2
Oligosaccharides	Glucoamylase, glucose oxidase	H_2O_2
Sucrose	Invertase, mutarotase, glucose oxidase	H_2O_2 , O_2

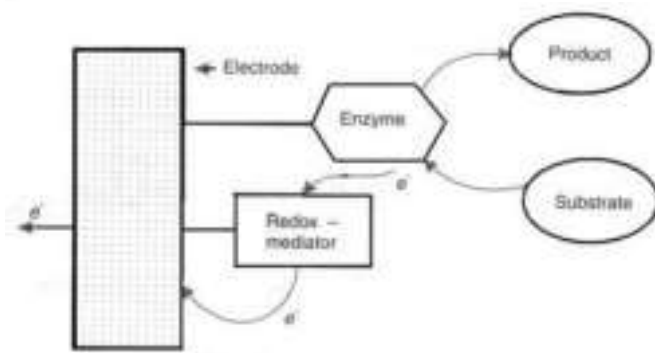


Fig 4. Mediated biosensor.

A major problem of such biosensors is their dependence on the dissolved O_2 concentration in the analyte solution. This may be overcome by using mediators, e.g., ferrocenes; these molecules transfer the electrons generated by the reaction directly to the electrode rather than reducing the O_2 dissolved in analyte solution. (these are called second generation biosensors). The present-day electrodes, however, remove the electrons directly from the reduced enzymes (without the help of mediators), and are coated with electrically conducting organic salts.

Enzyme Electrodes: Enzyme electrodes are a new type of biosensors which have been designed for the amperometric assay of potentiometric assay of substrates such as urea, amino acid, glucose, alcohol, and lactic acid. The electrode consists of a given electrochemical sensor in close contact with a thin permeable enzyme membrane capable of reacting with the given substrates. The enzyme is embedded in the membrane and produce O_2 , H^+ , NH_4^+ , CO_2 or other small molecules depending on enzymatic reactions. This is detected by the specific sensor. The magnitude of the response determines the concentration of substrates (Fig. 5).

5. Optical Biosensors: These biosensors measure both catalytic and affinity reactions. They measure a change in fluorescence or in absorbance caused by the products generated by catalytic reactions. Alternatively, they measure the changes induced in the intrinsic optical properties of the biosensor surface due to loading on it of

dielectric molecules like protein (in case of affinity reactions).

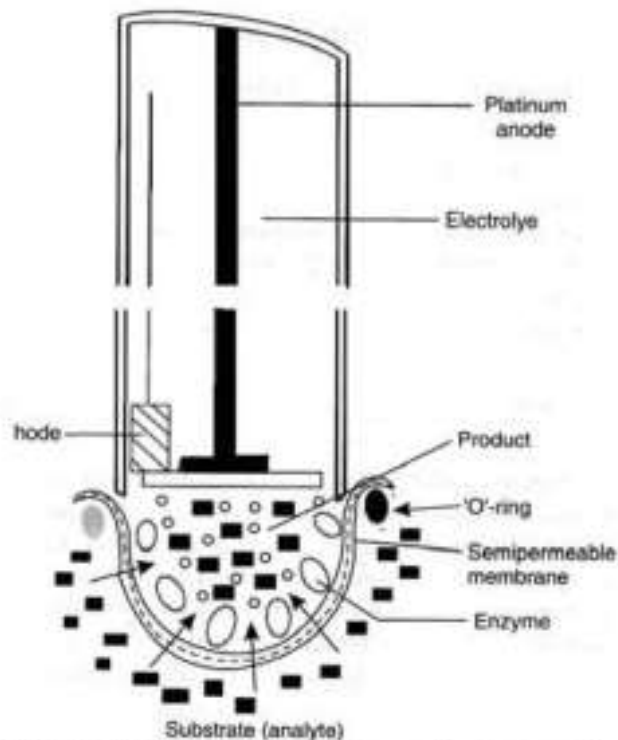


Fig 5 A simple biosensor combining on electrochemical electrode and an enzyme immobilized on to a semipermeable membrane.

A most promising biosensor involving luminiscence uses firefly enzyme luciferase for detection of bacteria in food or clinical samples. The bacteria are specifically lysed to release ATP, which is used by luciferase in the presence of O_2 to produce light which is measured by the biosensor.

Enzyme sensors based on optodes

Substrate	Bioreceptor	Product detected
Ethanol	alcohol dehydrogenase	NADH
Glucose	glucose oxidase	O_2
Urease	urease	ammonia
Lactate	lactate monooxygenase	pyruvate
Penicillin	penicillinase	penicillanic acid

6. Bioaffinity Sensor: Bioaffinity sensors are developed recently. It measures the concentration of the determinants, i.e. substrates based on equilibrium binding. This shows a high degree of selectivity. These are of diverse nature because of the use of radiolabelled, enzyme labelled or fluorescence- labelled substance. Principle of bioaffinity sensor is given in Fig.6.