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Contents

SINGLE CELL PROTEIN

By

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SEMINAR REPORT

Submitted in partial fulfillment for the requirement of the course PBT 651- Seminar

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DECLARATION

I, Geena Paul (2005-11-136) hereby declare that this seminar report entitled **"SINGLE CELL PROTEIN"** has been prepared by me, after going through the various references cited at the end of the report and has not been borrowed from any of my fellow students.

Vellanikkara

21 – 07 – 06

Geena Paul

(2005- 11- 136)

CERTIFICATE

Certified that the seminar report entitled **"SINGLE CELL PROTEIN"** for the course PBT 651 has been prepared by Geena Paul (2005-11-136) after going through the various references cited here under my guidance and supervision, and she has not borrowed from any of her fellow students.

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INDEX

INDEX OF TABLES

INTRODUCTION

One of the biggest problems facing the world today is population growth, especially in developing nations. There is deficiency of proteins and people suffer from malnutrition. The damage of agricultural crops leading to less availability of proteins to the common man. This has given rise to explore the feasibility new non –conventional resources of protein production. Among the various processes of supply protein, those based on microbial growth and microbial biomass have attracted the attention of scientific community. These proteins are grouped under single cell proteins (Gupta and Joshi, 2000).

Professor C.L Wilson coined the tem single cell protein at Massachusetts Institute of Technology, in 1966 to represent the cells of algae, bacteria, yeasts and fungi grown for their protein contents. Dried biomass of a single species of microbe that can be used as protein source in diet is know as single cell protein (Kumaresan, 2001)

Most of the microorganisms used as producers of protein grow as single or filamentous individuals rather than complex Multicellular organisms. Hence came the name single cell protein. SCP is expected to relieve protein deficiency in two ways. It can be directly used as human food supplement and as animal feed.

History

- Since ancient times people near Lake Chad, Africa and the Aztecs, harvested filamentous blue green algae, spirulina from the lake, dried in sun and used as food.
- The first industrial production of single cell protein was started during the First World War. They used 'Torula yeast' (Candida utilis) in soups and sausages.

- Interest in SCP peaked again during World War II and then during mid 1950s.
- By 1967, British Petroleum was producing SCP at an industrial scale,
- The product 'Toprina' was produced and marketed using gas oil as substrate in 1976.
- The Italian company Liquichimica built up a plant for the production of yeast proteins, amino acid, fatty acids and citric acids from n-paraffin.
- Since 1975 production of proteins from substrates other than n-paraffin such as methanol and cellulose was started.
- The British Petroleum Company, Imperial Chemical Industries, became involved in the production of single cell protein using methanol as the substrate to produce 'Pruteen'.
- Sulphite waste liquors from paper mills were used as substrate for SCP production using fodder yeasts.
- In1980s Ist pilot plant for the production of spirulina came on to stream.

(Sasson, 1984).

Importance of SCP

SCP is expected to relieve the protein deficiency in the following two ways.

- SCP can be used directly as human food supplement.
- It may be used in animal feed to at least partially replace the currently used protein rich soyabean meal and fish proteins and cereals that can be diverted for human consumption (Singh, 1998).

Why microbes are used for SCP production?

- Microbes can synthesize proteins much more rapidly than higher living systems (ie. Plants and animals).
- Microbes have a short generation time (eg. . 2-3 hours in bacteria, 1-3hrs for fungi, 2-6 hrs for micro algae).
- Microbes can be grown on media containing cheap sources of carbon and nitrogen.
- Microbes have high protein content (7-12 gms protein N per 100gms dry weight).

The commonly used microorganisms are algae, fungi, yeasts and bacteria.
They should be

- Nonpathogenic to plants, animals and man.
- Of good nutritional value.
- Easily and cheaply produced on large scale.
- Toxin free.
- Fast growing.
- Easy separation and drying.
- Microorganisms are more easily modified genetically than plants and animals. They are more amenable to large scale screening programmes to select for higher growth rate, improved amino acid content etc. and more easily subjected to gene transfer technology.
- Microorganisms can be grown in vast numbers in relatively small continuous fermentation processes using small land area and are independent of climate.
- Microorganisms can grow on a wide range of raw materials like low value wastes, cellulose etc.

Microorganisms used for SCP production

Algae

Members of genera *Chlorellla, Scenedesmus and Spirulina* are grown in ponds/ tanks.

- Substrates; CO_2 and sunlight
- Limiting factor in large-scale production is illumination.
- Algal SCP has about 60%crude protein, which is good in amino acid composition except Sulphur containing amino acids.
- They are suitable for animal feed as protein supplement.

The disadvantages of algal SCP are

- Rich chlorophyll content
- Low cell density (1-2 g dry wt /l)
- Serious risk of contamination
- Costly recovery methods for unicellular algae.

Filamentous fungi

Members are *Chaetomium cellulolyticum, Fusarium graminearum, and Pacilomyces varioti*

- They have been used to produce SCP mainly from polysaccharide hydrolysates. EG; starch hydrolysate, Sulphite liquor from wood pulp industries.
- These are grown as submerged cultures.
- They have crude protein content of 50-55% and the recovery of filamentous and pellet forms are easy by filtration.

The problems associated with fungi are

- Slow growth rate than bacteria and yeast.
- Contamination by yeast may frequent if sterility is not maintained (keeping the pH of the broth below 5 minimize contamination by bacteria)
- High nucleic acid content (up to 15% RNA).
- The strains have to be thoroughly evaluated for mycotoxin production.

Yeasts

Members of *Saccharomyces*, *Candida* and *Torulopsis* are suited for SCP production.

- The SCP has 55-60 %crude protein.
- Protein has good amino acid balance except for a deficiency in S-containing amino acids.
- Rich in B group of vitamins.
- The SCP is used both for human food and animal feed supplementation.
- The risk of bacterial contamination is low and recovery by continuous centrifugation is easy.
- Genetic Engineering to improve the strains
- Thaumatin is a sweet protein found in the fruit of a West African plant, *Thaumatococcus danielli*. It is 5000 times sweeter than 4% sucrose. A DNA coding for Thaumatin of *T.danielli* was introduced in to the yeast *Saccharomyces cerevisiae*. The genetically manipulated yeast accumulates Thaumatin in the cells.
- AMA-I is a nutritionally valuable protein found in grain Amaranthes.Genetic engineers at Jawaharlal Nehru University, New Delhi, and introduced AMA-I gene of Amaranthes in to the yeast *Saccharomyces cerevisiae*. The genetically engineered yeast produces AMA-I protein in the cells.

- Two yeast strains (*Candida tropicalis*) -NCYC 2705 and NCYC 2699 were isolated from *Caryota urens* for single cell protein production. The two strains contain essential amino acids amounting to 72-82% of total amino acid content. The have a high content of cysteic acid, which is normally found in low amounts in most of the microorganisms. (Wijeyaratne *etal*; 2000).

Disadvantages of yeasts as SCP are

- Slow growth rates than the fast growing bacteria
- High nucleic acid content (up to 15%)
- Deficient in S-containing amino acids.

Single cell protein diet of novel recombinant Vitellogenin yeast enhances growth and survival of first feeding Tilapia (*Oreochromis mossambicus*) larvae (Lim etal; 2005).

Bacteria

- Bacteria can produce SCP using a wide variety of substrates.
- Members which have been used for production at commercial scale are *Methylophilus methylotrophus* (using methanol), *Brevibacterium* sp (using C1-C4) hydrocarbons.
- SCP has very high crude protein (80%) of good amino acid composition.

Disadvantages of using bacteria in SCP production are

- High nucleic acid content (RNA 20 %)
- Sterility must be maintained during the production process.
- Risk of contamination by pathogenic bacteria is considerable.
- Recovery of cells is problematic

- Evaluation for endotoxin production is essential when gram-negative bacteria are used.

Table1. Some important features of different microorganisms and the SCP produced from them.

Feature	Algae	Bacteria	Yeast	Filamentous Fungi
Growth rate	Low	Highest	High	<Bacteria &Yeast
Substrate	Light, inorganic carbon sources, eg.CO2	A wide range of substrates.	Most substrates except hydrocarbons and CO2.	Limited substrates. (starchy & cellulosic materials)
PH range	Up to 11	5-7	5-7	3-8
Cultivated in	Open ponds; in sunlight	Bioreactors	Bioreactors	Bioreactors
Biomass recovery	Difficult and costly with unicellular algae	Problematic; improved methods are needed.	Easy by centrifugation	Easy for filamentous or pellet forms
Crude protein content	Upto 60%	80% or more	55-60%	50-55%
Nucleic acid content	-	Very high (20% RNA)	High (15 %RNA)	High (15% RNA)

(Singh, 1998)

Substrates

A variety of substrates ranging from inorganic carbon, industrial effluents, low cost organic materials like starch hydrolysate are used for SCP production.

These substrates can be divided in to two broad groups.

- Fossil carbon sources (non renewable)
- Renewable carbon sources.

But substrates like methanol and ethanol can be produced from both renewable and nonrenewable carbon sources.

Fossil carbon sources

- **Gaseous hydrocarbons**
- **Liquid hydrocarbons**
- **Methanol**
- **Ethanol**

Gaseous hydrocarbons

- C1-C4 gaseous hydrocarbons have been used for SCP production.
- Methane has been extensively studied.
- It is readily removed from the fermentation medium and supports high productivity in continuous processes.
- Methane utilizing bacteria are

Pseudomonas methanica

Methanomonas methanica

Methylococcus capsulatus

Pseudomonas methanitrificans

Use of methane for SCP production presents problems like

- Substrate limitation either due to methane & O_2.
- Heat generation necessitating efficient cooling.
- Hazards of explosion when >12% O_2 is used.

Liquid hydrocarbons

- Saturated, straight chain hydrocarbons called n-alkanes constitute 0-30% crude oil.

- N-alkanes having 9-18 carbons (C9-C18) are used as SCP substrates.
- Many bacteria, actinomycetes, yeasts and moulds are able to use liquid hydrocarbons.
- Pure n-alkanes having 10-23 carbons were utilized by British Petroleum to culture *Candida lipolytica* in a continuous process to produce 'Toprina'.
- The n-alkanes are almost completely utilized.
- Biomass recovery is easier and cheaper.
- This SCP is comparable to soyabean meal and fish meal.

Methanol

- Methanol can be produced from methane, coal, gas oil, wood and naphtha.
- Methanol is fully water soluble
- Used by many bacteria
- Little danger of explosion

A highly successful process by ICI (Imperial Chemical Industries Ltd) uses the bacterium *Methylophilus methylotrophus* for a continuous production process at 35-40°C. The SCP contains 71% protein and is marketed as Pruteen. It is used as milk substitute in calf feeding.

Ethanol

- Ethanol can be obtained from ethylene or from organic substrates by alcoholic fermentation.
- More acceptable as a substrate for SCP production for human use.
- Several bacteria, yeasts and mycelial fungi utilize ethanol.

Amco foods, U.S.A produces food grade SCP by growing *Candida utilis* (Torula yeast) in a 5000 t/ yr capacity plant.

Renewable Carbon Sources

Include

CO2, Molasses, whey, cellulose hydrolysate, starch hydrolysate, industrial effluents and cellulosic wastes.

CO2

- It is utilized by algae. They derive the required energy from sunlight.
- Spirulina is grown on commercial scale by Sosa Texcoco Co, Mexico-producing up to 5 tons dry biomass/ day.

Molasses

- Used for alcoholic fermentation and yeast biomass is obtained as a by-product.
- Baker's yeast (*Saccharomyces cerevisiae*) and torula yeast (*Candida utilis*) are produced on this substrate.
- S.cerevisiae is used as inoculum for dough fermentation in bakeries.

Whey

- Whey is liquid portion of milk remaining after the curd is separated during cheese production.
- Yeast (*Kluyveromyces fragilis, Candida intermedia*) are used for commercial scale production of SCP from whey.

Fermentation of whey using *Kluyveromyces (K.marxianus*) can be used for single cell protein production (Bargain ,1993).It consist of six stages-storage and pasteurization, ultra filtration, fermentation, separation of solids and liquids (precipitation by centrifugation), partial dehydration using heat

treatment(concentration by evaporation)and drying using a roller or spray drier. The protein obtained has a crude protein content of 54.33%, rich in essential amino acids, vitamins and minerals.

Fromagaries Le Bel, France produces about 2300 tons of SCP /year using *Kluyveromyces fragilis.*

Cellulose hydrolysate

- Cellulase obtained from fungi like *Trichoderma viride* has been used to hydrolyze cellulose and to produce glucose.
- Main advantage –large quantities of cheap substrate Major problems are
- Difficult and expensive process for complete hydrolysis of lignocelluloses.
- The extent of sugar destruction and by product formation during chemical hydrolysis.

Starch hydrolysate

- Expensive substrate for SCP production using *Fusarium graminearum.*
- SCP has fibrous, meat like texture .It is marketed as 'Mycoprotein '.
- The fungus grows at 30 ^{0}C on a variety of mono and oligosaccharides.
- Recovered by vacuum filtration. Then it is held at 60 ^{0}C for 20 minutes to activate the native RNAase to reduce the RNA content of SCP to about 1% of dry weight.

Industrial Effluents

- Effluents from many industries like breweries, distilleries, confectionery industries, potato and canning industries, Sulphite liquor from wood pulp mills are used for SCP production
- Contain large amount of carbohydrates and other organic compounds.
- The biological oxygen demand (BOD) of the effluent is reduced by 81%.

- SO2 is first removed from the liquor, pH is adjusted to 4.5 and inoculation is done with the selected microorganisms.
- Fungus utilizes both pentoses and hexoses and acetic acid present in the liquor. It is recovered by filtration.
- SCP has 52-57% crude protein and is used as animal feed.Besett Ltd, U.K uses *Candida utilis* to treat 1, 40,000 l confectionary effluent /day by continuous fermentation and produces 1.5 tons of dry yeast /day.

Agricultural and Cellulosic wastes

- Bagasse, straw, saw dust etc are used for SCP production.
- Material is pretreated thermally and chemically and is then fermented with the fungus *Chaetomium cellulolyticum*

Sugar cane bagasse pith dry pretreatment for single cell protein production (Rodriguez *etal*; 1992). Invitro digestibility and microbial growth were obtained with a mixed culture of *Cellulomonas flavigena; Xanthomonas* sp. Maximum digestibility was 76%using a dry pre treatment with NaOH (0.1g/g pith), a temperature of 50°C and moisture content of 80%.

Food processing industry waste

SCP can be produced from a number of food processing industry wastes like apple pomace, peach waste, cashew apple pomace, citrus waste extract, molasses, potato peels, cabbage wastes etc.

Table 2. Food processing waste used as SCP /animal feed after microbial fermentation

Waste	Microorganism utilized
Apple pomace	*Saccharomyces cerevisiae, Candida utilis, Aspergillus niger.*
Com cob	*Aspergillus niger*
Dried citrus peel	*Aspergillus niger*
Fodder beets	*Saccharomyces cerevisiae*
Orange peel and grape stalks	*Pleurotus ostreatus, Agrocybe aegirata, Armillarialla mellea*
Sugarcane bagasse	*Polyporus spp,Pleurotus sp ,Trichoderma sp*
Sugar beet pulp	*Trichoderma resei, Trichoderma viridae, Fusarium oxysporum*
Soyabean	*Rhizopus oligosporus*

(Gupta and Joshi,2000)

Production of SCP

For the production of SCP there should be

- Provision of carbon sources. It needs physical /chemical pretreatments.
- Sources of N, P and other nutrients also should be there to support optimal growth of selected media.
- Prevention of contamination by maintaining sterile or hygienic conditions.
- The selected micro organism is inoculated in a pure state.
- Adequate aeration must be provided and cooling is also necessary.
- The microbial biomass is recovered from the medium.
- Processing of the biomass for enhancing its usefulness and storability. Heat treatments are used during the final stages of harvesting to inactivate

heat sensitive organisms and to reduce RNA content. The cell must be broken to enhance the nutritional value of SCP.

Biomass recovery

- Bacteria- by flocculation and floatation combined with centrifugation.
- Yeast – centrifugation.
- Filamentous organisms – filtration

Spirulina

Spirulina is multicellular, filamentous, non heterocystous and non nitrogen fixing cyanobacteria.

Uses

- As protein, vitamin supplement as fortification in diets for malnourished people.
- In feeds of poultry, cattle, swine etc and in aqua culture.
- As a health food .Algal powder is incorporated in various preparations as a replacement for expensive vitamins and rare amino acids.
- Biologically important chemicals like β-carotene as pro vitamin A and food, drug, cosmetic colouring phycobiliproteins can be obtained.
- In anti cancer formulations, diabetes control, wound treatment and to promote skin metabolisms.
- Boost the immune system.
- Widely exploited in the manufacturing of beauty products such as anti wrinkling and antipimple creams, face masks and high protein shampoos.

Production

- Artificial, shallow (.5m) ponds- plastic lined or made of concrete with green house type covers.
- At harvesting spirulina is pulped from culture ponds through underground pipes and screened for particulate matter, followed the separation by stainless steel screens from culture medium.
- Wash three times with water and then vacuum filtration.
- Dry
- The bulk powder is packaged in foil laminate heat sealed bags with an oxygen absorbing pack sealed in bag.
- Yield – 2-3 kg dry wt /m^2 /year.(Gopalaswamy,2005)

Techniques of genetic engineering may lead to the isolation of strain with more favorable characteristics like faster growth rate, capacity to grow at optimal temperatures, higher total cell yield, and higher content of nutritionally important components.

Chemical composition of *Spirulina platensis*

Crude protein content -60 %

Crude lipids - 12%

Carbohydrates -15%

Nucleic acid content-<5 %

B-carotene- >.1%

Comparison of SCP with common food products

Table 3. Average percentage composition of microbial cells on dry weight basis

	Filamentous fungi	Algae	Yeast	Bacteria
Nitrogen	5-8	7.5-10	7.5-8.5	11.5-12.5
Fat	2-8	7-20	2-6	1.5-3
Ash	9-14	8-10	5-9.5	3-7
Nucleic acids		3-8	6-12	8-16

(Kihlberg, 1972)

Table 4. Nitrogen and protein content of microbial cells compared with selected foods of animals and plant origin

Source	Crude protein %
Filamentous fungi	31-50
Algae	47-63
Yeast	47-63
Bacteria	72-78
Milk	22-25
Beef	81-90
Egg	35.00
Rice	7.5-9.0
Wheat flour	9.8-13.5
Corn meal	7.0-9.4

(Gupta and Joshi, 2000)

Advantages of SCP

- SCP is rich in high quality protein, poor in fats.
- Year round production (except for algal processes)

- Microbes are fast growing and produce large quantities of SCP from relatively very small area of land.
- Use low cost substrates.
- When the substrate used for SCP production is a source of pollution, it helps to reduce pollution.
- Strains having high biomass yields and a desirable amino acid composition can be easily selected or produced by genetic engineering.
- SCPs are good source of vitamins, particularly B- group of vitamins.
- One of the feasible approaches to bridge the gap between requirement and supply of proteins.

Nutritional and safety evaluation

The safety and nutritional value of single cell protein sources must be established by rigorous testing. Between 1970 and 1974 the protein advisory group of UN system (PAG) developed a series of guidelines for testing novel sources of protein, the three of which were specifically directed at the production of SCP for human consumption. These have been reviewed and reissued as UN-PAG guidelines (Scrimshaw, 1985).

- Chemical composition of SCPs must be characterized in terms of protein, amino aids, nucleic acids, lipid, vitamin etc.
- Physical properties like density, particle size, texture, colour, storage etc should be determined.
- Microbiological descriptions eg: species, strain should be provided and information on contamination be also be given.
- The nutritional value should be evaluated on the target species and other species should also be included.
- The products for human use will be evaluated over a longer period using a multi staged process.
- Possible toxic or carcinogenic compounds must be assayed for. (Singh, 1998

Conclusion

It is imperative that new food sources be found in order that future generations of mankind are adequately fed. A food source that nutritionally complete and that requires a minimum of land, time, and cost to produce is highly desirable. In addition to meet these criteria, SCP can be produced from a variety of waste materials (Jay; 1986).

The two end uses of SCP are food for humans and feed for animals. The desired end use determines composition, protein quality, and requirements for purification including removal of trace amounts of substrate and undesirable contaminants.

The selection of micro organisms which are considered suitable for SCP production is crucial and depends upon the type of raw material available cheaply, abundantly and continuously.

The applications of SCP products in food include addition for nutritional value or for functional properties of the proteins or both. For human food use, the SCP product must have satisfactory nitrogen and protein contents, amino acid profiles, lipid contents, mineral and vitamin contents (Litchfield; 1977).

Before recommending a single cell protein, the chemical composition of dried biomass, toxicity, nutritional and energy values, digestibility, acceptability and safety should be considered by conducting animal experiments (Kumaresan; 2001)

Economics of SCP relates to the raw material prices and also the prices of alternative foods (fish and soyabean). Many of the processes developed so far have been with huge capital investments and sophisticated technology and that may not serve the purpose of supplying food to those who are hungry. The needed

is somewhere else and the production is occurring in countries, which are well fed. The SCP production should really yield in proper perspective (Jogdand, 1993).

So far, the research on single cell protein in India has been to a very limited extent, but it is an area of research, which used could show great potential to relieve protein deficiency and malnutrition. Efficient use of waste materials and reduction of pollution are added advantages in SCP. So the use of single cell protein appears to be one of the feasible approaches to bridge the gap between the requirement and supply of proteins.

Discussion

1. Most commonly used nitrogen source in SCP?

   Ammonia

2. What is the maximum permissible level of nucleic acid in SCP?

   The safe intake of nucleic acid for adult, healthy persons has been estimated to be 2g per day.

3. What about digestibility and amino acid content?

   It is almost completely digestible (84%).SCP is rich in amino acid content except for sulphur containing amino acids like methionin

4. What is the media for Spirulina production?

   Liquid effluents taken from well-digested human excreta, modified seawater and Zarrouk medium are used as economic media for Spirulina cultivation.

5. Whether industrial effluents are harmful?

   The most pronounced hazard with SCP consumption concerns the possible inclusion of carcinogenic compounds in the cells cultivated on crude oil fractions.

6. How SCP can reduce pollution?

   SCP can be produced from agricultural wastes and industrial effluents. Biological Oxygen Demand of the effluent can be reduced by 80%

7. Any method to remove nucleic acid?

   Processes for reducing the nucleic acid contents of SCP products include acid precipitation, acid or alkali hydrolysis, heat shock and incubation for endogenous nuclease action, and use of exonucleases.

8. Are there any comparative evaluation of conventional protein sources and SCP?

   SCP foods are on par with conventional protein sources. But its main competitors are soyabean meal (44% crude protein) and fish meal (60-65%).

9. Any institute in India conducting research on SCP?

CFTRI, Mysore (Central Food Technology Research Institute) is producing SCP in commercial level.

Murugappa Chettiar Research Centre (MCRC), Chennai has been trying to use Spirulina slurry along with the popular south Indian dishes like idly and dosa. It also uses Spirulina in purce and bread sandwich.

10. How the appearance of SCP food products improved?

- Addition of cellular product of yeast and bacteria (dry powder) to cookies, cakes, puddings and other processed foods in small amounts is quite feasible.
- Autolyzed or hydrolyzed yeast extract has been used as a flavoring agent to give a meaty or nutty flavor to foods.
- SCP like many other proteins can serve as a surface-active agent in emulsions, foams, gels and suspensions to maintain structure.

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ABSTRACT

The explosive increase in population and damage to agricultural crops has led to the deficiency of proteins and malnutrition in developing countries like India. This has led scientists and researchers to explore new non-conventional sources of protein production. Amongst the various processes of protein production, those based on microbial growth and microbial biomass have attracted the attention of the scientific community. These proteins are grouped under single cell proteins (Gupta and Joshi, 2000).

Dried biomass of a single species of microbe that can be used as a protein source in diet is known as single cell protein (Kumaresan, 2001).

Single cell protein can be used directly as food supplement or animal feed. A number of microorganisms like yeast, fungi, algae and bacteria can be employed for the production of single cell protein and each of them have their own advantages and disadvantages.

A variety of substrates ranging from low cost materials like cellulosic waste (straw), inorganic carbon, industrial effluents to high cost materials like starch hydrolysate are used for single cell protein production. The Single cell protein has 55-60% crude protein, which has good amino acid balance except for a deficiency in Sulfur containing amino acids (Singh, 1998).

Before recommending a single cell protein, the chemical composition of dried biomass, toxicity, nutritional and energy values, digestibility, acceptability and safety should be considered by conducting animal experiments (Kumaresan, 2001).

So far, the research on single cell protein in India has been to a very limited extent, but it is an area of research, which if used could show great potential to relieve protein deficiency and malnutrition. Efficient use of waste materials and reduction of pollution are added advantages in SCP. So the use of single cell protein appears to be one of the feasible approaches to bridge the gap between the requirement and supply of proteins.

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INDEX

INDEX OF FIGURES

Introduction

The classification and identification of living organisms is perhaps the oldest and most universal of the human sciences. Taxonomy is, and probably has been, an element of human culture, reflecting both pragmatic considerations and the deeper human penchant for ordering natural diversity. Although traditional taxonomy and taxonomic identification methods have provided a wealth of information about the organisms around us, the rate of progress is greatly exceeded by our growing need for fast and economical species identification and new species discovery.

DNA barcoding is useful to taxonomists who are trying to discover, distinguish and describe new species, and to anyone who is trying to assign an unidentified specimen to a known species. The earlier studies revealed that mitochondrial gene cytochrome c oxidase 1 (COI) seems to meet the criteria of a barcode extremely well for most eukaryotic animals. Scientists have proposed two barcode regions for plants, and there are efforts underway to find a barcode region (or regions) that will work across all land plants. A DNA barcode library together with rapid, portable methods for sequence analysis will empower enforcement of regulations for many protected species. DNA barcoding is being developed as a tool for taxonomic science, not a replacement for it.

Definition

DNA barcoding is a new and exciting tool for characterizing species of organisms of all life forms using a short DNA sequence from a standard and agreed-upon position in the genome(Hebert et al., 2003).DNA barcoding helps to discover, characterize and distinguish species, and to assign unidentified individuals to species.Barcoding has been equated with "molecular taxonomy" or the "DNA taxonomy" (Tautz et al., 2003).

Origin

The use of nucleotide sequence variations to investigate evolutionary relationships is not a new concept. Carl Woese used sequence differences in ribosomal RNA (rRNA) to discover archaebacteria, which in turn lead to the redrawing of the evolutionary tree and molecular markers (e.g., allozymes, rDNA,

and mtDNA) have been successfully used in molecular systematics for decades. DNA barcoding provides a standardised method for this process via the use of a short DNA sequence from a particular region of the genome to provide a 'barcode' for identifying species. In 2003, Professor Paul D.N. Hebert from the University of Guelph, Ontario, Canada, proposed the compilation of a public library of DNA barcodes that would be linked to named specimens. This library would "provide a new master key for identifying species, one whose power will rise with increased taxon coverage and with faster, cheaper sequencing".

Molecular tools used for DNA based species identification

- RFLP
- RAPD
- SSR-PCR
- AFLP
- Detection of SNPs
- DNA barcoding

Need for DNA barcoding

It took over two centuries for taxonomists to describe 1.7 million species, but we know this figure might be a gross under-estimate of the true biological diversity on earth (Blaxter, 2003). It represents a small minority of the estimated 10 to 100 million species alive today. Extrapolation of this past rate of discovery suggest that it will take another 1500 to 15,000 years to complete the global inventory of life through conventional approaches. All of this means that a rapid, accurate, automatable and globally accessible procedure for species delimitation and identification is badly needed now, and will become even more necessary in the future.

UPC Codes

The Universal Product Code system developed by the industrial sector to brand retail items employs 10 options at each of 11 positions to create 100 billion alternates.

Fig.1. UPC of a market product

DNA Codes

Just like UPC barcodes, the DNA sequences within each species are unique. A run of 15 nucleotides, with 4 options at each position, creates the possibility of 1 billion codes, a hundred-fold excess over the estimated number of animal species. Of course, specific nucleotides are fixed at some positions by selection. However, this constraint can be overcome by focusing on protein-coding genes, where every third position is generally free to vary because of the degeneracy of the genetic code. As a result, by examining a stretch of 45 nucleotides in these genes, one has the prospect of close to 1 billion alternates.

(Cytosine = Blue, Adenine = Green, Thymine = Red, Guanine = Black)

Fig.2. The DNA barcode of (a) Honeybee and (b) American Robin

Critera of a DNA barcode sequence

A genetic marker suitable for DNA barcoding needs to meet a number of criteria. First, in the study group, it needs to be sufficiently variable to discriminate among most species, but sufficiently conserved to be less variable within than between species. Second, priming sites need to be sufficiently conserved to permit a reliable amplification without the risk of false negatives when the goal is the

a reliable amplification without the risk of false negatives when the goal is the analysis of pooled samples, e.g. when the total of invertebrates from a soil sample is to be studied without separating individuals, or of environmental DNA such as sub fossil DNA remains from the soil. Third, the gene should convey sufficient phylogenetic information to assign species to major taxa using simple phonetic approaches. Fourth, its amplification and sequencingshould be as robust as possible, also under variable lab conditions and protocols. Fifth, sequence alignment should be possible also among distantly related taxa. Sixth,it should be known to be orthologous between specimens

Sequences used for DNA barcoding

- Nuclear small subunit ribosomal RNA gene (SSU) - (16S rRNA, 12S rRNA)
- Nuclear large subunit ribosomal RNA gene (LSU)
- Internal transcribed spacer section of the ribosomal RNA cistron (ITS) and the chloroplast ribulose biphosphate carboxylase large subunit (rbcL) genes for plants.
- Mitochondrial cytochrome c oxidase I (COI) gene for metazoan.

Mitochondrial DNA

Mitochondrial DNA (mtDNA, or less popularly, mDNA) is DNA that is located in mitochondria. This is in contrast to most DNA of eukaryotic organisms, which is found in the nucleus. Mitochondria are the parts of the cell that generate energy in the form of adenosine triphosphate (ATP).

Unlike most of the cell, the function of which is defined by nuclear DNA, mitochondria have their own DNA and are assumed to have evolved separately. Human mitochondrial DNA consists of 5-10 rings of DNA and appears to carry 16,568 base pairs with 37 genes (13 proteins, 22 tRNAs and two rRNAs) which are concerned with the production of proteins involved in cellular respiration. However many proteins found in the mitochondria are encoded by nuclear DNA: some, if not most, are thought to have been originally part of the mitochondrial DNA but have since been transferred to the nucleus during evolution.

mtDNA is typically passed on only from the mother during sexual reproduction (mitochondrial genetics), meaning that the mitochondria are clones. This means that there is little change in the mtDNA from generation to generation, unlike nuclear DNA which changes by 50% each generation. Since the mutation rate is easily measured, mtDNA is a powerful tool for tracking matrilineage, and has been used in this role for tracking many species back hundreds of generations.

COI PROTEIN

Structure

The COI protein, which ranges in length from 510-530 amino acids among different animal species, is divided into 25 structural regions. These regions consist of the amino and carboxyl terminals, 6 external loops that extend into the cellular cytoplasm, 5 internal loops and 12 alpha-helix segments that traverse the inner membrane of the mitochondrion. To simplify topographic discussions, we divide the protein into two sections (COI-5', COI-3'). The COI-5' section is 215 amino acids long in *Drosophila melanogaster* and extends from the amino terminal to the beginning of M6, the sixth membrane segment. The COI-3' section which extends from the point to the carboxyl terminal of the protein is 233 amino acids long in this species.

COI Amino Acid Variation

Although the COI protein has a critical metabolic function that is conserved across all life that employs oxidative metabolism, there is substantial variation in its amino acid composition. For example, in 1996, Lunt and his co-workers found variation in 125 of 522 amino acid positions in their comparison of COI proteins from 9 insect species. Levels of amino acid variation varied across the molecule with the carboxyl terminal showing the greatest variation. Among the 5 internal loops, I1 was least variable, while I2 and I4 were most variable. M1, M3, M4, and M12 were the most variable of the membrane segments, while M6 was least diverse. All of the external loops showed similar levels of variation except E4 that was invariant.

The target

Past phylogenetic work has often focused on mitochondrial genes encoding ribosomal (12S, 16S) DNA, but their utility in taxonomic analyses is constrained by the prevalence of insertions and deletions (indels) that complicate sequence alignments (Blaxter et al., 2005). The 13 protein-coding genes in the animal mt genome are a better target because indels are rare since most lead to a shift in the reading frame. There is no compelling *a priori* reason to focus on a specific gene, but the cytochrome c oxidase I gene (COI) does have two important advantages. Firstly, the universal primers for this gene are very robust, enabling recovery of its 5' end from the representatives of most, if not all, animal phyla. As well, COI likely possesses a greater range in phylogenetic signal than any other mitochondrial gene. In common with other protein-coding genes, its third position nucleotides show a high incidence of base substitutions. However, changes in its amino acid sequence occur more slowly than those in any other mitochondrial gene.

Components of DNA Barcoding projects

1) The Specimens:

Natural history museums, herbaria, zoos, aquaria, frozen tissue collections, seed banks, type culture collections and other repositories of biological materials are treasure troves of identified specimens.

2) The Laboratory Analysis:

Barcoding protocols can be followed to obtain DNA barcode sequences from these specimens. The best-equipped molecular biology labs can produce a DNA barcode sequence in a few hours for as little as $5 per specimen. The data are then placed in a database for subsequent analysis.

3) The Database:

One of the most important components of the Barcode Initiative is the construction of a public reference library of species identifiers, which could be used to assign unknown specimens to known species.

There are currently two main barcode databases that fill this role:

- The International Nucleotide Sequence Database Collaborative is a partnership among GenBank in the U.S., the Nucleotide Sequence Database of the

- The International Nucleotide Sequence Database Collaborative is a partnership among GenBank in the U.S., the Nucleotide Sequence Database of the European Molecular Biology Lab in Germany, and the DNA Data Bank of Japan. They have agreed to Consortium for Barcode of Life's (CBOL) database record for barcode records.
- Barcode of Life Database (BOLD) was created and is maintained by University of Guelph in Ontario. It offers researchers a way to collect, manage, and analyze DNA barcode data.

4) The Data Analysis:

Specimens are identified by finding the closest matching reference record in the database. CBOL has convened a Data Analysis Working Group to improve the ways that DNA barcode data can be analyzed, displayed, and used.

PROTOCOL FOR DNA BARCODING

Barcoding Animal Life relies on a number of protocols for obtaining COI DNA sequences.The high volume DNA analysis involves the following steps,

1. Specimen Collection/Preservation

Whenever possible, specimens should be killed in a DNA-friendly fashion (freezing, cyanide, immersion in ethanol), avoiding even brief exposure to killing/preservation agents such as ethyl acetate or formalin that damage DNA..

2.Specimen labeling:

The usefulness of DNA barcoding depends on linking the sequence to a specimen and its associated data (collector, taxonomic confirmation, date, georeference coordinates, etc.).

3. Tissue Sampling/Handling

All specimen samples should be handled on a clean working surface and all instruments should be acid or flame sterilized between each sample. In any

laboratory that seeks high production rates, it is critical to carry out all stages of barcode analysis in 96-well plates.

4. Genomic DNA Isolation/Purification

This stage involves breaking up tissues to make the DNA accessible for amplification. Different techniques are used depending on the quality of the individual tissue sample. In addition to standard methods, there are commercial kits (e.g. Sigma-Aldrich product number GDI-3) that are inexpensive and have high success in recovering DNA.

5. Genomic DNA Quantification

It is not usually necessary to quantify genomic DNA extracts because even a few copies of the target gene are sufficient for PCR amplification. However, the quantity of extracted DNA can be determined with a plate reader.

6. Amplification of Barcode Region

Amplification of the target DNA employs a technique called the PCR. This is accomplished by placing the DNA extractions in a specialized chemical environment and cycling them through a specific temperature regime. A heat activated DNA polymerase replicates the target DNA sequence with each cycle.Robust primers are available for mitochondrial COX1 gene(Sorenson *et al.*,1999)

7. PCR Product Cleanup

PCR products are often 'cleaned-up' to remove un-incorporated nucleotides and residual primers. If this step is omitted, it leads to degradation in the sequencing results for the first 50 or so bp.

8.Sequencing and Sequence Analysis

Many high-volume genomics facilities use either ethanol precipitation or magnetic bead protocols. At present, a Sephadex column based approach, which is available in a 96-well format, is used. Capillary sequencers have now largely displaced slab gel instruments, but ABI PRISM® 377 and 373 sequencers provide a low-cost sequencing solution for labs that seek to analyze no more than 50 templates a day. However, for higher production goals and greater automation, a multicapillary instrument is critical.

9. Sequence Editing

Whenever possible, barcode products should be sequenced bidirectionally if they are destined for inclusion in the barcode reference library. Bidirectional sequencing aids the generation of full length barcode sequences by avoiding problems in signal deterioration that often occur near the end of a read.

10. Sequence Alignment

Barcode of Life database is employed to organize sequence records and keep them in alignment. This software also allows results from different projects to be merged into a 'virtual project' enabling sequence comparisons and tree generation for specimens from different projects. As COI sequences rarely have indels or deletions, they can be easily aligned in any DNA alignment or editing software.

11.Databasing results

The Barcode sequences should be submitted to GenBank. Investigators of barcoding of marine organisms are encouraged to post results to the Ocean Biogeographic Information System (OBIS), including a link on OBIS species pages to the relevant sequence deposited in GenBank. In addition, a Barcode of Life database will soon be launched under the auspices of the Hebert laboratory that integrates sequence data with taxonomic and specimen information.

IDENTIFYING SPECIES BY DNA

1.Why barcode animal and plant species?

By harnessing advances in electronics and genetics, barcoding will help many people quickly and cheaply recognize known species and retrieve information about them, and will speed discovery of the millions of species yet to be named. Barcoding will provide vital new tools for appreciating and managing Earth's immense and changing biodiversity.

2.What are the benefits of standardization?

Researchers have developed numerous ways to identify species by DNA, typically tailoring the approach to answer a specific question in a limited set of species. Like convergence on one or a few railroad gauges, barcoding aims to capture the benefits of standardization. Standardization typically lowers costs and lifts reliability, and thus speeds diffusion and use. For barcoding, standardization should help accelerate construction of a comprehensive, consistent reference library of DNA sequences and development of economical technologies for species identification. The goal is that anyone, anywhere, anytime be able to identify quickly and accurately the species of a specimen whatever its condition. Results so far suggest that a mitochondrial gene barcode will enable identification of most animal species. For plants, mitochondrial genes do not differ sufficiently to distinguish among closely related species. Promising approaches to standardize plant identification using one or possibly two barcode regions are under development.

2.Why barcode animals with mitochondrial DNA?

Mitochondria, energy-producing organelles in plant and animal cells, have their own genome. Twenty years of research have established the utility of mitochondrial DNA sequences in differentiating among closely related animal species. Four properties make mitochondrial genomes especially suitable for identifying species.

Copy number: While each cell typically contains only 2 copies of nuclear DNA sequences, the same cell encompasses 100-10,000 mitochondrial genomes. Recovering mitochondrial DNA sequences succeeds much more often than nuclear sequences, especially from small or partially degraded samples. Greater success with smaller samples means lower processing costs.

Greater differences among species: Sequence differences among closely related animal species average 5- to 10-fold higher in mitochondrial than nuclear genes. Thus, shorter segments of mitochondrial DNA distinguish among species, and because they are shorter, less expensively.

Few differences within species: Intraspecific variation in mitochondrial DNA is low in most animal species. This may reflect rapid loss of ancestral polymorphisms due to maternal inheritance or selective sweeps following emergence of advantageous mutations. Regardless of cause, small intraspecific and large interspecific differences signal distinct genetic boundaries between most species, enabling

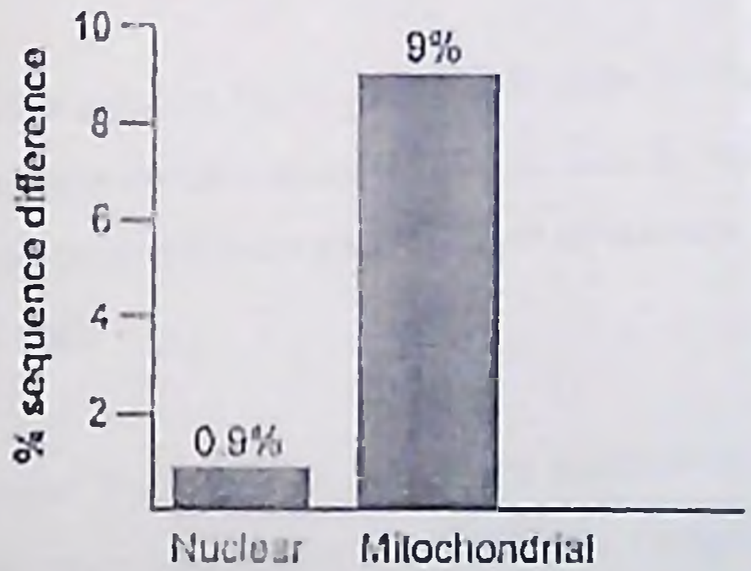

Fig.4.Average sequence difference in nuclear and mitochondrial DNA between human and chimpanzee

Absence of introns: In animals, mitochondrial genes rarely contain introns, which are non-coding sequences interspersed between the coding regions of a gene. Thus, amplification of mitochondrial DNA is usually straightforward. In contrast, amplification of coding regions of nuclear genes is often limited by introns, which may be long.

4. What are the main limits to barcoding encountered so far?

Groups with little sequence diversity: An example was found among a small number of corals and anemones from the marine phylum Cnidaria. The prevalence of such groups is not yet known, as researchers have analyzed only a few Cnidaria, and mitochondrial

DNA sequences do distinguish some closely related species from this group. A comparison of mitochondrial sequences from 2238 species in 11 animal phyla showed 98% of closely related species pairs had more than 2% sequence difference, which is enough for successful identification of most species.

Resolution of recently diverged species: Collections of closely related organisms that have recently passed the threshold to win the status of species challenge separation by any method, including morphology. In some cases, a mitochondrial barcode may narrow identification to two (or more) closely related species and no further. The frequency of species with shared barcodes is low in groups studied so far.

Hybrids: Identification systems based on a single gene (nuclear or mitochondrial) will not allow the certain identification of hybrids, that is, individuals whose male and female parent are from different species. Such specimens may be misidentified morphologically as well.

Nuclear pseudogenes: Pseudogenes, which are inactive copies of genes usually containing multiple mutations and/or deletions, can complicate identification by either mitochondrial or nuclear genes. Pseudogenes have proven a minor limitation to using a mitochondrial barcode in groups studied so far.

5. Why select the barcode sequence from within one gene?

With a few exceptions, animal mitochondria contain an identical set of genes: 13 protein coding, 2 ribosomal RNA, and 22 transfer RNA genes. While the order of the genes and their polarity (location on plus or minus strand) differ markedly among animal phyla, sequences from diverse organisms can be easily compared as

long as the barcode locality is limited to one gene. Staying within the boundaries of a single gene also eases development of broad range techniques for recovery of barcode sequences from diverse organisms.

6. Why standardize on COI for animals?

The mitochondrial protein-coding genes generally contain more differences than the ribosomal genes and thus are more likely to distinguish effectively among closely related species. Sequence comparisons among protein-coding genes are easier because they generally lack insertions or deletions frequently present in ribosomal genes. Among candidate protein-coding gene regions, the cytochrome *c* oxidase I (COI) locality contains sequence differences representative of those in other mitochondrial protein-coding genes. Possible gains in accuracy or cost from using a different protein-coding domain would likely be small in light of the general similarity of these regions. The COI region that is rapidly gaining currency represents approximately the first half of the gene and is 648 base pairs, a length easy to process in one "grab" with current technology and thus cheap.

Results to date indicate that this COI barcode is:

1) Easy to recover from diverse taxa, using a limited set of primers

2) Readily aligned for sequence comparisons

3) Effective in distinguishing among closely related animal species from a variety of invertebrate and vertebrate taxa

7. What do barcode differences among and within animal species studied so far suggest?

COI barcode sequences differ much more among than within species. For example, among 260 species of North American birds, differences between closely related species averaged 18-times higher than differences within species. Thus, a COI barcode alone should identify most bird species. Exceptions occur among some species that diverged very recently or hybridize regularly. Alternatively, low barcode differences between specimens attributed to different species may indicate synonymy, i.e., single species incorrectly split into separate taxa, or misidentified

specimens. On the other hand, large barcode differences of specimens within a species may signal the presence of species mistakenly lumped together by current taxonomy.

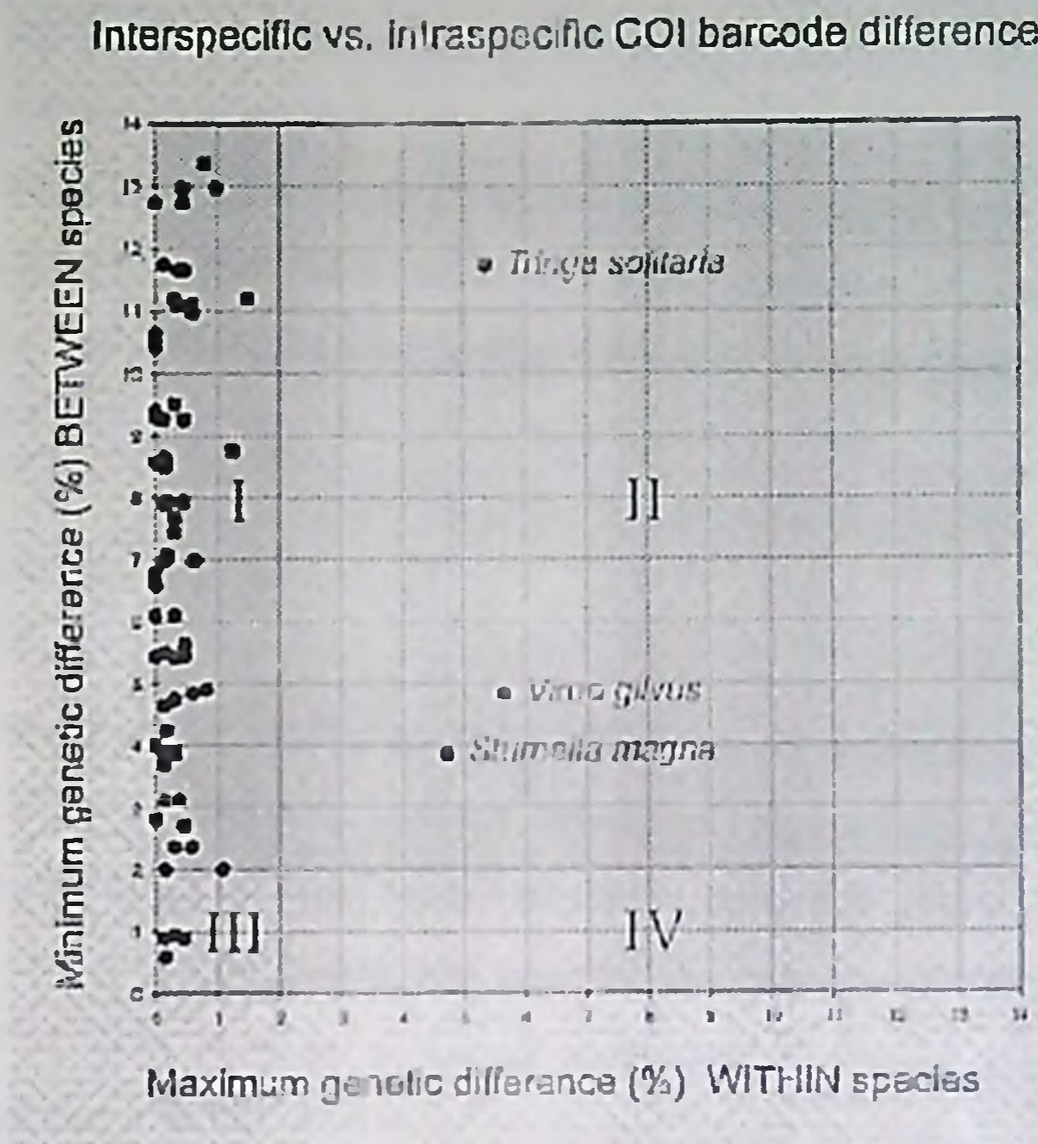

Fig.5. Results for COIbarcode difference of 73 species of North Ameriacan birds(Quadrants represent different categories of species).

8. What about humans?

Barcodes affirm the unity of the species *Homo sapiens*. Comparison of COI barcode sequences shows we typically differ from one another by only one or two base pairs out of 648, while we differ from chimpanzees at about 60 locations and gorillas at about 70 locations. Large intraspecific differences may signal the presence of hidden species, as for example in the recent recognition of two species of orangutan.

9. Can barcodes aid understanding history of animal and plant species?

While barcoding's goal is identification of specimens at the level of species, various rules also assemble groups of barcodes in "trees" suggesting evolutionary distances and relationships among species. For centuries biologists have worked to construct a tree of life or phylogeny showing the history of species. These efforts benefit from analysis of multiple characters, especially across long eras and varied groups. In the few cases examined so far, genetic distances among COI barcodes are largely congruent with understanding developed through traditional taxonomy, suggesting a library of barcodes will help evolutionary study.

10. Who is advancing barcoding?

The Consortium for the Barcode of Life (CBOL) is an international collaboration of natural history museums, herbaria, biological repositories, and biodiversity inventory sites, together with academic and commercial experts in genomics, taxonomy, electronics, and computer science. The mission of CBOL is to speed compilation of DNA barcodes of known and newly discovered animal and plant species, establish a public library of sequences linked to named specimens, and promote development of portable
devices for DNA barcoding.

More information is available at:

http://barcoding.si.edu http://www.barcodinglife.org

http:// phe.rockefeller.edu/BarcodeConference/index.html

TEN REASONS for BARCODING LIFE:

1.Works with fragments: Barcoding can identify a species from bits and pieces. When established, barcoding will quickly identify undesirable animal or plant material in processed foodstuffs and detect commercial products derived from regulated species. Barcoding will help reconstruct food cycles by identifying fragments in stomachs and assist plant science by identifying roots sampled from soil layers.

2.Works for all stages of life: Barcoding can identify a species in its many forms, from eggs and seed, through larvae and seedlings, to adults and flowers.

3.Unmasks look-alikes: Barcoding can distinguish among species that look alike, uncovering dangerous organisms masquerading as harmless ones and enabling a more accurate view of biodiversity. Mosquito control programs depend on accurate species identification, a task requiring great expertise, particularly for larval forms, which can provide early warning before adults hatch and can be treated with local measures. Once a reference library of DNA barcodes is established, DNA-based identification can be applied by many more personnel to more effectively target control measures and limit injury to non-harmful species.

4.Reduces ambiguity: Written as a sequence of four discrete nucleotides - CATG – along a uniform locality on genomes, a barcode of life provides a digital identifying feature, supplementing the more analog gradations of words, shapes and colors. A library of digital barcodes will provide an unambiguous reference that will facilitate identifying species invading and retreating across the globe and through centuries.

5. Makes expertise go further: The bewildering diversity of about 2 million species already known confines even an expert to morphological identification of only a small part of the plant and animal kingdoms. Foreseeing millions more species to go, scientists can equip themselves with barcoding to speed identification of known organisms and facilitate rapid recognition of new species.

6.Democratizes access: A standardized library of barcodes will empower many more people to call by name the species around them. It will make possible identification of species whether abundant or rare, native or invasive, engendering appreciation of biodiversity locally and globally.

7.Opens the way for an electronic handheld field guide, the Life Barcoder: Barcoding links biological identification to advancing frontiers in DNA sequencing, miniaturization in electronics, and computerized information storage.

Integrating those links will lead to portable desktop devices and ultimately to hand-held barcoders. Imagine the promise of a schoolchild with a barcoder in hand learning to read wild biodiversity, the power granted to a field ecologist surveying with a barcoder and global positioning system, or the security imparted by a port inspector with a barcoder linked to a central computer.

8.Sprouts new leaves on the tree of life: Since Darwin, biologists seeking a natural system of classification have drawn genealogical trees to represent evolutionary history. Barcoding the similarities and differences among the nearly 2 million species already named will provide a wealth of genetic detail, helping to draw the tree of life on Earth. Barcoding newly discovered species will help show where they belong among known species, sprouting new leaves on the tree of life. Barcoding the similarities and differences among the estimated 10 million species of animals and plants will help show where their leaves belong on the tree of life.

9. Demonstrates value of collections: Compiling the library of barcodes begins with the multimillions of specimens in museums, herbaria, zoos and gardens, and other biological repositories. The spotlight that barcoding shines on these institutions and their collections will strengthen their ongoing efforts to preserve Earth's biodiversity.

10.Speeds writing the encyclopedia of life: Compiling a library of barcodes linked to vouchered specimens and their binomial names will enhance public access to biological knowledge, helping to create an on-line encyclopedia of life on Earth, with a web page for every species of plant and animal.

Organisms barcoded

- Animals
- Birds
- Marine organisms
- Microorganisms
- Nematodes
- Plants

Barcoding animal life:

With millions of species and their life-stage transformations, the animal kingdom provides a challenging target for taxonomy. The results indicate that sequence divergences at COI regularly enable the discrimination of closely allied species in all animal phyla except the Cnidaria (Hebert *et al.*, 2003). In total, sequence divergences are examined in more than 13,000 congeneric pairs including representatives from 11 phyla. These results support, with the exception of a single phylum, the conclusion that species-level diagnoses can routinely be obtained through COI analysis.

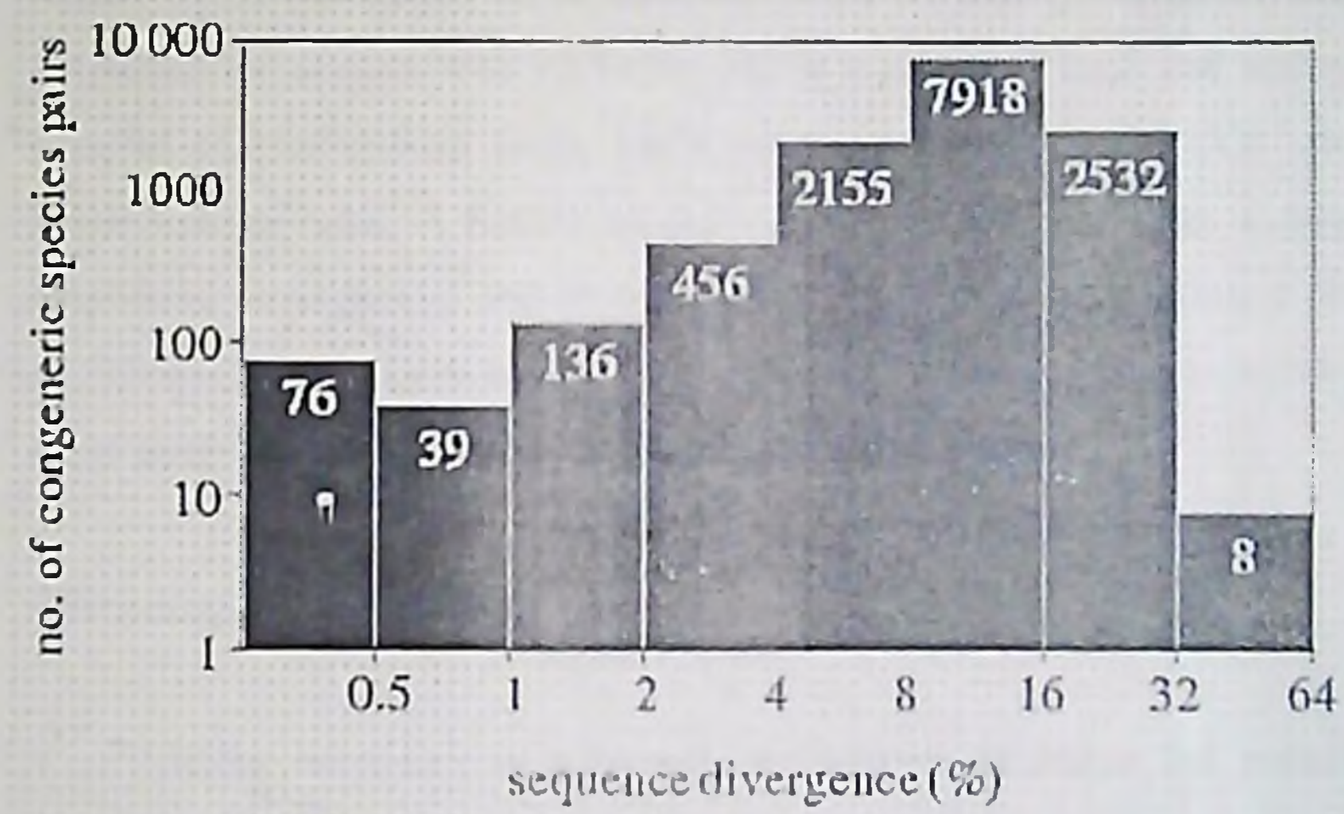

Fig.6. COI sequence divergences for 13,320 congeneric pairs of animal species belonging to 11 phyla.

Fish Barcode of Life Initiative (FISH-BOL)

FISH-BOL is a global effort to barcode fish to aid in species identification for all users, especially taxonomists. It will create a valuable public resource in the form of an electronic database that contains DNA barcodes, images, and geospatial coordinates of examined specimens. The database will contain linkages to voucher specimens, information on species distributions, nomenclature, authoritative

taxonomic information, collateral natural history information and literature citations.

All Birds Barcoding Initiative (ABBI)

So far, researchers have deposited 3308 avian COI barcodes from about 800 species, which represents 8% of world birds, to the Barcode of Life Database (BOLD)

Barcoding of Amphibians

Experimental evidences shows that the mitochondrial 16S rRNA gene fulfills the requirements for a universal DNA barcoding marker in amphibians. Amplification success was 100% for *16S* in a subset of fresh and well-preserved samples of Madagascan frogs, while various combinations of *COI* primers had lower success rates. A further mitochondrial gene that has been widely used in amphibian phylogenetic and phylogeographic studies is cytochrome b (Sumida *et al.*, 2001). This gene can easily be amplified in salamanders and archaeobatrachian frogs using primers that anneal with adjacent tRNA genes.

Barcoding Micro organisms

String barcoding is a recently introduced technique for genomic-based identification of microorganisms such as viruses or bacteria from among a set of previously sequenced microorganisms. Applications of this technique range from rapid pathogen identification in epidemic outbreaks to point-of-care medical diagnosis to monitoring of microbial communities in environmental studies (Borneman *et al.*, 2001). Here the whole genomic DNA sequence is used for creating a barcode. Identification is performed by spotting or synthesizing on a micro array the Watson-Crick complements of the distinguisher strings t1; : : : ; tk, and then hybridizing to the array the fluorescently labeled DNA extracted from the unknown microorganism. Under the assumption of perfect hybridization stringency, the hybridization pattern can be viewed as a string of k zeros and ones,

referred to as the barcode of the microorganism. DNA-BAR is software for selecting sets of distinguishers to be used in this type of identification assays.

Barcoding in plants

Hybridization and polyploidy have play a key role in plant speciation, complicating the reconstruction of their phylogenetic histories. Past works have revealed that rates of mitochondrial evolution are far slower in plants than animals, making COI an unlikely candidate. Plant mitochondrial genes exhibit lower nucleotide substitution than plastid or nuclear genes (Wolfe *et al.*, 1987;Yang *et al.*, 1999). Substitution rate in plastid genes rates about one quarter the rate observed in nuclear DNA. It is 10-20 fold less than mammalian mitochondrial DNA (Palmer and Herbon, 1988).

Two potential barcode candidates are the Nuclear Ribosomal Internal Transcribed Spacer Region (ITS) and plastid trnH-psbA intergenic spacer (Kress *et al.*, 2005). This locus meets the criteria that are necessary for a DNA barcode candidate, like

I. Significant species level genetic variability and divergence
II. An appropriately short sequence length (~450bp) so as to facilitate DNA extraction and amplification
III. The presence of conserved flanking sites for developing universal primers.

One major concern regarding the use of ITS is the presence of paralogous copies of ITS in some genera (Campbell *et al.*, 2005). It is also reported that ITS and trnH-psbA sequences are alignable within genera, but problematic above that rank. However, evaluation of other plastid coding regions not yet revealed a better candidate foe all land plants.

Other plastid loci tried are,

•trnL-F intergeneric spacer

•mat K (maturase- within trnK intron)

•ndh F(subunit of NADH dehydrogenase)

•atp B (beta subunit of ATPase)

•rbc L (RuBisCO large subunit)

A Tiered Approach in barcoding of plants

•First tier in which coding region common across the land plants provide resolution at a certain rank (Family or genus). The rbcL region meets most of the attributes for a first tier barcoding locus.

•Second tier in which more variable (coding/non coding) region provides resolution to the species level. Some of the plastid non-coding regions or trnH-psbA appear promising as second tier.

Case studies

Identification of birds

In an effort to find a correspondence between traditional species boundaries established by taxonomy and those inferred by DNA barcoding, Hebert and co-workers sequenced DNA barcodes of 260 of the 667 bird species that breed in North America (Hebert *et al.*, 2003). They found that every single one of the 260 species had a different COI sequence. 130 species were represented by two or more specimens, in all of these species. COI sequences were either identical or were most similar to sequences of the same species. COI variations between species (interspecific) averaged 7.93%, whereas variation within species (intraspecific) averaged 0.43%. In four cases there were deep intraspecific divergences, indicating possible new species. Three out of these four polytypic species are already split into two by some taxonomists. Their results reinforce these views and strengthen the case for DNA barcoding. Hebert *et al.* also proposed a standard sequence threshold to define new species, this threshold was defined as 10 times the mean intraspecific variation for the group under study.

Delimiting cryptic species

The next major study into the efficacy of DNA barcoding was focused on the neotropical skipper butterfly, *Astraptes fulgerator*. This species was already

known as a cryptic species complex, due to subtle morphological differences, as well as an unusually large variety of caterpillar food plants. However, several years would have been required for taxonomists to completely delimit species. Hebert *et al.* (2004) sequenced the COI gene of 484 specimens from north-western Costa Rica. This sample included "at least 20 individuals reared from each species of food plant, extremes and intermediates of adult and caterpillar color variation, and representatives" from the three major ecosystems where *Astraptes fulgerator* is found. Hebert *et al.* (2004) concluded that *Astraptes fulgerator* consists of 10 different species in north-western Costa Rica. These results highlight the potential of DNA barcoding in the discovery of new species, when used in conjunction with traditionally collected data

Identifying Flowering Plants

Kress et al.(2005) suggest that the use of the COI sequence "is not appropriate for most species of plants because of a much slower rate of cytochrome c oxidase I gene evolution in higher plants than in animals". A series of experiments was then conducted to find a more suitable region of the genome for use in the DNA barcoding of flowering plants. At the conclusion of these experiments, Kress *et al.* (2005) proposed the nuclear internal transcribed spacer region and the plastid trnH-psbA intergenic spacer as a potential DNA barcode for flowering plants. These results suggest that DNA barcoding, rather than being a 'master key' may be a 'master keyring', with different kingdoms of life requiring different keys.

Benefits of DNA barcoding

The direct benefits of DNA barcoding undoubtedly include:

(i) Make the outputs of systematics available to the largest possible community of end-users by providing standardized and high-tech identification tools, e.g. for biomedicine (parasites and vectors), agriculture (pests), environmental assays and customs (trade in endangered species);

(ii) Relieve the enormous burden of identifications from taxonomists, so they can focus on more pertinent duties such as delimiting taxa, resolving their relationships and discovering and describing new species;
(iii) Pair up various life stages of the same species (e.g. seedlings, larvae);
(iv) Provide a bio-literacy tool for the general public.

Barcode applications

-Biodiversity studies
-New species identification (e.g. in medicine, bacteriology, etc)
-Pest diagnostics in agriculture
-Disease diagnosis (e.g. in veterinary, parasitology, etc.)
-Quarantine

Advantages of DNA barcoding

-It is a taxonomic identification tool alternative or additional to morphology
-DNA sequencing is a rapid and relatively low cost technique
-Can process a great number of specimens at a time, thus is useful, for example, in biodiversity surveys
-Once a reference database is established, it can be applied by non-specialist.

Barcode limitations

- It is not always true that intraspecific variability is negligible, or at least lower than interspecific values
- There is no universal DNA barcode gene: No single gene will work for all taxa (e.g., COI is not appropriate for vascular plants, even for some animals).
- Barcode sequences should be generated from type specimens, thus relying on classical taxonomy.
- Single gene approach is less precise than using multiple genes; may introduce unacceptable error

- Some of the most attractive aspects rely on future technology, e.g., handheld sequencer

Summary

The Global Taxonomy Initiative works to overcome "the taxonomic impediment" — the lack of data concerning Earth's biodiversity, which limits our ability to manage living resources in a sustainable and responsible manner. The shortage of trained taxonomists and access to the essential information resources (especially museum and herbarium collections, taxonomic publications, databases on the Web) are most acute in developing countries where biodiversity is highest. DNA barcoding has the potential to increase access to taxonomic knowledge in all regions of the world. Databases of reference barcodes are connecting specimens to their correct species names, providing a direct route to species information associated with those names.

CBOL is working with GenBank and its partner DNA repositories (European Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ)) to construct a global library of reference barcode sequences. Each barcode record is linked to a voucher specimen in a collection, a valid species name, and the associated taxonomic literature. Connections are being built to the Global Biodiversity Information Facility (GBIF) and other biodiversity data portals. Through these efforts, an integrated information infrastructure for taxonomy is growing rapidly. And just as one might Google a species name today to find pictures or description of an individual insect, the time may come when we have star Trek style mobile computers that can read off barcodes and access species information in the field.

Discussion

1. Define species?

A species is a reproductively isolated population that shares a common gene pool and a common niche, or, a group of related organisms that share a more or less distinctive form and are capable of interbreeding.

2. What is the difference between DNA finger printing and DNA barcoding?

DNA finger printing is used for the individual level identification whereas DNA barcoding is used for the species level identification.

3. Why can't DNA barcoding be used for interspecific hybrids?

Identification systems based on a single gene (nuclear or mitochondrial) will not allow the certain identification of hybrids, that is, individuals whose male and female parent are from different species. Such specimens may be misidentified morphologically as well.

4. Why is called 'DNA barcoding' rather than 'mitochondrial coding'?

The barcoding make uses not only the mitochondrial DNA, but any DNA sequence from a standard position in the genome,which shows maximum variability between the species.It can be mitochondrial or chloroplast genome.But the early success has reported in animals using mitochondrial genome.

5. Why we select mitochondrial sequence for barcoding?

Mitochondrial genome has high copy number,shows minimum variability within species and maximum variability between species which is an essential feature required for DNA barcoding.Moreover, introns are absent in mitochondria which makes easy amplification.

6. Are there any works done in plants?

Yes, some works were conducted in *Atropa belladonna*.But the research for identification of a potential barcode region is going on.

7. Why we can't use mitochondrial gene sequences in plants?

The rate of mutation is very low in the case of plant mitochondrial DNA. Rsearches are going on to find out a barcode sequence that can be used for identification of all the plants.

8. What are transposons?

Transposons are sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition. In the process, they can cause mutations and change the amount of DNA in the genome.

9. Is there any special instrument used for DNA barcoding?

Till now there is no such instruments and scientists are using PCR amplification and sequencing software. Development of a hand held barcoder is on the horizon.

10. Why there are more than 4 colours representing ATGC?

The four colours represents the 4 bases present in DNA.The other colours represents the insertions / deletions and unrecognized bases in the DNA segment.

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ABSTRACT

Species identification underpins all of biological research. Existing morphology based diagnostic approaches are often both cumbersome to use and are effective only for certain life stages. Over the past 250 years, more than 1.7 million species of animals, plants and other organisms have been described. It represents a small minority of the estimated 10-to15million species alive today. This necessitates a rapid, accurate, automatable and globally accessible procedure for species delimitation and identification.

DNA barcoding is taxonomic methods, which uses a short genetic marker in an organism's genomic DNA to quickly, and easily identify it as belonging to a particular species. Mitochondrial DNA (mt DNA) has a relatively fast mutation rate, which results in significant variance in mt DNA sequences between species and comparatively less variance within species. A 648 bp region of the mitochondrial gene, known as cytochrome c oxidase I (COI), has been proposed as a potential 'barcode' in animals (Hebert *et al.*, 2003). However, COI is not a good candidate in plants because plant mitochondrial genes typically exhibit lower nucleotide substitution than plastid and nuclear genes (Yang *et* al., 1999). The two potential barcodes suggested in plants are nuclear ribosomal Internal Transcribed Spacer region (ITS) and the plastid trnH-psbA spacer (Kress *et al.*, 2005).

Just like UPC barcodes(Universal Product Code), DNA barcodes contain four alternate nucleotides at each position. DNA barcoding is advancing through the Consortium for the Barcode of Life (CBOL) and Barcode of Life Database (BoLD) (www.barcodinglife.org). Scientists are trying to see whether the technique will work and how it needs to be modified to include plants, if possible, many microbial species that may not be easily identified by the existing DNA barcoding system. DNA barcoding works for identifying organisms at different stages of life, such as the eggs and larvae of insects. It can easily distinguish between species that look alike.

Given the pace of advancement in technology, it is not unrealistic that in the span of a few years we may be using a barcoding tool for routine identifications, discovering new species, solving ecological puzzles, controlling the pathways of invasive species and for quality control in the food and herbal industries. Certainly in the course of development of a rigorous and comprehensive database, multiple new ecological and taxonomic hypotheses will emerge.

16.0

BIOPLASTICS

by

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MSc.Ag.Plant Biotechnology

SEMINAR REPORT

Submitted in partial fulfilment for the course

Seminar

PBT651

COLLEGE OF HORTICULTURE

KERALA AGRICULTURAL UNIVERSITY

VELLANIKKARA, THRISSUR - 680656

DECLARATION

I, **Ramya Haridas (05-11-139)** here by declare that this seminar report entitled **"Bioplastics"** has been prepared by me independently, after going through the various references cited herein and that I have not copied or adopted from any of the fellow students or previous seminar reports.

Vellanikkara
09 .07.2006

Ramya Haridas
05-11-139

CERTIFICATE

Certified that the seminar report entitled **"Bioplastics"**for the course PBT651 has been prepared by **Ramya Haridas (05-11-139)** after going through the various references cited here under my guidance and she has not copied from any of fellow students.

Vellanikkara

09 .07.2006

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INDEX

List of tables

List of figures

1. Introduction

The exponential growth of human population has led to the accumulation of huge amount of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically in such a way that the presence of non-biodegradable residues is affecting the potential survival of many species. For this reason, many countries have promoted special programmes directed towards the discovery of new commonly used materials from the biosphere and have the novel strategies aimed at facilitating the transformation of contaminants.

Biomaterials are natural products that are synthesized and catabolized by different organisms and that have many broad biotechnological applications. They can be assimilated by many species (biodegradable) and do not cause any toxic effects in the host (biocompatible), conferring upon a considerable advantage with respect to other conventional synthetic products. Biodegradable plastics are a new generation of plastics that are derived from renewable resources and can be converted to biomonomers by fermentation, which can be further converted by chemical synthesis to biodegradable polymers like polylactic acid (Narayan, 1998). Biomonomers can also be microbially transformed to biopolymers such as the polyhydroxyalkanoates

Bioplastics are a special type of biomaterial. They are polyesters produced by a range of microbes cultured under different nutrient and environmental conditions. These polymers which are usually lipid in nature are accumulated as storage materials (in the form of mobile, amorphous, liquid granules) allowing microbial survival under stress conditions. The number and size of the granules, the monomer composition, macromolecular structure and physicochemical properties vary depending on the producer organism (Leungo *et al*, 2003).

2. History

Significant commercialization of bioplastics only began in the middle of 19th century.

1869 – John Wesley Hyatt was looking for a substitute for ivory in the manufacture of billiard balls. In the same year patented a cellulose derivative for coating non ivory billiard balls.

1920 – Henry Ford experimented with using soyabeans in the manufacture of spare parts of car

1960 – one well established bioplastic that has survived the growth of the synthetic plastic industry is cellophane, a sheet material derived from cellulose. Production peaked during this period and still used in the packaging of candy, cigarettes and other articles

1989- The idea of producing PHA's in transgenic plants was first described

1990- successful production of PHB in various plants ranging from *Arabidopsis thaliana* to commercial oil seed crops and even cotton.

1996 – Monsanto began to sell PHB under the trade name Biopol

2000 & beyond – Demand for materials like plastics is continually increasing Monsanto's fermenters producing PHB from bacteria were closed down at the start of 2004. The focus is now on producing PHB from plants instead of bacteria

3. Why green plastics?

Green plastics are the focus of an emerging industry focused on making convenient living consistent with environmental stability.

- One reason to make a shift toward the use of green plastics is the availability of raw materials. Green plastics can be made using polymers that come from agricultural and marine feedstocks.
- These are abundant natural resources that are constantly being replenished
- Another favorable property of green plastics is their biodegradability, making them a natural material for use in such applications as compostable collection bags, such as for food or yard waste.
- They preserve nonrenewable resources - petroleum, natural gas, and coal and contribute little to the already burdensome problems of waste management.

4. Types of Biopolymers

There are two main types of biopolymers: those that come from living organisms and those, which need to be polymerized but come from renewable resources. Both types are used in the production of bioplastics.

4.1 Biopolymers From Living Organisms

These biopolymers are present in or created by living organisms. These include carbohydrates and proteins. These can be used in the production of plastic for commercial purposes.

Table.1 Examples for biopolymers from living organisms

Biopolymer	Natural source	What is it
Cellulose	Wood, cotton, corn, wheat and others	This polymer is made up of glucose. It is the main component of plant cell walls
Soya protein	Soyabeans	Protein which naturally occurs in soya plant

Starch	Corn, potato, wheat, tapioca and others	Carbohydrates, which are stored in the plant tissues. It is a polymer made of glucose and is not found in animal tissues
Polyesters	Bacteria	These polyesters are created through naturally occurring chemical reactions that are created by certain types of bacteria

4.2 Polymerizable Molecules

These molecules come from renewable natural resources, and can be polymerized to be used in the manufacture of biodegradable plastics.

Table.2 Examples of polymerizable molecules

Biopolymer	Natural source	What is it
Lactic acid	Beets, corn, potatoes and others	Produced through fermentation of sugar feed stocks. It is polymerized o produce lactic acid- a polymer that is used to produce plastic
Triglycerides	Vegetable oils	These form a large part of storage lipids found in plant and animal cells. These can be polymerized to produce plastics

5. How are biopolymers and bioplastics made?

There are two methods being researched and used to produce plastics from plants. The first uses fermentation, and the second relies on the plant to become the factory for plastic production. These two methods are outlined below.

5.1 Using Fermentation to Produce Plastics

Fermentation, used for hundreds of years by humans, is even more powerful when coupled with new biotechnology techniques. Fermentation is the use of microorganisms to break down organic substances in the absence of oxygen. Today, fermentation can be carried out with genetically engineered microorganisms, specially designed for the conditions under which fermentation takes place, and for the specific substance that is being broken down by the microorganism. There are two ways fermentation can be used to create biopolymers and bioplastics.

a) Bacterial Polyester Fermentation

Bacteria are one group of microorganisms that can be used in the fermentation process. Fermentation, in fact, is the process by which bacteria can be used to create polyesters. Bacteria called *Ralstonia eutropha* are used to do this. The bacteria use the sugar of harvested plants, such as corn, to fuel their cellular processes (Potter et al, 2002). The by-product of these cellular processes is the polymer. The polymers are then separated from the bacterial cells.

b) Lactic Acid Fermentation

Lactic acid is fermented from sugar, much like the process used to directly manufacture polymers by bacteria. However, in this fermentation process, the final product of fermentation is lactic acid, rather than a polymer. After the lactic acid is produced, it is converted to polylactic acid using traditional polymerization processes.

5.2 Growing Plastics in Plants

Plants are becoming factories for the production of plastics. Researchers created a *Arabidopis thaliana* plant through genetic engineering. The plant contains the enzymes used by bacteria to create plastics. Bacteria create the plastic through the

conversion of sunlight into energy. The researchers have transferred the gene that codes for this enzyme into the plant, as a result the plant produces plastic through its cellular processes. The plant is harvested and the plastic is extracted from it using a solvent. The liquid resulting from this process is distilled to separate the solvent from the plastic (Stenbuchel and Fuchstenbusch, 1998).

Eg: Growing plastics in corn

- The genes were isolated that enable the bacteria to make plastic
- These genes were inserted into the plastics to produce polyhydroxyalkanoates
- The stover (stalk and leaves) of the corn plant was targeted in order to create a crop that could be harvested for food and plastic

The research work was first conducted in the plant *Arabidopsis thaliana*. It belongs to the member of the brassicaceae family with 2n=10. It is considered as the model plant that is exploited in many genetic and molecular studies. *Arabidopsis* is well suited for genetic research because its life cycle is completed within one month (Slater *et al*, 1990).

Fig.1 *Arabidopsis thaliana*

Another important approach is to convert the plant sugar to produce plastic polymer called polylactide.The sugars are fermented into lactic acid using microorganisms. Then the lactic acid is chemically treated to link the molecule of lactic acid into polymers called polylactide (PLA) which is used in soda bottles and clothing fibres.

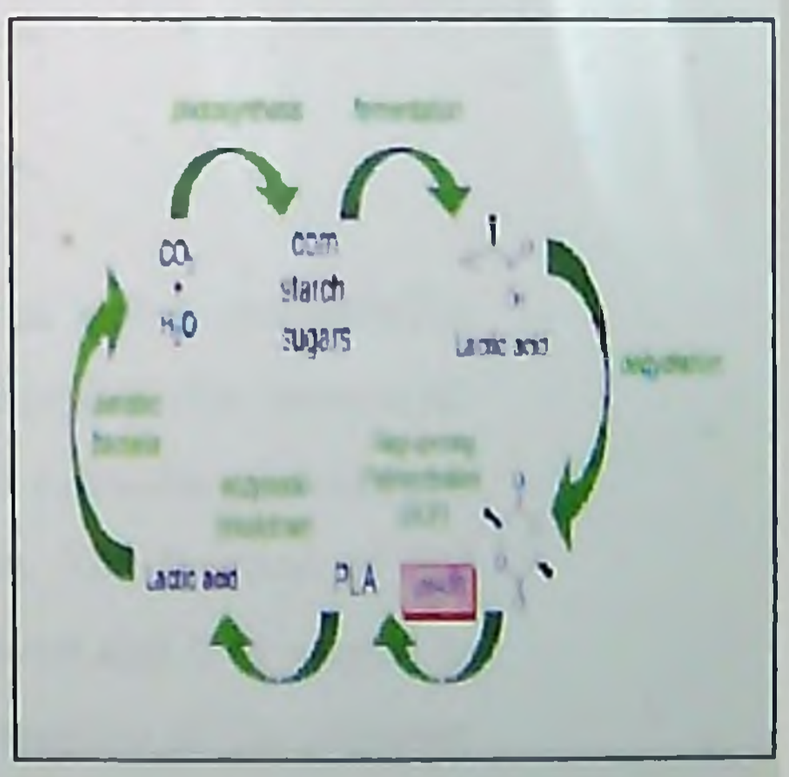
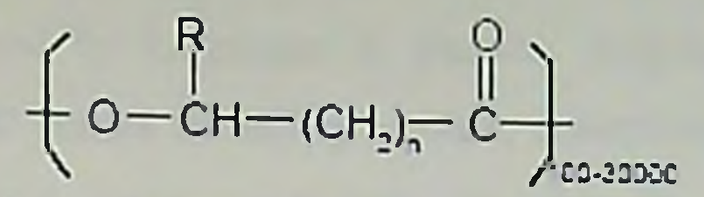


Fig.2 Production of PLA

6. Structure of PHA

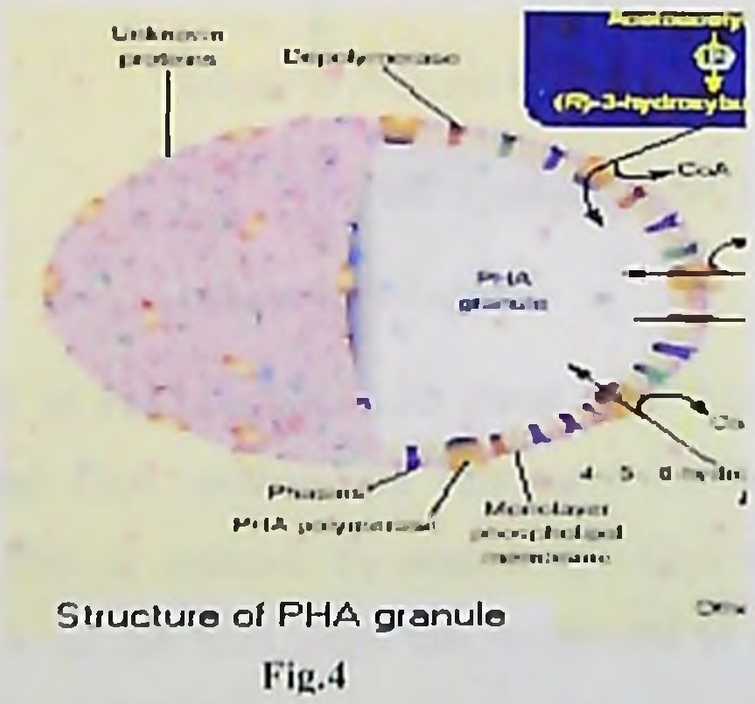
The structure of polyhydroxyalkanoates (PHA's) which forms the bioplastic is shown below.



n = 1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Fig.3 General structure of PHA

The length of R side chain alters the properties of the plastics and can vary from 0 carbon, 1 carbon or 2 carbon atoms up to long carbon chains. Polyhydroxybutyrate is the best characterized PHA and is found as intracellular inclusions in a wide variety of bacteria.



Structure of PHA granule

Fig.4

PHA's are accumulated intercellularly (as amorphous mobile polymers) in granules of different sizes. They are surrounded by a phospholipids monolayer (PM) containing phasins polymerase, a depolymerase and cytosolic proteins non specifically attached to the granule (Sudesh *et al.* 2003). The function of PM envelope has not yet well established although it is believed that it is needed to avoid the contact of PHA's with water (preventing the transition of the polyester from amorphous liquid state to a more stable crystalline form) and that it act as a protective barrier (avoiding cellular damage caused by the interaction of PHA

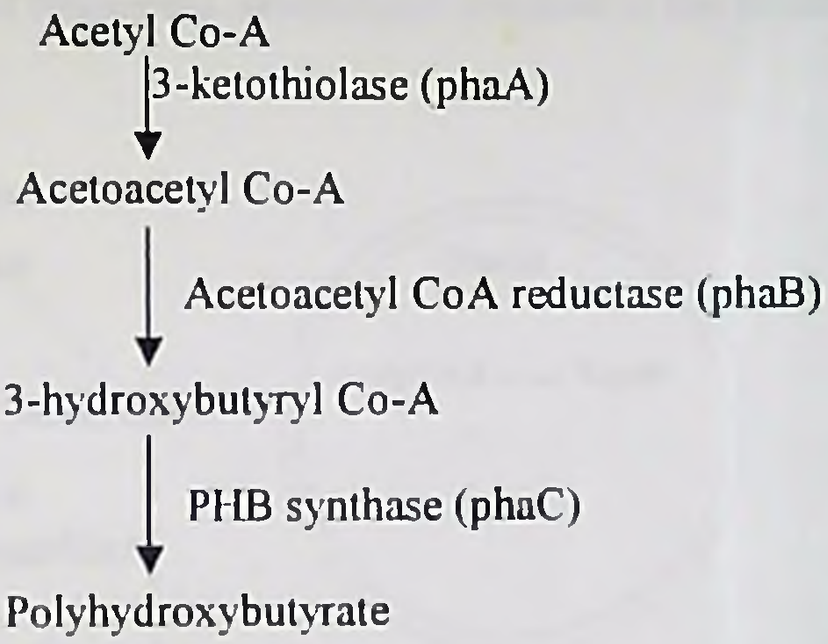
with internal structures or with cytosolic proteins. Phasins are low molecular weight protein (accumulated to high levels during PHB synthesis) that enhances PHB production by binding to the granules (York et al, 2001).

7. PHB

PHB was first described by Lemoigne in *Bacillus megaterium*. Microbes belonging to more than 90 genera including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolize these polyesters

8. Biosynthesis of PHB:

The pathway for PHB synthesis is shown below



The biosynthesis of PHB requires only three steps from acetyl CoA. In the first step, two acetyl CoA residues are condensed by 3-ketothiolase to form acetoacetyl CoA. This is reduced by acetoacetyl CoA reductase to give 3-hydroxybutyryl CoA. In the final stage this compound is polymerized by PHB synthase (Hulsman *et al*, 1991).

PHB and other related polyhydroxyalkanoates are aliphatic esters with thermoplastic properties. *Alcaligenes eutrophus* and many other species of bacteria produce polyhydroxyalkanoates as a carbon reserve when grown in a medium with an excess of carbon but limited in an essential nutrient such as nitrogen or phosphorus. PHB is synthesized as 0.2-1μm and may accumulate upto

80% of the dry weight. In *Alcaligenes eutrophus*, the enzymes responsible for the synthesis of PHB from acetylCoA are encoded by three different genes phaA, phaB and phaC respectively. The first step of the pathway, production of acetoacetyl CoA occurs in the cytoplasm of the plant.

A wide variety of microorganism can hydrolyze polyhydroxyalkanoates polyesters to monomers, which are metabolized as a carbon source. Therefore polyhydroxyalkanoates have attracted interest as a potential source of renewable biodegradable plastics (Anderson and Dawas, 1990). The cost of PHB produced by bacterial fermentation is significantly higher than the cost of starch or oil from agricultural plants. Therefore the feasibility of producing PHB in plants was initially explored in a pilot experiment in which the expression of acetoacetylCoA reductase and PHB synthase of *Alcaligenes eutrophus* in the cytoplasm of transgenic *Arabidopsis* resulted in the accumulation of granules of PHB.

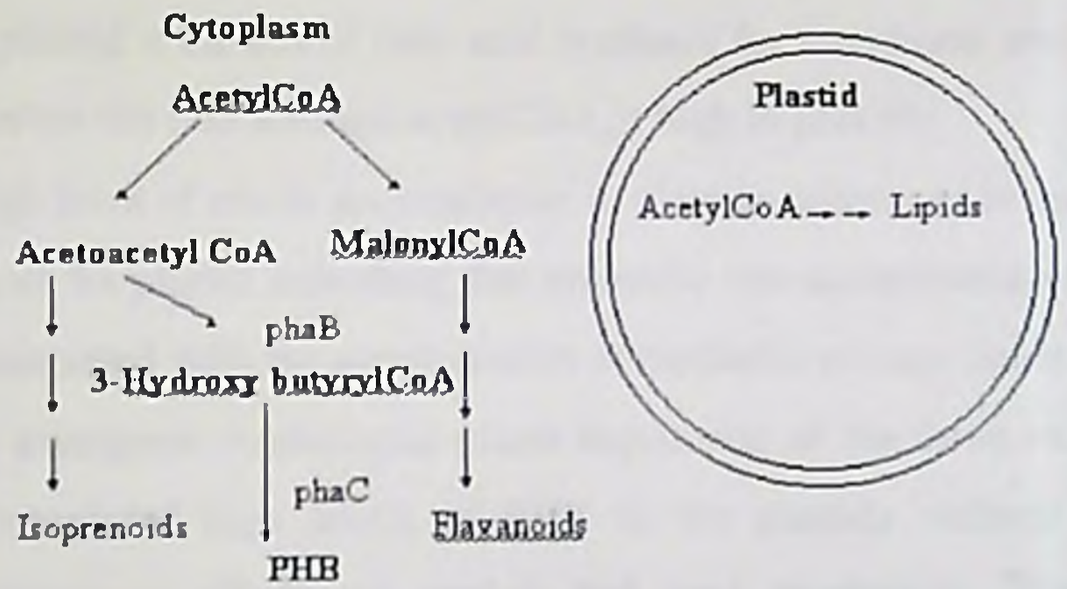


Fig.5 Metabolism of PHB in cytoplasm

The presence of 3-ketothiolase activity in the plant cytoplasm removes the necessity to transfer three genes into the plants. The phaB and phaC genes from *Alcaligenes eutrophus* were transformed into *Arabidopsis* without protein targeting sequence

The main metabolic role of cytoplasm acetylCoA in higher plants is thought to be as the precursor for mevalonate synthesis. Thus the low yield of PHB and the growth inhibition observed in PHB producing plants were hypothesized to be due to depletion of cytoplasmic acetyl CoA pool. A possible strategy for overcoming

this problem is to change the sub cellular location of PHB production. So the biosynthesis of PHB was targeted to the plastids of the plant by fusing to a sequence encoding the transit peptide plus N-terminal fragment of Rubisco small subunit protein and expression was directed by CaMV 35S promoter (Poirier *et al*, 2002).

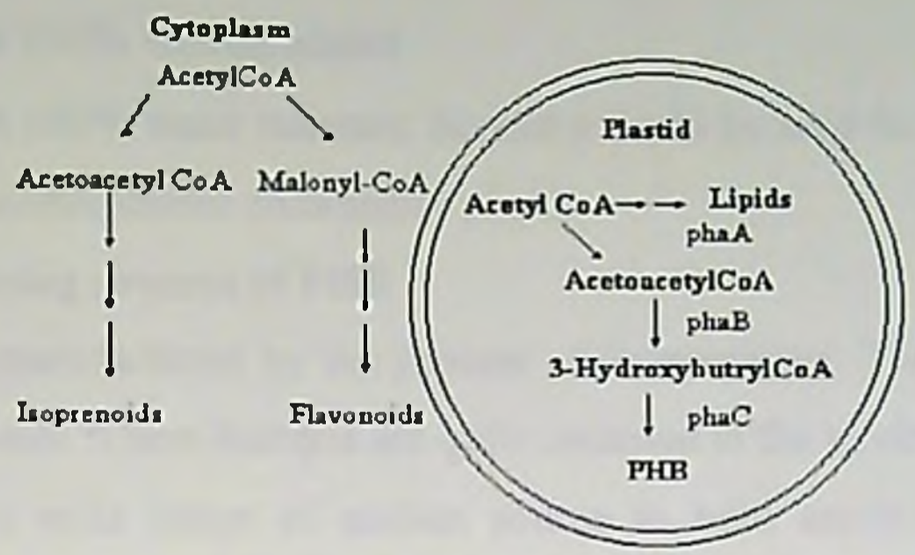


Fig.6 Metabolism of PHB in plastid

9. Advantages of plastid over cytoplasm:

- The plastid is the site of fatty acid synthesis for membrane and storage lipids. Therefore the flux through acetylCoA is high in plastids.
- A high level of starch accumulation in plastids seems not to interfere with the function of the plastid indicating that organelle can accommodate the physical distortion associated with the accumulation of insoluble storage lipids.

The transgenic Arabidopsis plants expressing all the three enzymes in the plastids accumulated high levels of PHB in the plastids without any readily apparent deleterious effects on growth and seed production. The bioplastics accumulated as0.2-0.7μm granules in the plastids to levels of up to 14% plant dry weight and cytoplasm resulted in 100 fold increase in the amount of PHB that has been accumulated.

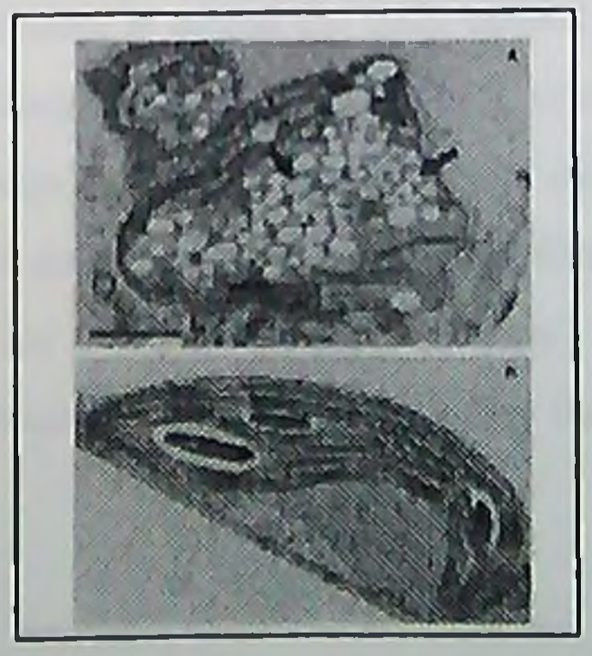


Fig.7 PHB granules in the plastids of *Arabidopsis thaliana*

10. Properties of PHB

- PHB is a stiff and brittle
- They show high degree of crystallinity
- It has high melting point
- They are 100% biodegradable
- They are 100% water resistant. So that it could be used for similar applications as conventional commodity plastic

11. Manufacturing process of PHB

PHB is manufactured by the process of fermentation. The microorganism used is *Alcaligenes*. These bacteria are quite common in the environment and they can grow on a wide range of carbon source in both aerobic and anaerobic conditions. This strain grows very efficiently on glucose and it is safely handled in large quantities.

For balanced growth bacteria needs a carbon source, an energy source, nitrogen, phosphorus, sulfur, trace elements water and oxygen. If one nutrient is limited bacteria cannot produce amino acids and proteins. As a result they cannot grow. PHB production exploits these facts.

To begin the fermentation process *Alcaligenes eutrophus* is inoculated into a fed batch reactor containing a balanced glucose medium. All the nutrients are in excess except phosphorus. The medium's phosphate content is limited to support only a certain growth. The phosphate content decreases as the culture grows such that the culture eventually reaches phosphate starvation. Up to this point in the fermentation very little PHB has accumulated in the cell. But in the stage two of the process in which the glucose is added the cells cannot convert the glucose to amino acids or protein because of the low phosphate availability. Consequently the dry weight of the biomass rises significantly as the cells convert the glucose fed to PHB causing massive amounts of PHB to accumulate in the cells. The PHB concentration can account for up to 80% of the biomass's total dry weight at the end of the fermentation process. Carbon sources other than glucose can also be

used. Much work is under way to assess the use of various agricultural by products like molasses and sugarbeet (Luzier, 1994).

12. Polymer extraction

The last stage of PHB production involves separating the polymer from the cells. To do this a solvent or aqueous extraction can be used. In aqueous process, the cell walls are broken and the polymer is then extracted and purified. The aqueous process is less expensive but the process reduces molecular weight. Solvent extraction can produce the copolymer weights of one million whereas typical molecular weights of aqueous extracted copolymers are in the range 600,000 range. But the solvent process presents some safety concerns. The objective is to produce a consistant usable clean product wile keeping the production costs down.

13. Biodegradability

PHB degrades microbially active environments. Microorganisms colonize on the surface of the polymer and secrete the enzymes, which degrades PHB into different fragments. The cells use these fragments as a carbon source for their growth.Biodegradation rates depend on a variety of factors including surface area, microbial activity of the disposal environment, temperature, moisture levels and the presence of other nutrient materials. PHB is not affected by moisture alone. The environment must be microbially active.

PHB degrades in a wide range of environments. Degradation occurs most rapidly in anaerobic sewage and slowest in seawater. In aerobic environment, the end products are Co2, H2O and humus. Under anaerobic condition CH4 is also produced. No harmful byproducts or intermediates are produced during the degradation process.

14. Research works in other crops

- Researchers have recently succeeded in transferring plant producing genes from bacteria into oilseed rape plant. These were transferred into single multigene vectors to transform oil seed rape. PHB was found to accumulate in mature oil seed leucoplasts to levels upto 7.7% of fresh seed weight.

- Researchers also developed biodegradable plastic containers made from starch. This plate is used to make bowls, coffee cups, packaging and plastic films for wrapping materials
- Another discovery is to make plastics from potato waste that is high in starch. The starch is broken down to glucose and bacteria is added causing fermentation into lactic acid.
- The production of bioplastics in cotton fibres has been another interesting discovery. The cotton fibres contain β-ketothiolase activity. The genes were transferred into cotton by particle bombardment of seed axis meristem. Clusters of small PHB granules were found in the cytoplasm of the fibre cells.

15. Biolac

Other bioplastic, which is easily biodegradable are polylactides and polyglycolides. Researchers at the university of Wisconsin, USA have produced biodegradable polymers from lactic acid by fermentation of whey permeates. It can be used to manufacture bioplastics, as food preservatives, as flavour enhancer, acidulant and in pharmaceutical industry.

16. Industrial production

There are three important limitations in the bulk production of bioplastics.

- The special growth conditions required for the synthesis of these compounds (usually unbalanced nutrient conditions that cause slow growth).
- The difficulty involved in synthesizing them from inexpensive precursors.
- The high cost of their recovery

17. Applications

- They are used for the fabrication of bottles, fibres, latex and several products of agricultural commercial or packaging interest
- These polyesters have been employed for medical applications such as sutures, implants, surgical pins etc
- They are used as packaging films (for food packages), bags, containers, paper coatings etc
- Biodegradable carrier for long term dosage of drugs, medicines, insecticides, herbicides, or fertilizers

- Disposable items such as razors, utensils, cosmetics, shampoo bottles, cups etc
- PHA act as the starting material for many chiral compounds which are used in the synthesis of some drugs or insect pheromones
- Used as mulch films which are laid over the ground to control the weed growth and retain moisture

18. Industrial production of PHA and other biodegradable plastics

Japan is one of the world leaders in bioplastic manufacture. Demand for bioplastics in Japan rose from 4,000 tons in 2000 to 6,000 in2001 and to 10,000 tons in 2003 with an anticipated growth to account for approximately 10% of plastic production within the ten years (Byron, 1987).

Table.3 list of companies producing bioplastics

No	Company	Areas of interest
1	ZENECA seeds (UK)	Production in transgenic plants
2	ZENECA Bio-products	Production by *Alcaligenes eutrophus*
3	Monsanto (USA)	Production in transgenic plants
4	Metabolix Inc (USA)	Production in transgenic plants
5	Polyferm Inc (Canada)	Production from cheap substrates
6	Bioscience Ltd (Finland)	Medical applications

19. Conclusion

The world consumes approximately 93% fossil resources for energy production while only 7% are used for the production of various organic chemicals including solvents and plastics (Sharma *et al*, 2004). Replacement of fraction of synthetic plastics with the biodegradable polymer produced from renewable resources is thus likely to have a small impact on the overall consumption of fossil fuels. Nevertheless greater use of biodegradable plastics could significantly attribute to help solve problems associated with the environmental pollution and waste management.

Biopolymers and bioplastics are the main components in creating a sustainable plastics industry. These products reduce the dependence on non-renewable fossil fuels and are easily biodegradable. Together this greatly limits the environmental impacts of plastic use and manufacture. Also the characters such as being biodegradable make plastics more acceptable for long term use by the society.

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Discussion

1 Which is the presently available natural polymer?

- Rubber

2 Any works in India

- So far no works has been reported. But some initiative works have been done at TamilNadu Agricultural University

3 How far it has come to commercial uses?

- It has been commercialized in Japan, Germany, United States, Italy etc

4 Why there is low acceptance?

- Mainly due to its high production costs

5 Can they be used to make all sorts of products?

- They can be used to make all sorts of products as ordinary plastics. The only difference is that the bioplastics are biodegradable

6 Is there any future for these bioplastics?

- Now this is at its infant stage. But with proper manufacturing process by reduction in the production cost there is immense scope for bioplastics to be acceptable by the society

7 Are there any manufacturing units in India?

- In India these have not been commercialized. So there are no manufacturing units.

8 Why there are no manufacturing units?

- The reason behind is mainly high production costs and other risk factors involved in the production

ABSTRACT

The exponential growth of human population has led to the accumulation of huge amounts of non degradable waste materials like plastics. Conventional plastics are made from oil and do not degrade easily thus contributing to the growing heaps across the world. This led scientists to search for alternative sources of plastics that are degradable.

Biodegradable plastics are a new generation of plastics that are derived from renewable resources and can be converted to biomonomers by fermentation which can be further converted by chemical synthesis to biodegradable polymers like polylactic acid. Biomonomers can also be microbially transformed to biopolymers such as the polyhydroxyalkanoates.

In the bacterium *Alcaligenes eutrophus*, three genes encode the enzymes necessary to catalyze the synthesis of PHB (polyhydroxybutyrate) from acetyl Co-A. These were targeted into the plastids of higher plants and these plants accumulated PHB up to 14 per cent of the dry weight as 0.2-0.7μm granules within plastids. In contrast to the earlier experiments in which the expression of PHB biosynthetic pathway in cytoplasm led to a deleterious effect on growth, the expression of PHB biosynthetic pathway in plastids had no obvious effect on growth and fertility of the transgenic plants and resulted in 100 fold increase in the amount of PHB that has accumulated (Poirier *et al.*, 2002).

Biopolymers such as PHB are naturally produced by the bacteria from agricultural raw materials. They can be processed to make a variety of useful products where their biodegradability and naturalness are quite beneficial (Luzier, 1992).

The environmental attributes of being annually renewable and biodegradable, in contrast to the current petroleum based products will be a major drive to the entry of biodegradable plastics based on agricultural feed stocks into the market place (Narayan, 1998). Eventually bioplastics will help us to reduce our dependence on petroleum thereby reducing negative impacts on the environment and acceptable for long term use by society.

BIOPHARMING

By

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(2005-11-140)
M.Sc. Plant Biotechnology

SEMINAR REPORT

Submitted in partial fulfillment for the requirement of the course PBT 651- Seminar

CENTRE FOR PLANT BIOTECHINOLOGY AND MOLECULAR BIOLOGY
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DECLARATION

I, **Anjali Divakaran** (2005-11-140) hereby declare that this seminar report entitled **"BIOPHARMING"** has been prepared by me, after going through the various references cited at the end of the report and has not been borrowed from any of my fellow students.

Vellanikkara
28-8-06

Anjali Divakaran
(2005-11-140)

CERTIFICATE

Certified that the seminar report entitled **"BIOPHARMING"** for the course PBT 651 has been prepared by Anjali Divakaran (2005-11-140) after going through the various references cited here under my guidance and supervision, and she has not borrowed from any of her fellow students.

Vellanikkara
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INDEX

INDEX OF TABLES

INDEX OF FIGURES

INTRODUCTION

Imagine a situation where we wouldn't have to depend on tablets to cure diseases. Wouldn't it be wonderful if we could replace pills with say, a bowl of cornflakes or potato chips perhaps? This could become a reality in the near future by the help of biotechnology.

Plant biotechnology, which was initially developed to improve agricultural products, is now being used to manufacture various proteins, which find application in human health. Modern biotechnology has resulted in a resurgence of interest in the production of therapeutics agents using botanical sources.

One of the more intriguing ideas that has emerged from the biotechnology industry is "biopharming", that standard crops can be genetically engineered to "grow" pharmaceuticals (drugs). Not only do these pharmaceutical crops have the potential to reduce the cost of manufacturing drugs, if the technique of using food as a drug and vaccine delivery system could be perfected, providing drugs and vaccines to people in developing nations would be greatly simplified.

A bioreactor may refer to any device or system that supports a biologically active environment. Transgenic plants have significant potential in the bioproduction of complex human therapeutic agents due tot ease of genetic manipulation, lack of potential contamination with human pathogens, conservation of eukaryotic cell machinery mediating protein modification and low cost of biomass production.

Transgenic plants can be used for the inexpensive production of large amounts of safe, functional recombinant molecules like vaccines and pharmaceuticals. In recent years, several proteins have been successfully produced in plants, which include human serum albumin, haemoglobin,

monoclonal antibodies, viral or bacterial antigens (vaccine), etc (Sharma *et* al., 2004).

WHAT IS BIOPHARMING?

Biopharming is an experimental application of biotechnology in which genetic engineering is used to create plants that can produce pharmaceutical proteins and chemicals. Biopharming allows the production of compounds of commercial interest in domesticated crops. Pharmaceutical and biotech companies see biopharming as a less expensive way to produce large quantities of pharmaceutical chemicals and other potent, biologically active substances.

ADVANTAGES OF PLANT BIOREACTORS

Plant bioreactors have several advantages. They are:

- It is simpler, cost-effective and faster to produce transgenic plants than transgenic animals.
- Plant pathogens do not infect humans or animals.
- They require shorter period for maturity – they usually mature after one season of growth, and so lesser time is required to bring the product to the market.
- It is possible to produce large amounts of genetically engineered products from large biomass producers like corn and tobacco.
- Proteins can be stored indefinitely in seeds and tubers with little reduction in activity – seeds have low moisture content and so indefinite or long-term storage is possible without damage to the pharmaceutical protein.
- Commercialization would become a viable option because of the lower upfront capital requirement.

Traditional pharmaceutical manufacturing on the other hand has many disadvantages like,

- Limited production capacity due to reasons like
    - Limited cell growth rate and unit production
    - Production usually requires animal cells or microorganisms, so separation of the protein of interest is more cumbersome.
    - Possible viral and prion contamination.
- Production occurs in large vats or fermenters, and these require more space.
- High amounts of energy are required and this makes the procedure expensive.
- Plant expansion is very expensive.

HISTORY OF BIOPHARMING

The first report of human antibodies produced in plants was by During (1988), and was expanded to include secretory antibodies by Hiatt *et al.* (1989). The first report of a protein being produced in plants for the specific purpose of extraction, purification and sale of that protein was by Hood *et al.* (1997), which detailed the production of avidin, an egg protein with several important properties. Aprotinin, one of the first molecularly farmed pharmaceutical proteins to be produced in plants, may be soon used on medical patients for wound closure and to suppress the systemic inflammatory response during surgery (Horn *et al.*, 2004).

CHOICE OF PLANTS

In selecting a crop for production one must decide if working with a domesticated species would be preferred over use of a wild species. Native plants only grow well in specific environments because of light, temperature and soil conditions. Wild species have an advantage in that they will be unlikely to be mistaken for food crops and therefore unlikely to be inadvertently mixed with the

food supply. Unfortunately, they would be more likely than food crops to outcross with native species.

One consideration for foreign protein production is whether to use an open-pollinated or a self-pollinating plant, an issue for either domesticated or wild species. For most domesticated crops, self-pollination usually means that the seed planted by the grower can be saved every year and replanted the following season. This is in contrast to open-pollinated crops that are produced as hybrids. For these, subsequent production is not from plants in the production fields but from parent seed stocks.

After plant tissue is harvested and transported to its designated location, it must be stored for some time before it is processed. Leaves and fruits contain water, making storage problematic. In contrast, if a plant storage organ is used, e.g., seeds or tubers, the plant part is in a dormant state with little metabolic activity, and storage is easy.

The amount of protein as a percentage of total biomass can range from less than 1% to over 40% depending on the plant and tissue source. This feature is critical because in addition to obtaining relatively high percentages of total soluble protein for ease in purification, the overall cost of tissue processing and extraction is directly related to the amount of necessary biomass to obtain the required product amount.

For orally delivered therapeutic proteins of vaccines, a food crop that has GRAS (Generally Recognized as Safe) status would be best, and for protein stability and ease of transport a grain would be a good choice (Hood, 2004).

BIOPHARMING SYSTEMS

There are currently four methods of protein production from plants:

Stable nuclear transformation

This is the most common method to date, and this method has produced majority of the products available in the marketplace today. This system requires a method for transferring the foreign genes into the plant cells. Usually, *Agrobacterium tumefaciens* mediated transformation or particle bombardment method is used for the stable integration of genes into the host plant genome.

This method is especially advantageous when performed in a crop species such as grains. In this case the protein product normally accumulates in the seed, which can be harvested in a dry state and stored till processing (Delaney, 2002). Moreover, there is ease of transport without refrigeration. This method becomes disadvantageous in some cases when used in crops such as corn, where out crossing is a possibility.

Plastid transformation

The first plastid transformation system was described using tobacco. Tobacco appears to be the only species in which plastid transformation has been established routinely.

In this case, protein expression levels exceeding 40% on a dry weight basis have been reported when tobacco chloroplasts were transformed. Moreover, since plastid genes are not usually transmitted though pollen, out crossing is not a major concern.

The disadvantages of this method are the problem of diminishing protein stability over time and the problem of tobacco being a highly regulated crop.

Transient transformation of a crop species

This system depends on the ability of recombinant plant viruses such as tobacco mosaic virus (TMV) to infect tobacco plants and then transiently express a target protein in the plant tissue. The protein will accumulate in the interstitial spaces. The interstitial fluid can then be collected by centrifugation under vacuum.

TMV is highly amenable for genetic manipulation and the infection process is rapid. This method is probably the ideal system for a large amount of custom proteins that are needed in relatively small amounts.

This method would be unsuitable for any protein needed in large (kg) quantities, and the product must be processed immediately as storage will cause degradation of the plant tissue.

Stable transformation of a plant species that is grown hydroponically

In this system, transgenic plants containing a gene coding for the target protein are grown hydroponically in a way that allows release of the desired product as part of the root exudate into the hydroponic medium.

Hydroponics refers to the soil-less culture of plants. Plants grown hydroponically are contained in a greenhouse setting and so have reduced fears of unintentional environmental release. Purification of the desired product is considerably easier since no tissue disruption is needed and the quantity of contaminating proteins is low. But this method is not useful in case of proteins needed in large quantities and greenhouse facilities are costly and difficult to operate (Horn *et al.*, 2004).

Stable transformation is characterized by the integration of the target gene into host-plant genome. Selection of transformed tissues requires inclusion of genes that allow identification of the transformed cells. Selectable markers can provide selection pressure on plant tissues resulting in death of non-transformed cells, or through the starvation of unwanted cells because selective growth of transformed cells is supported. Selectable markers have recently been deemed as undesirable traits because of their perceived danger to public health. Thus, a discriminating assessment of marker gene value versus risk should be undertaken.

THE PRESENT

Biopharming is presently being used for the production of vaccines, recombinant proteins, high value pharmaceuticals and plantibodies.

Recombinant Antibodies or Plantibodies

Transgenic plants offer advantages or the production of recombinant antibodies since plants can be grown on a large scale and the antibodies can be harvested from these crops.

Plants are preferred for the production of recombinant antibodies compared to microorganisms and animal cells due to reasons like,

- Low cost of production of raw material on an agricultural scale
- Efficient transformation technology and speed of scale-up
- Correct assembly of multimeric antibodies unlike bacteria – plants have similar pathways of protein synthesis, secretion, folding and post-translational modifications
- Increased safety, as plants do not serve as hosts for human pathogens
- High yields of homogenous products for various applications
- Storage of transformed plant line as seeds almost indefinitely under ambient conditions.

Microorganisms are unsuitable for recombinant antibody production because or reasons like,

- Low or no expression when the gene is introduced
- Impaired folding pathway and limited capacity for accurate post translational modification of eukaryotic proteins
- In vitro refolding and fermentation of the inaccurately produced proteins is labour and cost intensive
- Problem of contaminating endotoxins.

The expression of full size antibodies (Hiatt *et al.*, 1989) and antibody fragments (Owen *et al.*, 1992) has been successfully achieved in plants. Intensive research over the past few decades has resulted in effective gene transfer procedures, with subsequent recovery of transgenic plants through *in vitro* regeneration. Transfer of the foreign gene into plant cells can be performed using *Agrobacterium* mediated transformation, particle bombardment, electroporation of protoplasts or using viral vectors.

Agrobacterium mediated gene transfer is restricted to dicotyledons. Biolistic delivery of genes is therefore used for monocotyledons such as wheat and corn. However, rice can be transformed by *Agrobacterium* and methods are being developed or transforming other monocotyledons.

Recombinant antibodies have been targeted to the following components of plant cells:

- Intracellular space
- Chloroplasts
- Endoplasmic reticulum

If long-term storage is required, seed specific expression with seed specific promoters are targeted. Plant based production of recombinant antibodies requires only a virus infected or transgenic plant, sunlight, mineral salts and water.

Modern agricultural practice enables easy scale-up, rapid harvesting and processing of large quantities of leaves or seeds.

Antibodies are an ideal model for the expression of therapeutic or diagnostically important proteins in plants. Plantibodies can be used for therapeutic and diagnostic purposes, for the modulation of the function of a corresponding antigen (immunomodulation), engineering resistance to pathogens or blocking of pathogen development in plants and for use as biofilters to entrap environmental pollutants to reduce the load of environmental contaminants or for bioremediation (Kumar *et al.*, 2003).

Edible Vaccines

The phrase "edible vaccine" was coined by Charles Arntzen. Edible vaccines have received considerable attention from researchers in both academia and industry.

The concept of edible vaccines is that people take their dose of medicines as part of their diet. Vaccines administered using needles (parenteral delivery) do not usually give a good immune response in the mucosal tissues of the vaccine recipient. Mucosal surfaces, such as the mouth and the urinogenital tract are the primary ports of entry of most disease organisms. Edible vaccines have been shown to induce excellent mucosal immune responses.

Korban and colleagues (2002) at the University of Illinois have reported a plant-based oral vaccine against respiratory syncytial virus (RSV) in tomato fruit. HSV is a viral pathogen hat causes respiratory diseases and is a leading cause of viral lower respiratory tract illness in infants and children worldwide (Horn *et al.*, 2004).

E. coli LT-B in corn, Rotavirus capsid protein VP6 (Rotavirus produces an enteric toxin) in potato and *Vibrio cholerae* Toxin B subunit in tomato are examples of edible vaccines.

Dry grain (corn, rice, wheat) may prove to be a superior delivery system for edible vaccines since the antigen will remain at a constant level for an extended period of time without refrigeration (Streatfield, 2001). Vaccines require a known dose of the antigen and this would be difficult to accomplish using fresh or dried fruit such as potato, tomato or banana.

MAJOR CROPS USED

The major crops used for biopharming include:

- Tobacco
- Potato
- Maize/corn
- Tomato
- Soybean

Other crops like alfalfa and safflower are also being tested for suitability.

Tobacco

Tobacco is considered as the choice plant and the ideal bioreactor. Tobacco is an ideal plant-bioreactor because the pharmaceutical protein is produced in its leaves and the leaf yield is about 40 tons/acre. Therefore the production system does not require flowering. The leaves contain 10% protein and about 2000 kg protein/acre/year can be produced. Tobacco is also a prolific seed producer with about 1 million seeds/plant. The first transgenic plant synthesized product is a tobacco derived antibody targeting gum disease (Gadani *et al.*, 1995).

The biopharmaceuticals produced from transgenic tobacco are as follows: -

a) Antibody against dental caries

Dental caries are caused by *Streptococcus mutans*. This organism binds to the teeth and causes cavities and tooth decay. This antibody is effective against a

surface antigen of *Streptococcus mutans*. It has been formulated in toothpastes.

b) SimpliREDTM HIV diagnostic reagent (Schunmann *et al.*, 2002).

This is the first report of an antibody fusion protein expressed in transgenic plants for direct use in medical assay. A fusion protein is a combination of a single chain antibody and an epitope of HIV virus. This reagent is expensive and laborious to produce by conventional means, since chemical modifications to a monoclonal antibody are required. This fusion protein was found to be fully functional in crude extracts. This reagent is used for the detection of HIV-1 antibodies in human blood.

c) Human papilloma virus type 16 major capsid protein (Varsani *et al.*, 2003).

This protein acts an antigen to produce immune response to human papilloma virus. Its production shows the feasibility of using transgenic plants for production of HPV vaccines.

d) Specially modified human GC (Glucocerebrosidase) or Cederase (Sharma *et al.*, 2004).

Deficiency of GC causes Gaucher's disease, which is characterized by enlargement of liver, spleen and lesions in bones. The effective treatment for Gaucher's disease is enzyme replacement therapy of glucocerebrosidase or Cederase. Cederase is considered as the most expensive drug in the world. The annual cost of therapy per patient is approximately $100,000 to $300,000. This enzyme is being produced in tobacco by *Agrobacterium* mediated transformation and sufficient amounts are also being produced. It represents one of the first active human enzymes produced in transgenic plants.

e) scFv- alpha HER2 (Human epidermal growth factor receptor-2) in cancer therapy (Galeffi *et al.*, 2005).

Human epidermal growth factor receptor –2 (HER2) is an oncogene involved in abnormal cell growth in breast cancer and is considered for the development of new cancer therapies. The expression of a scFv-alpha HER2 has been produced in tobacco and *Nicotiana benthamiana*. The single chain antibodies extracted and purified from the plant tissues showed positive results in diagnosis and therapy.

Potato

Biopharmaceutical production in potato is directed towards the potato tubers. The biopharmaceuticals produced from potato are as follows.

a) Production of human serum albumin.

Human serum albumin is a protein source. It is identical to human blood, and it is used for a variety of diagnostic and therapeutic purposes like

- formulation for therapeutic proteins
- vaccine formulation and manufacturing
- development of mammalian cell cultures
- infertility treatments
- coating of medical devices
- drug delivery
- in vivo diagnostics

b) Human beta – amyloid peptide (Hyunsoon *et al.*, 2003).

The human beta-amyloid peptide is associated with Alzheimer's disease therapy.

c) Expression of Rotavirus capsid protein VP6 (Yu and Langridge, 2003).

Rotavirus VP6 is an enteric viral pathogen. Mice fed with potato tubers producing the rotavirus capsid protein showed production of both serum Ig and intestinal IgA antibodies.

d) *Escherichia coli* Labile Toxin (LT-B) (Sharma *et al.*, 2004).

Escherichia coli Labile Toxin LT is responsible for causing diarrhoea in humans. It is composed of a 27 kDa A subunit and five 1.6 kDa B subunits. The B pentamer (LT-B) has been used as a vaccine component, as antibodies against this would block toxin activity. LT-B was expressed in transgenic potato.

Maize

Corn is by far the most popular substrate for biopharming. The biopharmaceuticals produced from corn are as follows.

a) Aprotinin

Aprotinin is a protein thant inhibits serine proteases. This activity modulates and lessens the systemic inflammatory response associated with bypass surgery. So there is decreased need for blood transfusions and reduced bleeding. Current material supplied to the market is native aprotinin extracted from bovine lungs. With growing concern regarding bovine spongiform encephalopathy (BSE) and other animal pathogens, a high market price in surgery and wound healing, and limitations on natural sources of aprotinin, a non-animal derived, lower cost and reliable source adds significant value to the user.

b) Human lactoferrin

Human lactoferrin is a natural defense iron-binding milk protein that possesses anti-bacterial, anti-fungal and anti-inflammatory properties.

c) *E. coli* heat labile enterotoxin LT-B and cholera toxin (CT) (Chikwamba *et al.*, 2002).

LT-B is a multimeric protein that presents an ideal model for an edible vaccine, displaying stability is the gut and inducing mucosal and systemic immune responses.

d) Avidin (Horn *et al.*, 2004).

Avidin is a glycoprotein found in egg white. It is the first protein produced in plants for extraction, purification and sale. It is used as an immunological reagent. Sigma-Aldrich Company is currently marketing the product.

e) Human gastric lipase (Horn *et al.*, 2004).

The lack of human gastric lipase is a protein involved in the condition known as exocrine pancreatic insufficiency characterized by the inability to digest food lipids.

f) Trypsin (Horn *et al.*, 2004).

Corn- derived Trypsin (TrypZean) is the first large-scale protein product from transgenic plant technology. Trypsin is a protease used in processing of biopharmaceuticals. Availability of maize-derived trypsin helps to meet the demand for animal-free reagents.

<u>Tomato</u>

Biopharmaceuticals produced from tomato:

a) *Vibrio cholerae* Toxin B subunit vaccine (edible and heat-labile) (Sharma *et al.*, 2004).

Vibrio cholerae is a bacterial pathogen causing cholera in humans. The transgenic tomatoes expressing the vaccine can be eaten fresh and can be available in all seasons.

b) Rabies virus coat glycoprotein gene (Ko and Koprowski, 2005).

Rabies virus epidemics are problematic throughout the world and adequate treatment has been hampered by the worldwide shortage and high cost of prophylactic antibodies.

Alfalfa

The use of transgenic plants as vectors for the expression of viral and bacterial antigens has been increasingly tested as an alternative methodology for the production of experimental vaccines. The production of transgenic alfalfa plants containing the genes encoding the polyprotein P1 and the protease 3C of foot and mouth disease virus have been reported. The immunogenicity of the expressed products was tested using a mouse experimental model. Parenterally immunized mice developed a strong antibody response and were completely protected when challenged with the virulent virus. This report demonstrates the possibility of using transgenic alfalfa to express proteins effective as experimental immunogens (Dus-Santos *et al.*, 2005).

Barley

The SimpliREDTM HIV diagnostic reagent, which is a fusion protein used for detecting HIV-1 antibodies in human blood has been expressed through biopharming in transgenic barley (Schunmann *et al.*, 2002).

Safflower

Safflower (*Carthamus tinctorius*) is being evaluated as a crop for the production of plant-made pharmaceuticals using an oleosin fusion protein system. The potential for transgenic gene flow from *C. tinctorius* to wild or weedy relatives was studied. Cytogenetic and phylogenetic studies indicated that the cultivated safflower has the potential to hybridize with only six wild relatives

worldwide of which only two hybridized to give fertile hybrids. Locations where wild species of Carthamus have not been naturalized may provide biologically isolated locations for the cultivation of a transgenic safflower crop. It is because of this possibility that safflower is undergoing evaluation for use in biopharming (McPherson *et al.*, 2004).

Table.1. Ratings for selected crops as suitable protein production systems

Crop Type	Product Safety	Environmental safety	Lab ease	Growing Ease	Harvest Transport Storage	Process Purification	By-Product Credits
Leafy crops							
Tobacco	C	A	A	B	C	B	C
Fruits							
Tomato	A	B	A	B	C	C	C
Cereals							
Maize	A	B	B	A	A	A	A
Mustards							
Canola	B	C	A	A	A	B	B
Legume seeds							
Soybeans	B	B	C	A	A	A	B

A-best performance

B-moderate performance

C-least desirable performance

The advantages and disadvantages for a variety of a single representative of several plant types when considering production characteristics and their ability to be a host production system are summarized in Table 1. No one type of plant rises to the top as the clear choice. This suggests the possibility that an existing crop could be modified specifically for recombinant protein production. A cultivated crop could be altered to have a higher protein content that could translate into higher amounts of recombinant protein.

For orally delivered therapeutic proteins or vaccines, a food crop that has GRAS (Generally Recognized As Safe) status would be best. For protein stability and ease of transport a grain would be a good choice (Hood, 2004).

Table. 2. Biopharmaceuticals and crops

Potential application/indication	Plant	Protein	Method
Anticoagulants			
Protein C Pathway	Tobacco	Human protein C (serum protease)	AMT
Indirect thrombin inhibitors	Tobacco, oil seed, Ethiopian mustard	Human hirudin variant 2	AMT
Recombinant hormones /proteins			
Neutropenia	Tobacco	Human granulocyte-macrophage colony stimulating factor	AMT
Anemia	Tobacco	Human erythropoietin	AMT
Antihyperanalgesic by opiate activity	Thale cress, oil seed	Human enkephalins	AMT
Wound repair/control of all proliferation	Tobacco	Human epidermal growth factor	AMT
Hepatitis C & B treatment	Rice, turnip	Human interferon – α	AMT
Liver cirrhosis	Potato, tobacco	Human serum albumin	AMT
Blood substitute	Tobacco	Human hemoglobin	AMT
Collagen	Tobacco	Human homotrimeric collagen- I	AMT
Protein/ peptide inhibitors			
Cystic fibrosis, liver disease and hemorrhage	Rice	Human α – 1 antitrypsin	Particle bombardment
Trypsin inhibitor for transplantation surgery	Maize	Human aprotinin	Particle bombardment

Hypertension	Tobacco/ tomato	Angiotensin-1-converting enzyme	AMT
HIV therapies	*Nicotiana benthamiana*	α – trichosanthin form TMV-U1 subgenomic coat protein	AMT
Recombinant enzymes			
Gaucher's disease	Tobacco	Glucocerebrosidase	AMT

STEPS INVOLVED IN BIOPHARMING

Production practices for recombinant proteins refer to the growing, harvesting, transport, storage and tissue processing of the crop, as well as the extraction and purification (Table 3).

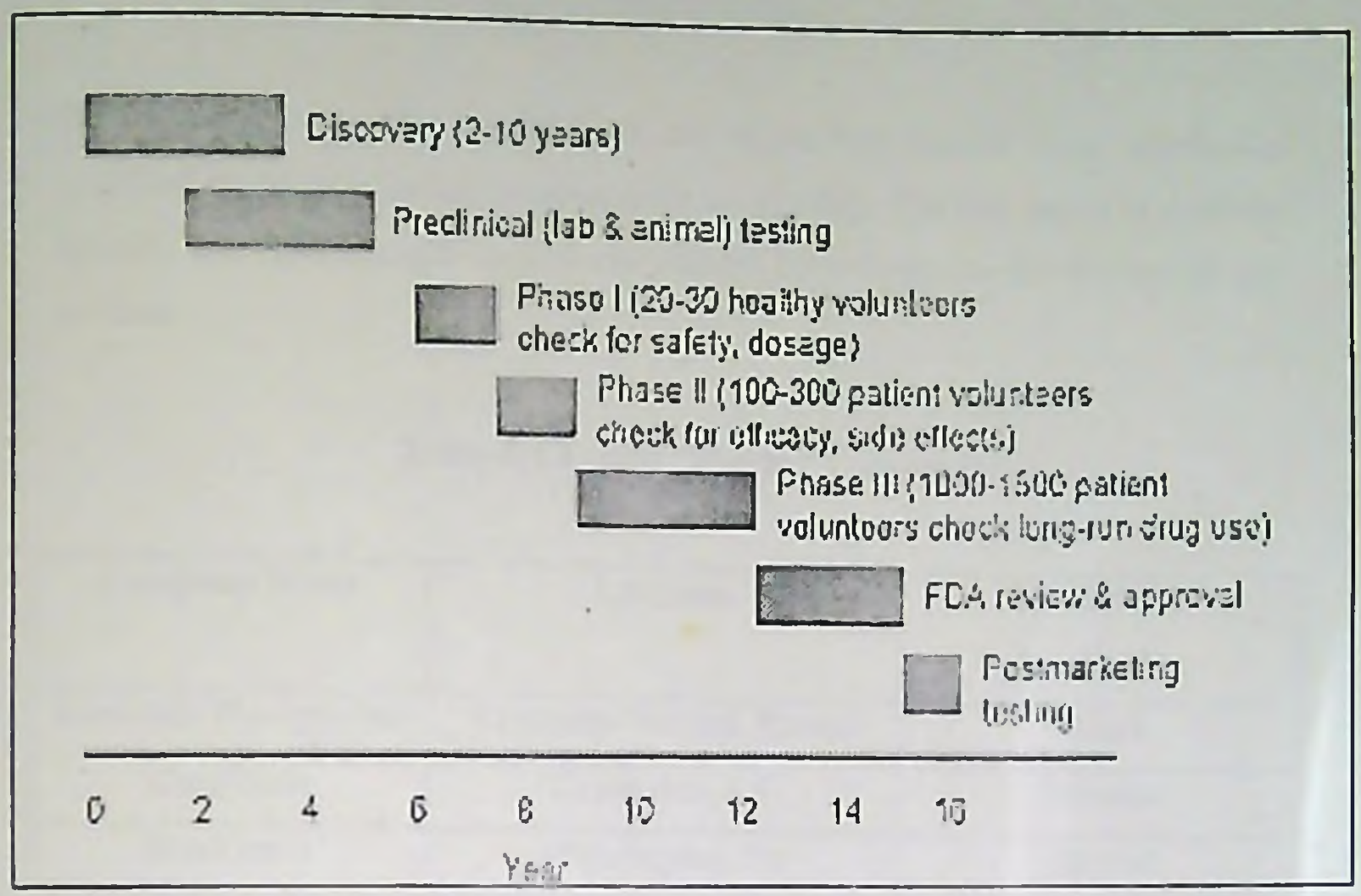
Table. 3. Plant based production steps

Production step	Consideration
Growing	Requirements Geographic Limitations Seasonal Limitations Recombinant Protein Yield Confinement
Harvesting	Mechanical Issues Time Sensitivity
Transportation	Temperature sensitivity Cost
Storage	Temperature sensitivity Protein stability in tissue
Tissue Processing	Stability in tissue Potential for Enrichment
Extraction	Protein stability Biomass Quantity
Purification	Interfering agents CGMP

The key features of the best production system include a potential for low cost of goods, maintenance of protein integrity, flexibility with regard to time and temperature for harvest and maintenance of product as well as environmental safety (Delaney, 2002; Nikolov and Hammes, 2002).

The next phase of biopharming is product development.

Fig.1. Stages in product development



Among the stages in product development (Table.4), the clinical trial stage is the most expensive stage of bringing a drug into the market. It consumes around 80% of the total capital inputs.

Other issues that require attention hen bringing a product to the market are:

Legal issues: a consideration of legal issues surrounding any new industry is important In case of biopharming, legal considerations include such things as patent protection of intellectual property and licensing of patented technology to provide freedom to operate. Consideration should also be given to a strategy for invention versus licensing.

Regulatory issues: regulatory issues are becoming more complex. Regulations that are based on scientific principles rather than reaction to public fears are the goal of scientists as well as the regulatory agencies.

Public acceptance: the public must see a positive benefit from genetically modified crops in order for acceptance to be a reality. The risk factor is product-specific and confinement procedures should be tailored to the hazard of the product.

Table.4. Current "Pharm" Companies

Company Name	Location	Species For Production
Meristem Therapeutics	Clermont-Ferrand, France	Maize
CropTech	Charleston, S.C	Tobacco
PlantGenix	Philadelphia, Pa	Various
Large Scale Biology	Vacaville, California	Tobacco
Monsanto Protein Tech	St. Louis Mo.	Maize
SemBioSys	Calgary, Alberta, Canada	Safflower
Medicago	Quebec City, Quebec, Canada	Alfalfa
Ventria	Sacramento, California	Rice
Epicyte Pharmaceutical	San Diego, California	Maize
Planet Biotechnology	Hayward, California	Tobacco
Prodigene	College Station, Texas	Maize

DANGERS OF BIOPHARMING

Biopharming crops are now undergoing open-field trials. This raises the question of whether food crops contaminated with biopharmed crops could harm human health. Scientific reviews indicate that biopharming poses a number of risks Tobacco human health and the environment. It may:

- Spread new and unwanted compounds into the air, water and soil, as well as into foods and beverages, potentially creating a public epidemic of disease, and allergic reactions such as life-threatening anaphylactic shock.
- Pose occupational safety hazards, since chemicals from biopharm crops may cause harm Tobacco humans through inhalation, unintended ingestion and skin contact.
- Persist in the environment and bioaccumulate in wildlife, plants and soil microorganisms leading to both acute and chronic toxicity and major ecological disruption.
- Contaminate wild and food plants with alien genetic material through crossbreeding. Contamination of food crops with biopharmaceutical crops has already occurred. In the fall of 2002, USDA had Tobacco quarantine and destroy500, 000 bushels o soybeans in Nebraska meant for human consumption that had been contaminated with corn genetically engineered to produce a pig vaccine.

CONCLUSION

Biopharming is a uniquely powerful tool for low-cost mass production of pharmaceuticals and biologics. The findings to date indicate that plants can be exploited as bioreactors and cost-effective alternative Tobacco microbial and animal systems for the production of biomolecules. The pharmaceutical products from animal sources are feared for the possible association with microbes, which could be pathogenic to humans. Plants offer a safer system for the production of human serum albumin and other such products.

It is, however possible that the effect of some biomolecules on plant health may come in the way of viable production systems. In view of this, each case would have Tobacco be tested experimentally. Proper regulatory mechanisms are required for deriving full benefits from this extremely powerful and useful tool. Therefore, rather than a complete ban, regulation and control is the need for the day.

DISCUSSION:

1. **What is the difference between "biopharming" and "molecular farming"?**
   "Biopharming" refers to the production of pharmaceuticals from plants using transgenic technology, while "molecular farming" refers to the production of pharmaceuticals, industrially important proteins, enzymes and bioplastics from plants using transgenic technology.
2. **What are the criteria for selection of plants for biopharming?**
   Plants are selected based on the use of the particular pharmaceutical to be produced. For example, for long-term storage, a seed based system would be more effective than a leaf tissue based system. If a particular product is required in large quantities, a system like tobacco would be more effective because of the high quantities of protein production in leaves and the large leaf yield.
3. **How are biopharmaceuticals better than conventional pharmaceuticals?**
   Biopharmaceuticals lack potential contamination from human pathogens, while there may be chances for contaminating endotoxins and other toxins in conventional pharmaceuticals. Moreover the proteins can be stored indefinitely in seeds with little reduction in biological activity.
4. **Do antibiotics come under biopharming?**
   Biopharming does not include antibiotics. Antibiotics are substances produced by one microorganism against the action of another microorganism.
5. **Which is the best method for biopharming? Is biopharming good?**
   Stable nuclear transformation using *Agrobacterium tumefaciens* or particle bombardment is the best method for biopharming. Biopharming is definitely good because it could be the way to deliver pharmaceuticals to the masses.
6. **How do edible vaccines act?**
   Edible vaccines are actually weak antigens that produce the immune response. When they come into contact with the mucosal tissues they produce mucosal immune response, thereby inducing resistance to the particular disease.

7. **Is biopharming costlier or cheaper than conventional methods of production?**

   The initial costs of obtaining the transgenic crop will be high, but once the technique is established, biopharming is much cheaper than conventional methods of production.

8. **Are there any commercial products?**

   The antibody against dental caries has been formulated in toothpastes. This antibody is produced from tobacco.

9. **Can "golden rice" and biopharming be related with each other?**

Biopharming involves extraction of the pharmaceutically important proteins from transgenic plants. Golden rice which has been developed through transgenic technology is meant for enhanced nutritional importance rather than for extraction of any commercial product.

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ABSTRACT

Plant biotechnology, which was initially developed to improve agricultural products, is now being used for the production of heterologous proteins, which find application in the pharmaceutical industry (Hood, 2004). This has given rise to the concept of "biopharming". Biopharming involves the production of pharmaceutical proteins in plants by the use of biotechnology, genetic engineering in particular.

Plant based production of biopharmaceuticals is a potentially viable industry with tremendous potential. Plants allow large amounts of biomass and can be easily and inexpensively produced (Hood, 2004). Biopharming through transgenic plants require the lowest capital investment of all production systems and they are easily scalable by increasing production acreage. The currently used methods for producing biopharmaceuticals include stable nuclear transformation using *Agrobacterium tumefaciens* or particle bombardment, plastid transformation, transient transformation of a crop species and stable transformation of a plant species that is grown hydroponically (Horn *et al.*, 2004).

Selection of suitable plants is an important consideration in biopharming. The currently used systems include corn, tobacco, potato, tomato, alfalfa, soybean and barley. Various other crops are also being tested for efficacy. Biopharming is now being used for the production of vaccines, recombinant proteins, high value pharmaceuticals and plantibodies (Sharma *et al.*, 2004). Serum albumin and *Escherichia coli* LT-B from potato, Trypsin and Avidin from maize and Cederase and the SimpliREDTM HIV diagnostic reagent from tobacco are examples of biopharming products.

Biopharming could reduce the cost of manufacturing drugs, and thereby the provision of drugs and vaccines to people in developing nations would become very easy. Biopharming has its disadvantages also, but in spite of all that transgenic plants show ample promise for use as bioreactors for the production of valuable therapeutics.

BIOPHARMING

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M.Sc. Plant Biotechnology

SEMINAR REPORT

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MOLECULAR BIOLOGY
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KERALA AGRICULTURAL UNIVERSITY
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BIONANOTECHNOLOGY

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M.Sc Plant Biotechnology

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LIST OF FIGURES

LIST OF CONTENTS

INTRODUCTION

Advancement of science and technologyhave allowed for the broadening of horizons and miniaturization of extremely complex devices. The new revolutionary technologies has resulted in explosion of entirely new industries in health care, medicine, food and nutrition, environmental management, chemical synthesis and agriculture

Nanotechnology is a catch all phrase for materials and devices that operate at the nanoscale. In the metric system of measurement, "Nano" equals a billionth and therefore a nanometer is one billionth of a meter. A possible way to interpret this size is to take the width of a hair, and imagine something ten thousand times smaller. (Foster, 2005).

Nanotechnology is the ability to build and shape matter one atom at a time. The idea of nanotechnology was first presented by physicist Richard Feynman. In a lecture entitled "Room at the Bottom," he unveiled the possibilities available in the molecular world.

It is a nanotechnology that is closely modeled after our own macroscopic technology. This new field has been dubbed *molecular nanotechnology* for its focus on creating molecules individually atom-by-atom. K. Eric Drexler has proposed methods of constructing molecules by forcibly pressing atoms together into the desired molecular shapes, in a process dubbed "mechanosynthesis" for its parallels with macroscopic machinery and engineering. Nanotechnology would explode to fill every need and utterly change our way of life.

BIONANOTECHNOLOGY

Bionanotechnology is the term coined for synthetic technology based on the principles and chemical pathways of living organisms, ranging from genetic-engineered microbes to custom made organic molecule. It encompasses the study , creation and illuminations of the connections between structural molecular biology and molecular nanotechnology , since the development of nanomachinery might be guided by studying the structure and function of natural nano machines

found in the living cells(Sammer *et al.*, 2005). Bionanotechnology seeks to modify and find technological uses of natural nano components like nano motors of ATP synthase and things like using the scaffold of enzyme complex of cellulosomes for adding new enzymes to make "nanosomes".

Biotechnology uses the ready-made assemblers available in living cells to build thousands of custom-designed molecules to atomic specifications, including the construction of new assemblers. This has lead to myriad applications, including commercial production of hormones and drugs, elegant methods for diagnosing and curing infectious and genetic diseases, and engineering of organisms for specialized tasks such as bioremediation and disease resistance.

Biotechnology has grown, and is still growing, with each new discovery in molecular biology. Further research into viral biology has led to improved vectors for delivering new genetic material. An explosion of enzymes for clipping, editing, ligating, and copying DNA, as well as efficient techniques for the chemical synthesis of DNA, has allowed the creation of complicated new genetic constructs. Engineered bacteria now create large quantities of natural proteins for medicinal use, mutated proteins for research, hybrid chimeric proteins for specialized applications, and entire new proteins.

FROM BIOTECHNOLOGY TO BIONANOTECHNOLOGY

Biotechnology grew from the use of natural enzymes to manipulate the genetic code, which was then used to modify entire organisms. The atomic details were not really important—existing functionalities were combined to achieve the end goal. Today, we have the ability to work at a much finer level with a more detailed level of understanding and control. We have the tools to create biological machines atom-by-atom according to our own plans.

Nanotechnology and bionanotechnology are entirely new concepts, invented late in the twentieth century, and biotechnology has only been around for a few decades, so the scope of these fields is still being defined. Machines in our familiar world have moving parts in the range of millimeters to meters. As machining capabilities improved, tiny machines, such as the movement of a fine watch, extended the precision of machining to a fraction of a millimeter. Computer technology, with its ever-present pressure to miniaturize in order to improve performance, has driven the construction of tiny structures to even smaller ranges,

with micrometer-scale construction of electronic components and tiny machines, like tiny gears, created at the Sandia National Laboratories. Bionanotechnology operates at the smallest level, with machines in the range of 10 nm in dimension. The bacterium builds thousands of different bionanomachines, including a working nanoscale assembler, the ribosome. Because these bionanomachines are composed of a finite, defined number of atoms, they represent a limit to the possible miniaturization of machinery.

BIONANOMACHINES

Modern cells build thousands of working nanomachines, which may be harnessed and modified to perform our own custom nanotechnological tasks. Modern cells provide us with an elaborate, efficient set of molecular machines that restructure matter atom-by-atom, exactly to our specifications. And with the well-tested techniques of biotechnology, we can extend the function of these machines for our own goals, modifying existing biomolecular nanomachines or designing entirely new ones.

These are true nanomachines. Each one is a machine built to nanoscale specifications, with each atom precisely placed and connected to its neighbors. Human body is arguably the most complex mechanism in the known universe, and most of the action occurs at the nanoscale level. These nanomachines work in concert to orchestrate the many processes of life—eating and breathing, growing and repairing, sensing danger and responding to it, and reproducing(Denns, 2003).

Remarkably, many of these nanomachines will still perform their atom-sized functions after they are isolated and purified, provided that the environment is not too harsh. They do not have to be sequestered safely inside cells. Each one is a self-sufficient molecular machine. Already, these nanomachines have been pressed into service. They have been developed by the process of evolution (instead of intelligent design), which places unfamiliar restrictions on the process of design and the form of the final machine. Bionanomachines are also selected to perform their tasks in a very specific environment and are subject to the unfamiliar forces imposed by this environment

MODERN BIOMATERIALS

Four basic molecular plans were developed through evolution over 3 billion years ago and are still used by all living things today. Modern cells use proteins, nucleic acids (such as DNA), polysaccharides, and lipids for nearly all tasks. A handful of other small molecules are specially synthesized for particular needs, but the everyday work of the cell is performed by these four basic plans. Of course, in bionanotechnology we are not forced to stay within these existing plans, but there are many advantages to exploring them first. Most notably, we can use the thousands of working natural bionanomachines as a starting point to build our own practical nanotechnology.

GUIDED TOUR OF NATURAL BIONANOMACHINERY

Nature has already realized many of the dreams of nanotechnology. Thousands of bionanomachines have been selected and perfected by evolutionary optimization to perform nanoscale tasks accurately, consistently, and under specific control. These bionanomachines use all of the engineering tricks used in our familiar macroscale machines: construction from many tight-fitting parts, hinges for bending, rotating axles and bearings, digital information storage, chemical adhesion and chemical power.

BIONANOMACHINES IN CELL

Proteins

Protein is the most versatile of the natural biomolecular plans. Protein is used to build nanomachines, nanostructures, and nanosensors with diverse properties. Proteins are modular, constructed of a linear chain of amino acids that folds into a defined structure. The longest protein chain (thus far) is titin with over 26,000 amino acids, and peptides with less than a dozen amino acids are used as hormones for cell signaling.

The rigidity of the amide group is essential for the construction of nanomachinery with defined conformations. The rigid amide limits the number of conformations available to the chain. The chemical diversity of the different side chains provides the real advantage of proteins as a structural material, allowing them to be used for many different functions. The 20 side chains used in natural proteins are chemically and structurally diverse. By arranging them in the proper order, the structure of the protein may be shaped and stabilized. Then particularly

reactive side chains may be placed at key locations to perform the desired function. Bionanotechnology is exploiting the potential of proteins in every way imaginable.

Nucleic Acids

Nucleic acids are modular, linear chains of nucleotides, ranging up to hundreds of millions of nucleotides in length. Two forms are commonly used: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Nucleic acid chains are far more flexible than protein chains, so nucleic acids adopt a wide range of conformations. The structure is largely determined by the interactions of the bases in each nucleotide. The uniform chemical properties of the nucleotides limit the functions of nucleic acids.

They are specialized for applications in nanoscale information storage and retrieval. Each nucleotide encodes two bits of information. Information is duplicated and read through specific interactions of each nucleotide with a specific mate. Despite these limitations, the ribosome, which is DNA carries a library of information. Biological information is stored at remarkable density. A single bacterial cell, barely a micrometer in largest dimension, stores 70 Kbyte of information in its genome. A typical compact disk uses a similar space to store a single bit of information. Biological information is stored in a form that is chemically stable and redundant for ease in repair. This medium is used in one mode to store blueprints for construction and in a second mode to control synthesis. Every aspect of the structure of DNA is used to carry information. Inside the double helix, the genetic information is stored by using a specific set of hydrogen bonds. Perhaps the most important molecule in the cell, is composed predominantly of RNA.

Lipids

Some of the largest structures built by cells are composed not of large macromolecules like proteins or nucleic acids but instead by a fluid aggregate of small lipid molecules. The lipids used by living cells have been designed to aggregate into a defined set of useful infrastructures. They are small molecules that combine two different chemical characteristics into a single molecule. They are composed of a polar or charged group, which interacts favorably with water, attached to one or more carbon-rich chains, which strongly resist dissolving in

water. This dual character causes them to act much like protein chains when placed in water

Polysaccharides

Polysaccharides are the most heterogeneous of the four molecular plans. Sugars, the building blocks of polysaccharides, are covered with hydroxyl groups. The polymers are created by connecting the hydroxyl groups together, offering many possible geometries for polymerization. In nature, many different linear and branched polymers are constructed for different needs .

Ribosomes

Ribosomes are complete factories for information-driven nanoassembly of proteins. The factory performs a modular assembly, reading information in a linear storage medium and arranging 20 different modules into a linear chain. Any length and any sequence of modules may be created at will, simply by creating the appropriate set of instructions. Ribosomes are fully general: Any protein may be created using a standard set of starting and stopping instructions and a standard coding scheme for the blueprint of the desired product.

BIOMOLECULAR MOTORS

In bionanomachinery, however, motors are remarkably rare. Much of the work of nanoscale transport and motion is accomplished by diffusion and capture, without the need for directed motion. Diffusion is so fast for proteins in cell-sized enclosures that no additional mechanisms are necessary. Motors are brought to bear in larger tasks of motion at the micrometer level, such as the separation of chromosomes and the remodeling of cell organelles, and all the way up to meter scales, with the contraction of our muscles. However, there are a few remarkable exceptions where nature does use motors for nanoscale tasks, such as the rotary motor in ATP synthase.

ATP Synthase

ATP synthase is a rotary motor and generator. ATP synthase performs an energy transformation, converting electrochemical energy into chemical energy and vice versa. It consists of two motors driven by electrochemical gradients, which is bound inside a lipid membrane This motor is composed of a rotor

composed of a cyclic ring of proteins and a stator The stator guides the flow of hydrogen ions across the membrane and transforms it into motion of the rotor. Second is a chemically powered motor, driven by the breakage of the unstable molecule ATP. This motor is composed of a ring of six proteins with an eccentric axle through the center . Cleavage of ATP forces a change in the shape of the surrounding proteins, driving rotation of the axle. The whole complex may be used in either direction. The electrochemical motor can drive the chemical motor, creating ATP in the process, or the chemical motor can be powered by breakage of ATP, turning the electrochemical motor and creating a gradient(Pantalonia *et al.*,2001)

Fig.1.ATP synthase of bacterial flagellum

Sacomere- Unit Of Muscle Contraction

Actin and myosin form an engine of contraction. Muscle cells are filled with a huge array of interdigitated myosin filaments and actin filaments. A small section is shown here, with myosin filaments in pink and actin filaments in gray. Chemical energy is converted into mechanical work by myosin. The many myosin heads climb along the neighboring actin filaments, powered by ATP. In a contracting muscle, each myosin head may perform a power stroke five times a second, moving along the actin filament about 10 nm with each motion(Goodshell, 2004). About 2 trillion myosin power strokes are needed to generate the force to hold a baseball in your hand, but your biceps have a million times this

many, so only a fraction of the myosin in a muscle is exerting force at any given time.

BIOMOLECULAR DESIGN AND BIOTECHNOLOGY

Current methods of biotechnology excel at modification. This is a powerful capability that leverages the extensive body of working nanomachinery that is available from natural sources. We can introduce specific changes into the plans for a given protein, or we can splice together the plans for several different proteins, creating a hybrid molecule with combined function. Using these modified plans, we can then engineer bacteria to produce large quantities of the mutant or chimeric protein. Thousands of academic and industrial laboratories are using these methods for medicine, bioremediation, and countless other applications(Rost and Sander,1996).

Recombinant DNA Technology

Recombinant DNA technology is the core capability of bionanotechnology. This technology allows us to construct any protein that we wish, simply by changing the genetic plans that are used to build it. Two natural enzymes— restriction enzymes and DNA ligase—are the keys to recombinant DNA technology, allowing us to edit the information in a DNA strand . Before the discovery of these enzymes, researchers modified the genetic code of living organisms by using biology's own tools of mating and crossing or by random mutagenesis with chemicals or ionizing radiation. Today, researchers modify the genetic code rationally at the atomic level.

DNA May Be Engineered with Commercially Available Enzymes

Researchers use a wide variety of natural biomolecules for handling DNA. Well-characterized protocols and commercial sources for these enzymes are available, so these processes are available to any modest laboratory. A few of the most important are:

(i) Restriction enzymes are isolated from bacteria. Over 100 types are available commercially. Each one cuts DNA at a specific sequence of bases. Typically, restriction enzymes are composed of two identical subunits, so they attack DNA symmetrically and cut at specific palindromic sequences.

(ii) DNA ligase reconnects broken DNA strands. When two sticky ends anneal, DNA ligase is used to reconnect the breaks.

(iii) DNA polymerase creates a new DNA strand by using another strand as a template, creating a double helix from a single strand. It is used to fill single-stranded gaps and to copy entire pieces of DNA.

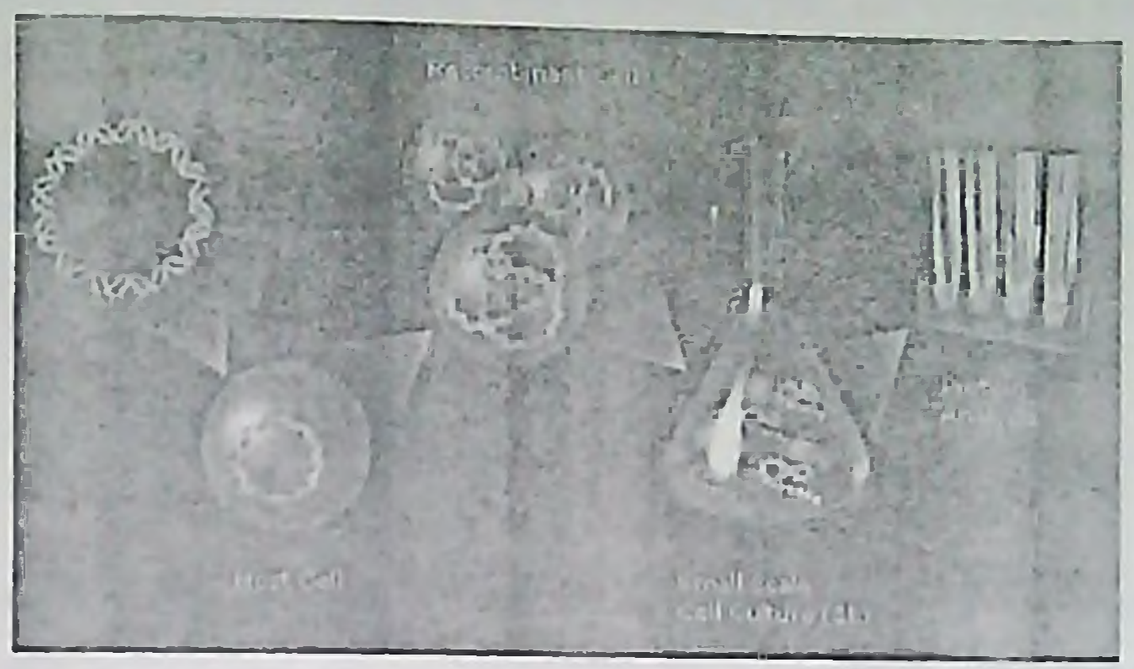

Fig. 2. Recombinant DNA technology

Chemical synthesis of DNA perfectly complements these natural bio-molecular tools for manipulating DNA. Current methods allow the automated synthesis of DNA strands about 100 nucleotides in length. Two complementary strands are easily constructed and annealed in solution to form a double helix. Short oligonucleotides are routinely synthesized and are available commercially.

Once engineered DNA strands are built, we need methods to use them to create custom proteins. Proteins are conveniently made in engineered cells using *expression vectors*, plasmids that contain the gene specifying the protein along with a highly active promoter sequence. The promoter, which is often taken from a virus, directs the engineered cell to create large quantities of messenger RNA based on the plasmid DNA in the vector. The cell then synthesizes the protein based on this messenger RNA. Bacteria are the most widely utilized host cells that are engineered for protein production. Engineered bacteria create large amounts of protein, often comprising 1–10% of the total cellular protein. Also, bacteria are easy to grow, and inexpensive fermentation methods allow growth of high densities of bacterial cells with modest resources.

Proteins may also be created without the help of living cells, by isolating the protein production machinery and performing the reactions in the test tube. The first step of protein production, the transcription of DNA into a messenger RNA, is now routine with purified RNA polymerase. However, the second step, the synthesis of proteins based on purified messenger RNA in cell-free systems, is still a technical challenge. In some cases, extracts of the cell cytoplasm, containing the protein synthesis machinery along with everything else, are effective. Extracts can, however, encounter problems with limited energy supply and the presence of protease and nuclease enzymes that cleave the products and RNA message. Specialized continuous-flow cell-free systems have been developed to overcome this problem.

Site-Directed Mutagenesis

Site-directed mutagenesis is used in these cases to modify the amino acid sequence of a protein by making specific changes in the existing gene encoding it. In this way, we can make atomically precise changes in the structure of a protein, altering structure and function. A wide variety of methods are available for modifying existing genes. Some of these methods are so reliable that prepackaged kits are available from commercial sources. Site-specific mutations are conveniently introduced into existing genes with specially designed oligonucleotides.

Fusion Proteins

Recombinant DNA techniques are also used to combine entire genes, forming a larger fusion protein that combines the functionality of all of the pieces. Special care must be taken when designing the linkage site, so that the fused proteins will not block one another when folding into their active structures. Fortunately, many natural proteins are very robust and perform their functions even when fused to another large structure. Fusion proteins can harness the natural delivery mechanisms in cells

BIOMOLECULAR STRUCTURE DETERMINATION

X-ray crystallography

X-ray crystallography currently provides the most detailed information on atomic structure. The experimental information obtained from a crystallographic analysis is a three-dimensional map of electron densities. This map shows the observed density of electrons at each point in the crystal lattice.

NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is the workhorse for determining molecular structure in chemistry. Data from NMR spectroscopy characterizes the local environment of atomic nuclei inside molecules.

Electron Microscopy

Electron microscopy has a long and venerable history in all aspects of nanoscale science. It is perhaps the most intuitive approach to imaging macromolecular objects, because it is so similar to light microscopy. Theoretically, electron microscopes should be able to see subatomic structure, but practical limitations—imperfections in the magnetic optics and prob

Atomic Force Microscopy

Atomic force microscopy, developed in the early 1980s, is a newcomer relative to the other techniques described here. The approach is more akin to touch than to vision. A sharp probe is scanned over the surface of the sample, recording the height at each point and yielding a topographic map of the surface. Of all of the methods for determining molecular structure, this provides the most direct connection between our world and the atomic world.

MOLECULAR MODELLING

In designing new bionanomachines, computation works hand in hand with experimentation, often in an iterative manner. Molecules are designed in the computer, and the best ideas are synthesized and tested. The lessons learned are then applied in the next round of computational design, and so on. This approach has been highly successful in rational drug design, most notably leading to many of the powerful drugs used to treat AIDS. Computation also often allows exploration

of systems that are experimentally inaccessible, providing predictions and directing further research and development.

Computer graphics revolutionized the study of biomolecules and now is an indispensable tool for all of the molecular sciences. Computer graphics allows us to visualize the unfamiliar shapes, properties, and interactions of molecules in a manner that is familiar and intuitive. Molecular graphics provides the first window onto a new project, allowing the researcher to explore and understand the molecules that will be built or modified.

Today, computer graphics hardware and software are sufficiently fast to allow interactive representation of even the largest biomolecules. Excellent commercial and free software is available for visualizing molecular structures. Some of the most popular packages include:

(i) RasMol. A compact, self-contained program for the display of molecular structures. A flexible scripting language

allows choice of representation styles and coloration and selective display of portions of a molecule.

(ii) Protein Explorer. Another compact molecular display program.

(iii) Chime. A Java plug-in for display of molecular structure within HTML pages.

Molecular modeling techniques allow the researcher to build any desired molecule based on the known molecular geometry of the component atoms. Molecular mechanics then applies a mathematical force field to this three-dimensional structure to define the interactions between each atom. The system may be used in several ways.

Many of the recent successes in bionanotechnology involve the design of new functionality into natural biomolecules. The closer we stay to known structures, of course, the greater our confidence that the prediction will be realized in the biomolecule. The workhorse for design in many laboratories is simple molecular modeling, designing changes by hand on the computer graphics screen. These techniques are available off the shelf, and they allow researchers to build in new structure and then minimize the structure, looking at how well the modifications fit into the overall structure.

Many of these techniques are being developed in the field of computer-aided drug design. The goal is to design a drug molecule that perfectly fits into the active site of a target bionanomachine, blocking normal function. Some approaches

begin by docking thousands of small fragments, each composed of 5 or 10 atoms. The best fragments are then linked to fragments that bind in neighboring portions of the active site, to form larger drug molecules. Another approach starts with a "seed" molecule that binds in the middle of the active site. The drug is then grown into a larger molecule by adding atoms one at a time until the active site is totally filled. These methods can design excellent candidates for new drugs but may occasionally run into problems with overeager researchers who design exotic molecules that are impossible for any chemist to build.

STRUCTURAL PRINCIPLES OF BIONANOTECHNOLOGY

Our first goal in nanotechnology is to build a stable nanostructure. Only then, after we can arrange atoms the way we want, can we can start thinking about what jobs these structures might do. To achieve this basic goal, we must understand the forces that link atoms together inside a nanostructure. These forces are different than anything in our familiar world. First of all, we can't shape atoms into any arbitrary form.

Fortunately, scientists have been laying the groundwork for this goal for centuries. Chemists have discovered a wealth of information on the structure and stability of molecules and have perfected methods for con- structing them from their component atoms. Biologists, on the other hand, have studied the atomic details of thousands of working nanomachines, each constructed by using only the basic principles discovered by chemistry. We can analyze the ways that existing bionanomachines achieve stability and function and then use this information to develop a basic nanoscale toolkit for designing and constructing our own nanomachinery (Whitesides and Gryzbowski 2004).

Natural Bionanomachinery Is Designed For A Specific Environment

Natural bionanomachines are made to function inside cells. They have been optimized for this environment and may not function optimally, or even at all, when placed in different environments. Hydrogen Bonds Provide Stability and Specificity .Hydrogen bonds play a central role in the stability of bionanomachines, and in their interactions with one another. Hydrogen bonds are like Velcro; they are reusable fasteners that may be connected and broken according to need. Hydrogen bonds are weaker and less directional than covalent

bonds, but they are slightly stronger than the typical thermal energy, so they are stable in biological contexts. Because they are not as stable as covalent bonds, they are not as difficult to break.

The most important limitation is the need for water. Bionanomachines are designed to be stable when surrounded by water. The unusual properties of water, described below, are harnessed to stabilize biomolecular structures. Except in rare cases, bionanomachines cannot be designed or analyzed in other solvents or in vacuum, because they only show their true structure and function when placed in water. The biological environment is also limited to a narrow range of temperatures. Typical bionanomachines perform best at temperatures of about 37°C, although in special cases biomolecules may be designed to perform at temperatures up to 90°C. Bionanomachines are designed to be stable at this temperature. Natural bionanomachines are also constructed to be stable over a typical biological time scale. Most bionanomachines are expected to be functional for mere seconds, and bionanomachines are only rarely built to last more than a year. These machines are built quickly, used for a specific task, and then demolished, providing raw materials for building the next machine. Planned obsolescence is the rule.

HIERARCHICAL STRATEGIES FOR CONSTRUCTION OF BIONANOMACHINES

(i) Covalent synthesis has been used to create many small organic molecules that mimic biological function. Eg.,The molecule has been desiged whichis a mimic of the heme group in hemoglobin and shows similar binding properties for oxygen.

(ii) Covalent polymerization has been used to create novel structural materials, ranging from tough plastics to elastic rubbers. A nylon chain, composed of small repeating units, is shown here.

(iii) Self-organizing synthesis has been used to create liposomes for delivery of drugs. The lipids associate randomly to form a dynamic aggregate.

(iv) Self-assembly is used to build the most complex biological machinery. The viral capsid shown here is comprised of 60 identical subunits arranged in perfect icosahedral symmetry.

Covalent synthesis

The first strategy is *sequential covalent synthesis*. Atoms are directly bonded into covalent molecules of the desired shape. Of the four hierarchical strategies, this one is the most similar to manufacturing techniques in our macroscale world. The product is designed, and then the components (atoms) are placed together piece by piece to build up the structure. This is exactly what synthetic chemists do. Synthesis of molecules such as vitamin B12 and taxol, with up to several hundred atoms, shows the upper limits of molecules that are currently feasible by synthetic chemistry. The advantages of covalent synthesis lie in the diversity that is achievable. Atoms may be combined in nearly any combination, including highly strained shapes and unlikely combinations of atoms, given, of course, that the methods for proper positioning and bonding of the atoms are available.

Covalent polymerization

The second strategy is *covalent polymerization*. Structures are built of modular units, which are linked into linear or branched chains. The synthesis may be performed in bulk, to form a mixture of chains. Plastics such as polyethylene are an example. Or polymerization may be controlled one step at a time, creating identical chains each time. The chemical synthesis of DNA by solid-phase techniques and the synthesis of DNA in cells are examples—in both cases, exactly the same chain, identical at the atomic level, is produced each time.

Self organizing synthesis

The third strategy is *self-organizing synthesis*. Modular units are again applied, but the nanostructures are formed by noncovalent association of units. Familiar examples include molecular crystals, such as sugar crystals or protein crystals, and liquid crystals used in computer displays. In cells, examples include the micelles and bilayers formed by lipids. Many current applications termed "nanotechnology" fall under this category, such as nanospheres and nanocomposites. Note the difference between self-organization and the previous two levels of the hierarchy. In covalent synthesis and polymerization, the engineer links atoms together in any desired conformation, which doesn't necessarily have to be the energetically most favored position. Self-organizing molecules, on the other hand, adopt a structure at a thermodynamic minimum, finding the best

combination of interactions between subunits but not forming covalent bonds between them. In self-organized structures, the engineer must predict this minimum, not merely place the atoms in the location desired.

Lipids Self-Organize into Bilayers

Lipids are small molecules composed of a water-soluble chemical group connected to one or more carbon-rich tails. A few variations on this common design, described in Chapter 2, are used in cells, but many other variations on this basic chemical architecture are possible for use when designing a new application. Because of their long hydrocarbon tails, lipids are highly insoluble in water. Self-organization is driven, as in protein folding, by the need to shelter these carbon-rich tails from water. Each lipid has a distinctive *critical concentration.* When placed in water at concentrations greater than the critical concentration, lipids associate to shield the hydrophobic segments from water. The critical concentration is very low and is lower for lipids with longer carbon chains. For instance, a typical lipid with 16 carbon atoms in each chain will have a critical concentration in the picomolar range.

Self assembly

The fourth strategy is *self-assembly.* Whitesides defines self-assembly as "the spontaneous assembly of molecules into structured, stable, non covalently joined aggregates." *Protein folding:* the spontaneous folding of a protein chain into a stable, globular structure. Self-assembly: the assembly of globular subunits into defined multichain complexes. Both processes involve searching of many possible conformations until the thermodynamic minimum is found, powered by random thermal fluctuations. Highly specific interactions define the geometry of the final structure. Of all the lessons that may be learned from nature, the use of spontaneous self-assembly to construct nanomachines is arguably the most important(Whitesides *et al.,* 1998) This mode of construction is utterly foreign to our macro scale technologies. Familiar manufacturing is dominated by willful, directed construction of objects based on specific blueprints that specify the three-dimensional form of the product. Cells, on the other hand, bring all of the necessary components together and let them self-assemble into the product

Protein Folding

Nature uses an information-efficient method for construction of proteins. Natural proteins are designed to form stable globular structures. The amino acid sequence of each protein is tailored to provide a collection of carbon-rich amino acids to form a stable core, taking advantage of the hydrophobic effect. They also include many charged and hydrogen-bonding amino acids, which tie the chain together into a stable bundle. Everything is in precisely the right place. But perhaps more remarkably, proteins are designed. By current estimates, only a small fraction of the total number of possible amino acid sequences will fold to form stable structures. Researchers estimate that there are about 1000 ways to fold a protein chain to form a stable structure. However, this does not mean that there are only 1000 protein sequences that fold into stable structures. A wide range of similar, homologous sequences fold into similar structures, differing only in local details.

Many biomolecules require some assistance when folding and maturing. Cells contain a collection of biomolecules, collectively known as *chaperones*, that assist in folding, as well as a wide variety of biomolecules that process the proteins into mature forms . This is important to keep in mind when engineering an organism to create a protein. Different organisms have different systems for assisting in folding and maturation, so the end product may be different.

Once proteins fold into globular structures, they are prone to unfolding. Extremes of temperature, salinity, pH, and other environmental factors promote unfolding and loss of activity in proteins. One of the earliest industrial applications of protein engineering was to stabilize enzymes for use in non-biological environments. One goal of this work is to create enzymes that are stable at extreme temperatures. Fortunately, we can look to nature for clues as to how to proceed.

Proteins May Be Designed to Self-Organize with Lipid Bilayers

Of course, a perfectly sealed membrane is useful for only the most limited applications. Methods are needed to communicate and transport materials across the membrane. In natural systems, these functions are performed largely by specialized proteins that interact with the membrane. Membrane proteins associate with lipid bilayers in a variety of useful manners. In the simplest case, a lipidlike group may be attached to a protein surface. This lipid will insert into the bilayer, tethering the protein to the surface of the bilayer. Many other proteins are designed

with hydrophobic segments that span the membrane or insert on one side of the membrane. Some contain a single stretch of approximately 20 amino acids of predominately hydrophobic character. These form an -helix, shielding the hydrophilic peptide groups inside and displaying the hydrophobic sidechains outside. This structure will pass through the membrane perpendicular to the plane, exposing the two ends of the protein on opposite sides of the membrane. Many other proteins are built with a hydrophobic girdle that orients the protein within the membrane, like a bather surrounded by an inner tube.

FUNCTIONAL PRINCIPLES OF BIONANOTECHNOLOGY

Information-driven synthesis is essential for any practical technology. To make a technology successful, we must be able to assemble our available raw materials into many final products, based on instructions held in a blueprint. Otherwise, we must create a new assembler for each new product, which would pose too great an investment of resources to allow practical development of new machines and new methods. Once the investment is made in producing an informaion-driven assembler, anything may be constructed simply by giving it the appropriate instructions.

Nucleic Acids Carry Genetic Information

In the natural process of protein synthesis, the blueprint for building proteins is held in DNA strands. The information is encoded in the sequence of the four bases in DNA. The code is very simple and universal on Earth. Information is transferred from one nucleic acid strand to another through the specific interaction of bases(Cox, 2001).

Ribosomes Construct Proteins

Ribosomes are nanoassemblers that construct proteins according to the information in RNA . The process is complex, because the genetic code is an informational code and not a chemical code. The transcription of DNA to RNA uses a chemical code: Each DNA base chemically matches an RNA base. All that RNA polymerase needs to do is display the DNA strand and connect the RNA bases when they bind properly to the strand. The translation of RNA to protein, on the other hand, is not based on chemical interaction between the RNA and the

protein amino acids. Instead, it is a translation of one chemical language into another. And, as with all translations, a dictionary is needed for matching words in one language with their proper counterparts in another. In all cells, the dictionary is composed of *transfer RNA*, a collection of translator molecules made of RNA. These L-shaped adapters have a segment at one end that recognizes the information in RNA and a segment at the other end that is chemically connected to the proper amino acid.

Information Is Stored in Very Compact Form

The flow of information from DNA to RNA to protein demonstrates that information may be densely stored at the nanoscale.Photosynthetic organisms also contain effective molecules that harvest light and transfer it to reaction centers . These proteins are packed with chlorophyll and carotenoid molecules that absorb light of many wavelengths. The energy is then transferred from molecule to molecule by resonance energy transfer, until it reaches the special pair of chlorophylls in the reaction center, where the excited electron is quickly shuttled away. Energy from light is also harnessed to do physical work. For instance, the protein bacteriorhodopsin transports protons across a membrane by using power provided by the absorption of light, and the light-sensing protein

Electrical Conduction and Charge Transfer Have Been Observed in DNA .

DNA contains many aromatic bases stacked one atop the next. The quantum mechanical orbitals of these bases overlap, creating a pathway for the flow of electrons. DNA is a potential candidate for the design of nanoelectronic devices, with several advantages. The synthesis of DNA is routine, and customized assemblages, anything from single nanowires to complex networks, may be designed and synthesized. This is a beautiful idea in concept, but the details of electrical conduction and transfer in DNA are still hotly debated. Several processes have been observed.

Electrochemical Gradients Are Created Across Membranes

Nanoscale energy may be stored by using a concept similar to batteries and capacitors. The idea is to separate charged objects into two separate compartments, so that one holds more negative charge and one is more positive. In

a capacitor, electrons are pumped from one metal plate to the other, building up a negative charge on one side. Then the flow of electrons back can be used to power an electrical machine. In cells, ions are typically used instead of electrons. An enclosed space is created, surrounded by a membrane that is impermeable to ions. Then ions are pumped across the membrane, creating an electrochemical gradient. The flow of ions back across the membrane is then used to perform chemical or mechanical work.

MICROSCALE INFRASTRUCTURE IS BUILT FROM FIBROUS COMPONENTS

Helical Assembly of Subunits Forms Filaments and Fibrils

Filaments are created by designing a protein with a binding site for other copies of itself. The use of a self-associating globular protein to form a filament has several attractive properties. Because a filament is composed of a number of modular subunits, it provides a ready scaffolding with many identical attachment sites for other structures. Filaments are also designed for rapid assembly and disassembly, allowing rapid response to the changing needs of a given application. Because the interactions are similar to typical protein recognition sites, the integrity of the filament may be modified by binding of ligands, ions, or other proteins. Helical protein filaments are indeed found throughout nature and are some of the most plentiful and widespread proteins in cells.

By careful design of the location of the self-association sites on each individual subunit, any type of filament may be generated . If the sites are on opposite sides of the subunit, the subunits will stack like a string of beads, forming a tenuous, extended filament. If the association sites are shifted slightly, a spring-shaped helix will be formed. Then, if additional self-association sites are engineered, the spring will close down to form a sturdy cylinder. Examples at both ends of this range of design are known.

Actin filaments are an example of an extended filament. Actin is the most common filament-forming protein in our cells, forming the infrastructure of the cytoskeleton and much of the infrastructure used for cell motility. Actin associates to form a directional helical structure with two different ends. When growing, actin monomers add to one end 10 times faster than the other, leading to directional growth of the filament. Actin filaments are highly dynamic in living

cells, and are continually built and disassembled minute by minute according to need. Growth of each filament is regulated by the binding of ATP, which promotes growth, and a collection of fila-ment-stabilizing and filament-severing proteins.

Cells Make Specific and General Adhesives

Adhesives are a remarkably cost-effective and general method of construction. If designed correctly, adhesives can be used to join many dissimilar materials with a stress-resistant bond. Adhesives require two design criteria: They must form a strong, intimate interaction with the surfaces being connected, and they must be cured into a tough solid, so that the join itself is stable. In our water-filled world, however, adhesives are often compromised by the presence of water or water vapor. Water can form a thin layer on surfaces, blocking the interaction of adhesive with surface. Water can also attack already-glued areas, destroying the glue or infiltrating along the seam between adhesive and substrate.

Traffic Across Membranes

Cells require an infrastructure for containment, because biological nanosystems are typically composed of many individual freely interacting parts. If we choose to design nanomachinery with the same paradigm, we will also need effective containers. Membranes are the primary structures used for containment in natural biological systems. They have attractive properties: Lipid membranes are flexible, self-healing, and impermeable to the molecules that must be contained. However, use of membrane-enclosed spaces creates a new problem: the need to transport objects across the barrier. A perfectly sealed membrane is useless in most applications. In answer to this challenge, cells build a wide variety of active and passive transport systems to traffic molecules across membranes.

BIONANOTECHNOLOGY TODAY

Bionanotechnology is a reality today; in fact, it is a booming field. It's an exciting time to be working in bionanotechnology. Everything is new, and new discoveries are reported every day. It is a particularly exciting time to be working in this field because bionanotechnology today is driven by clever people. A clever new idea can open an entirely unforeseen avenue of research and application. The first glimmerings of nanomedicine are allowing researchers to make tailored

changes to the mechanisms of the human body, correcting defects and curing disease. Familiar biomaterials, such as wood, bone, and shells, are providing the principles needed to create materials tailored at the nanoscale to fit our needs.

Some of the earliest experiments were performed on the enzyme ribonuclease by Bernd Gutte. He set out to find a minimal chain that would still fold and perform the cleavage reaction. He began with the structure of natural ribonuclease, which contains 124 amino acids, and noticed that the active site is composed of only a small core structure of the protein. Many of the loops extending from this core seemed to be unnecessary. So he saved the core structure and then clipped off all of the large loops that extend from the core, replacing them with smaller loops. He was able to design an enzyme that included only 63 amino acids but still folded and performed the catalytic reactions

Proteins Are Being Designed from Scratch

Of course, to take full control of bionanotechnology, we must be able to design a protein. We must be able to predict the proper amino acid sequence that will fold into any desired structure. Currently, we can predictably make small local changes to known protein structures with homology modeling, to tailor the local characteristics of a protein. However, to allow the necessary freedom to explore entirely new applications, we need to have the ability to design proteins using only basic principle.

Jane and David Richardson were among the first to attempt this challenge. They designed two proteins based on the basic folding patterns of natural proteins. But instead of using homology modeling with the existing protein structures, they attempted to design a novel amino acid sequence with no homology to any existing proteins. Their first protein, "Felix," was modeled after proteins composed of bundles of four -helices. It contains 79 amino acids and includes 19 of the 20 amino acid types. When synthesized, it was found to be soluble and showed a high content of -helix when analyzed by circular dichroism spectroscopy. However, they found that it is only marginally stable, and when analyzed by NMR, it shows a significant amount of disorder. Their second protein, "betabellin," was modeled after proteins with two sandwiched -sheets. After a dozen redesigns providing valuable lessons at each step, a stable, soluble version has been obtained.

Proteins May Be Constructed with Nonnatural Amino Acids

Natural proteins are limited to the 20 natural amino acids, occasionally augmented with a few modifications performed after the protein is made. Although this provides a remarkable breadth of function, some applications need additional chemical or structural diversity. Natural proteins typically use prosthetic groups to perform these extended functions. However, prosthetic groups can be complicated to synthesize and to incorporate into proteins. So, quite naturally, researchers have developed methods to extend the range of amino acids that are incorporated into proteins(DeGrado *et al*.,1999).

Harnessing biomineralisaton

Many dreams of nanotechnology center around exotic materials, such as diamond or carbon nanotubes, with exotic properties. Visionaries often design nanomachines or nanorobots that strongly resemble macroscopic machines, shrunk to nanoscale sizes. So naturally they look for tough materials that mimic the strong metal, glass, and plastic materials that we use in our everyday machinery.

Natural biomaterials, on the other hand, are built according to a different paradigm. Organisms are constantly changing, growing, and responding to environmental changes, so biomaterials are dynamic constructions. They are built for a given need and then quickly disassembled when needs change(Pekta *et al.*, 1998). The structures inside cells may only last for minutes before they are remodeled. Even the most sturdy biological structures, such as bones, undergo continual repairs and reshaping by cells that systematically dissolve and rebuild build them part by part. Remarkably few natural biostructures, such as bone, shells, and wood, remain useful after the signs of life have left; however, they have been used since the very beginning of human technology. We now have the tools to reshape these natural biomaterials and to use the principles of their construction to build our own biologically inspired nanoscale materials.

Researchers can now create glassy amorphous silica on demand, using biological processes. In one application, researchers have looked to sea sponges for methods. These sponges build spicules composed of a thin strand of protein surrounded by silica. The proteins are termed silicateins, and it was found that they can catalyze the formation of amorphous silica by using tetraethoxysilane as a starting material. Looking to simplify the process, several simple polypeptides

were tested for similar activity. It was found that large polymers, composed of blocks of 10 to 30 cysteine amino acids separated by blocks of several hundred lysine amino acids, could perform the same reaction. The blocks of cysteine align the tetraethoxysilane molecules and catalyze the polymerization, and the lysine amino acids are needed to make the entire chain soluble. Remarkably, different forms of silica could be formed under different conditions . In the absence of air, hard, transparent spheres were formed. However, in the presence of oxygen, the cysteine amino acids form disulfide linkages. When these were used to form silica, packed columns of silica were formed.

Minerals Are Combined with Biomaterials for Special Applications

When additional strength or altered properties are needed, minerals are added to biomaterials. More than 60 different types of minerals have been discovered in biological systems. In bones, teeth, eggshells, and seashells, minerals are incorporated to add strength, but crystals also play less familiar functional roles. For instance, the inertia of small otolith crystals is used to sense gravity, and the alignment of small of magnetite crystals is used to sense the magnetic field of Earth. The optical properties of single crystals are also exploited, albeit more rarely than in our commercial world: Trilobites used crystals of calcite in their eyes, and some scarab beetles owe their metallic luster to crystals of uric acid.

Biomineralization is a fascinating process that is just starting to reveal its secrets. The growth of crystals on demand requires control of four processes. First, a space within the biological matrix is created to allow growth of the mineral. Second, ions are transported into the space, often at very high concentrations. Third, crystals or aggregates of the mineral are nucleated at the desired locations. And finally, the growth and orientation of the mineral must be carefully controlled to produce the desired size and shape. By using different approaches to these steps, different types of minerals are tailored for specific applications.

Sea urchins show even more control. As they create their spiny shells, they create a structure composed in large part from a single crystal of calcite. A large vesicle is created by fusing many cells together. The single crystal is then nucleated and its growth carefully controlled, presumably through use of specific proteins that alter the growth rate along different crystal faces. The result is a plate or spine up to a centimeter in size composed of a single crystal. Similarly, our

bones are created by the nucleation and growth of single crystals of dahllite inside a matrix of collagen.

NANOMEDICINE TODAY

One of the great promises of nanotechnology is increased control over our personal health. As our understanding of the human body has deepened, so has our understanding of disease, opening the door to therapies for treating disease. The art of surgery builds on centuries of knowledge in anatomy. The benefits of hygiene and antiseptics were made clear with the discovery of microorganisms. Today, with our growing knowledge of the atomic-scale structure of our bodies, we can now exert control at the nanoscale level(Chang, 1999).

Nanomedicine is a natural application for bionanotechnology. After all, the human body is designed for maximal function of biological molecules. This is ideal for nanomedicine, because we can use the raw materials that nature has given to us. We have incredible disease-fighting systems to use as examples. The immune system gives us tools for seeking out pathogens and quickly dispatching them. The blood clotting system gives us the tools to patch major damage in a matter of seconds, and the processes of wound healing show us how to forge lasting repairs. Now, we have the ability to tailor these tools to perform functions that nature has overlooked.

Thus far, a targeted approach to nanomedicine has been the most successful. A single target is chosen that causes (or contributes to) the disease state. A specific nanoscale device is then created to find that target and correct its function. A familiar example of this approach is aspirin. When we take aspirin, we are flooding our body with nanomachines, each composed of only 20 atoms. This nanomachine contains a recognition element that seeks out an overactive pain signaling protein and a warhead that attaches to the protein and temporarily stops its action. With aspirin, we are controlling our own bodies at the nanoscale. Currently, drug therapy is our most effective method for targeting a pathogenic organism or a given cell type or disease state.

Computer-Aided Drug Design Has Produced Effective Anti-AIDS Drugs

Rational drug design is a major triumph of current nanomedicine. A target is chosen in the pathogenic organism, and the target is characterized at the atomic level. Then, using this nanoscale information, a team of scientists engineers a molecule that specifically attacks the target, blocking its action.

The team typically includes a biologist, who performs the characterization of the target and tests trial drugs; a chemist, who synthesizes drugs; and a computational chemist, who designs and optimizes drugs to bind to the target. Through successive cycles of design, synthesis, and testing, drugs are discovered, perfected, and then used in therapy.

HAART (highly active antiretroviral therapy) is a successful example of rational drug design. HIV is arguably the best-characterized organism known to science, and this knowledge has been aggressively pressed to service in the fight against AIDS. In a matter of a decade, AIDS has changed from a uniformly deadly disease to a manageable disease in many cases, because of the nanoscale design of effective anti-HIV drugs.

Immunotoxins As Targeted Cell Killers

In many therapies, we want to kill entire cells. For instance, cancer is caused by cells that are growing without control, so in cancer therapy we want to find these cells and kill them while sparing the surrounding normal tissue. What we need is a nanoscale scalpel to seek out cancer cells and remove them.

Many natural toxins, made by plants and bacteria, seem like good candidates for this function. They often kill only specific cells. This is important, after all, so that the organism making the toxin does not kill itself in the process. Many of these toxins are built from two components. One component binds to target cells, and the other component is the poison that kills the cell. Unfortunately, these toxins still attack many types of cells in an organism and would kill cells throughout the body if used in therapy. In nanomedicine, we require an even more specific approach, designing toxins that target a single cell type, such as the cells in a tumor.

Drugs May Be Delivered with Liposomes

For decades, researchers have explored the use of nature's own packaging system for custom delivery. Artificial membranes, under appropriate conditions, form small, closed vesicles called *liposomes*, composed of a lipid bilayer that encloses a small droplet of water. Methods are available for creating liposomes in a range of sizes, from about 20 nm to 10 m. They are useful in many applications. Because they have a water droplet trapped inside, they can carry water-soluble cargo such as proteins, but because they are composed of lots of carbon-rich lipids, they can also carry water-insoluble cargo inserted into the membranes. They are at home in the human body, because they may be created with naturally occurring lipids. Liposomes are nontoxic, nonimmunogenic, and biodegradable.

Although liposomes have not turned out to be the answer to all drug delivery problems, they are attractive delivery mechanisms in a number of useful applications . They are particularly useful for delivering drugs that are very potent and very toxic. In addition, liposomes can increase the lifetime of drugs that are rapidly cleared from the blood. Liposomes are also particularly effective in some cases for targeting drugs to given locations, for instance, for delivering drugs to macrophages in the blood. These immunoliposomes are cleared from the blood by macrophages, which engulf and digest them, releasing the drugs directly inside the cell in the process.

Artificial Blood Saves Lives

Countless lives have been saved by blood transfusions, but transfusions of whole blood have a few disadvantages. Whole blood has a short shelf life and must be carefully matched for blood type. Also, the possibility of viral contamination is always a cause of concern, even though HIV and hepatitis viruses are no longer a problem because of effective screening of blood. This has spurred research in creating artificial blood for use during surgery or in emergencies.

Purified hemoglobin has many potential advantages as a blood substitute. It may be used on any patient without the need for blood typing, because the blood type sugars are found on the surfaces of red blood cells, not on the hemoglobin molecules. Purified hemoglobin may be sterilized to remove pathogens, and it may be stored for more than a year. However, the first experiments showed that natural

hemoglobin, by itself, is not a useful blood substitute. In 1937, it was found that transfusion of purified hemoglobin delivered oxygen successfully but was highly toxic to the kidneys. Hemoglobin is normally a tetramer but dissociates into dimers when free in the blood. These dimers are rapidly filtered by the kidney, where it accumulates to toxic concentrations.

Researchers are now looking to bionanotechnology for answers, engineering hemoglobin for use in blood replacement. The first approach was to create a hemoglobin complex that is stable in the blood. Purified hemoglobin was cross-linked, using small chemical agents like glutaraldehyde to connect lysine amino acids on the surface(Moore, 2000). Two forms were successful: poly-hemoglobin, in which several hemoglobin molecules are linked into a larger complex, and cross-linked tetrameric hemoglobin, in which specific cross-links were created between subunits within the tetramer. A more targeted approach was taken with recombinant DNA methods. A modified hemoglobin was designed that contains two hemoglobin subunits fused into one chain . This then forms a stable complex similar to the tetramer, but with two of the subunits covalently linked together. In all of these cases, the resultant protein was large enough to resist filtering by the kidney but retained excellent oxygen-carrying capabilities.

Gene Therapy Will Correct Genetic Defects

With our growing understanding of the human genome, we now have the possibility of understanding and correcting specific genetic defects such as diabetes or cystic fibrosis at their source. Gene therapy is an area under intense research, with the hope that useful therapies will be produced in the near future. Tempering this excitement, researchers and policy makers have proceeded slowly. Because gene therapy seeks to make changes at the heart of our humanity, it raises profound ethical questions. Each generation will have to decide which changes are ethically acceptable.

Several approaches are being explored. The most obvious is to correct a missing or defective protein. The correct gene is added to the cell, where it then creates the active protein. This will provide a way to correct cells that have lost their ability to create insulin or an essential enzyme. One might also incorporate a new gene, with entirely new properties, to correct a problem. For instance, researchers are

developing methods to incorporate toxic genes into cancer cells, so that the cancer cells make poisons and kill themselves.

General Medicine Is Changing into Personalized Medicine

Nanomedicine is changing the face of medicine, moving in small steps toward a new paradigm of *personalized medicine*. Based on the genetic makeup of each individual patient, therapies may be tailored to prescribe the most effective forms of treatment and minimize potential side effects with the particular variants of enzymes found in each person's cells.

Nucleic Acids with Novel Functions May Be Selected

With the discovery of natural *ribozymes*—RNA molecules with catalytic ac-tivities—and the recent discovery that the ribosome uses its RNA in the catalytic reaction, the possibilities of DNA and RNA as functional molecules are becoming clear. So, naturally, researchers have searched for methods to find RNA molecules with novel functions. SELEX (Systematic Evolution of Ligands by EXponential enrichment) has been the most successful approach to this goal. It mimics the process of natural evolution to identify oligonucleotides with specific functional properties. The process and its variants have been used to identify *aptamers*, nucleic acids that specifically bind to ligands, as well as custom ribozymes

Antibodies May Be Turned into Enzymes

Based on a clever concept, antibodies have been used to create customized enzymes that catalyze reactions of interest. As described in Chapter 5, enzymes often catalyze a chemical reaction by stabilizing the transition state, the unstable molecular structure on the reaction path between the reactants and the products. Remarkably, an antibody that binds to this same transition state will often act as an enzyme. This selection is conveniently accomplished by injecting a molecule that mimics this transition state into an animal and gathering the antibodies that are formed. The trick is finding the proper mimic. We need a molecule that is similar in shape and chemical character to the transition state but is stable under the conditions of antibody selection.

ATP is made with an artificial photosynthetic liposome

Artificial organisms will require a source of chemical energy. Researchers at Arizona State University have looked to natural photosynthesis for inspiration and have created a simplified light-powered system that creates ATP with remarkable efficiency . Their clever approach creates an artificial mimic of the light-capturing mechanism found in natural photosynthetic reaction centers. They created a three-part molecule. At the center is a porphyrin that absorbs light, similar to the less stable chlorophyll molecules used by nature. At one side, they connected a naphthoquinone. Soon after absorbing a photon, the porphyrin transfers an electron to this quinone. On the other side, they attached a carotenoid. This carotenoid then donates an electron back to the porphyrin. This creates an excited state with an electron on the quinone and a positive charge a long distance away on the carotenoid. Normally, this decays in less than a nanosecond back to the ground state. But, quite remarkably, they have designed a system to capture the excited state, just as in natural photosynthesis, using it to create ATP.

HYBRID MATERIALS

When we think of technology, we think of automobiles, cellular phones, computers, and other durable products of mechanical and electronic engineering. Abundant technologies are available, ranging from the micron level up, that harness diverse materials, from glass, plastic, and metal, to lubricating oils and flexible rubber, to conductors, semiconductors, and superconductors. Interfacing these technologies with the more delicate materials of bionanotechnology is yielding some exciting early fruit.

- Bionanomachines combined with silicon devices.
- Nanoscale conductive metal wires may be constructed with DNA.

Technino - Israel Institute of Technology developed nanoscale wire composed of silver grains deposited along a strand of DNA.

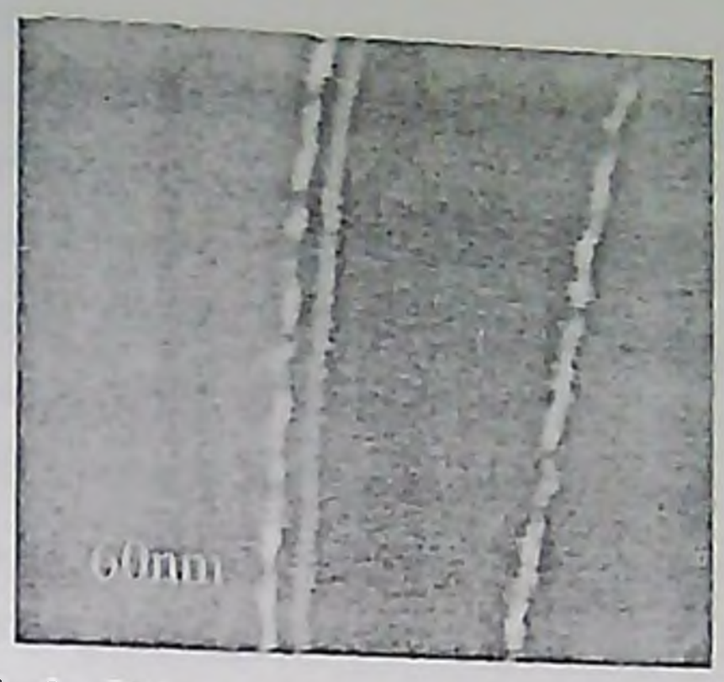

Fig.3. DNA carbon nanotube

Engineered Nanopores Detect Specific DNA Sequences

Researchers at Texas A&M University have designed a biosensor that can detect short strands of DNA, about seven nucleotides in length . The sensor is based on the bacterial protein *hemolysin.* Hemolysin is composed of seven protein chains that form a pore through lipid bilayers. In nature, this is used as a toxin. As a biosensor, hemolysin is embedded in a membrane separating two chambers. An electrical potential is applied across the membrane, which draws ions through the pore from one chamber to the other. The current through this pore is monitored, and when the nanopores are blocked an abrupt change in the current is easily detected.

DNA COMPUTERS

Molecular computers have been a dream, and a goal, of nanotechnology from its inception. The possibility was introduced by Richard Feynman in his seminal lecture, and numerous schemes have been proposed ever since. The first successful man-made molecular computers have taken their lead from biology, using biology's premier information-carrying molecule.

DNA is used widely in natural biological systems as a data storage medium, but quite surprisingly, it is used in only limited ways for computation inside cells. The regulation of gene expression is a form of computation, where various promoter, repressor, and enhancer proteins bind to the DNA and their effects are summed to determine whether a particular gene will be expressed. This computation, however, is relatively wasteful of resources. A new protein is made

for nearly every new logical operation, and the space within the DNA genome that is used for computation, encoding these proteins and providing binding sites for them, may be very large(Adleman, 2003)

Staggeringly large computations may be performed with DNA computers. A typical simulation may sift through 10^{20} different potential solutions to a problem, selecting the single one that is correct. Because of the small size of the individual molecules, this requires only a manageable amount of material. The computation is also massively parallel. Once the computation is set up, all of the possible solutions are examined simultaneously. Finally, because each component is molecule-sized, the computations are cheap and energy efficient. Critics have warned, however, that as the problems are scaled larger, the details of manipulating large quantities of DNA will become a problem.

BIOMOLECULAR SENSING

Our environment is full of interesting properties, such as light, sound, and pressure, that we would like to monitor, but which have very little effect on most physical objects. To sense changes in these environmental properties, we need to transduce the changes into a form that we can recognize. In biological systems, sensing is often performed by a receptor protein, which senses changes in some environmental property and then transduces this stimulus into a signal that may be recognized by the biomolecular signaling machinery. Most often, the sensory function induces a change in shape or a change in the charge distribution of the receptor protein, which is then recognized and amplified, ultimately making intracellular changes or initiating a nerve impulse.

The challenge for bionanotechnology is to harness these receptors when separated from their biological context. These receptors use sensitive recognition and amplification schemes to transduce a small molecular change in the receptor into a large cellular or nervous response(Anson, 2001). In some cases, the application to bionanotechnology will be straightforward. For instance, some receptors are channels that open on sensing. These channels may be used in any application that has two reservoirs separated by a membrane. The receptors of vision and smell, however, require a sophisticated protein-protein signaling scheme, so applications will necessarily be more complex.

BIOSENSORS

Many applications in medicine, environmental analysis, and the chemical industry require sensitive methods for sensing small organic molecules. Our sense of smell and taste are designed to perform exactly this task, and the immune system recognizes millions of different molecules. Recognition of small molecules is a specialty of biomolecules, so they provide an attractive approach to the creation of specific sensors. Two components are needed: the recognition element and some mechanism for readout once the recognition element has found its target. Often, the recognition element may be taken unchanged from the biological source. The challenge is to design a suitable interface to a macroscale readout device.

Antibodies Are Widely Used as Biosensors

Antibodies are nature's premier biosensors, so it comes as no surprise that the development of diagnostic tests using antibodies has been one of the major successes of biotechnology. Perhaps the most familiar example is the simple test used to determine blood type. This is the simplest possible form of immunotesting, taking advantage of two properties of antibodies: their specificity and their ability to cross-link targets. The blood type test is composed of a collection of antibodies that recognize specific sugars on the surfaces of red blood cells. The antibody is added to the blood, and if the particular blood type is present on the cells, the antibodies bind to the surface, sticking many cells together. The result is a clumping of cells that is easily seen with the naked eye.

Biosensors Detect Glucose Levels for Management of Diabetes

Glucose biosensors are some of the most successful biosensors on the market today. People living with diabetes require convenient methods for monitoring glucose levels. Implanted sensors and noninvasive sensors are under development, but currently the most accessible approach is a handheld biosensor that analyzes a drop of blood.

The biosensor relies on the fungal enzyme glucose oxidase, which combines glucose and oxygen to form gluconic acid and hydrogen peroxide. A sensor can be designed to detect the amount of hydrogen peroxide formed. In the 1960s, Leland C. Clark had the clever idea to hold the enzyme very close to a platinum electrode with a membrane, so that the chemical changes could be followed by watching changes in the current at the electrode. This idea proved

effective, and a series of laboratory-sized instruments were developed based on the sensing of peroxide.

Nanoparticles

Nanoshells typically have a silicon core that is sealed in an outer metallic core. By manipulating the ratio of wall to core, the shells can be precisely tuned to scatter or absorb very specific wavelengths of light. For example, gold encased nanoshells have been used to convert light into heat, enabling the destruction of tumours by selective binding to malignant cells.

Meanwhile, researchers at the University of Texas at Austin have described a means of using nanospheres for oral drug delivery. These nanosphere carriers are derived from hydrogels, which are highly stable organic compounds that swell when their environment becomes more acidic. They have been successfully formulated into controlled-release tablets and capsules, which release active compounds when the hydrogel body swells.

Finally, nanotechnology is finding new applications in the area of toxin removal. Colloidal dispersions have been demonstrated to remove potentially lethal compounds from the bloodstream, including high concentrations of lipophilic therapeutics, illegal drugs, and chemical and biological agents.

Vigilent vector - Could Insert Genes That Sense, Prevent Heart Attacks

The University of Florida team used a harmless virus to deliver a combination of genes to animal heart tissue that protected the tissue from heart attacks, according to an article in the February issue of Hypertension, a journal of the American Heart Association. The virus sensed when the heart tissue began to experience hypoxia, or oxygen deprivation, and switched on the protective genes, which prevented the damage and scarring, called ischemia.

The UF team used the adeno-associated virus, a commonly used gene carrier, to insert the cardio-protective gene switch. The approach is a classic example of "bionanotechnology," or manipulating basic elements of biology such as DNA and proteins for research and therapeutic purpose.

Silicon-based drug delivery system

A novel approach to treating inoperable liver cancer using bracytherapy, which involves the implantation of a drug that delivers short-range radiotherapy to a tumour. The new product, called BrachySil, uses the firm's BioSilicon technology to deliver radioactive phosphorous (32-P) to the tumour and subject it to beta radiation, killing off the cancer cells.

pSivida reported encouraging interim results from the Phase IIa trial in November 2004, but has just revealed new data from a second cohort of patients that underscore the efficacy of the approach.

Results from this second group of four patients, 12 weeks after their BrachySil treatment, revealed an average tumour regression by volume of 80 per cent, determined by CT scanning. These data reinforce the results from the first four patients, which found up to 60 regression of tumours.

Modification of therapeutic proteins

Companies developing protein drugs have attached polymers such as polyethylene glycol (PEG) to their protein drugs to extend their half-life in the body and reduce the number of injections needed. PEG is attached to the protein drug in random fashion and at variable numbers of sites on each molecule. This technology will allow PEG or other modifying molecules to be attached in a specific location, reducing the risk that the activity of the drug will be interfered with, and potentially improving the effectiveness of the drug.

Novel way of delivering drugs into the eye

At present, most ophthalmic drugs are delivered via eye drops, but it is estimated that 95 per cent of the activity of medication delivered in this way is lost as the eye drops mix with tears and drain into the nasal canal. This can also cause side effects. Now, researchers at Singapore's Institute of Bioengineering and Nanotechnology (IBN) claim to have solved this problem by developing polymeric contact lens material that can be loaded with active compounds.

The technology allows different types of eye medication to be incorporated into the lens' mixture, and initial studies suggest that glaucoma medications, antibiotics and anti-inflammatory drugs would all be suitable for delivery in this way.

Antimicrobial fabrics with nanopatiele coatings

Antimicrobial fabrics find many technical applications in medical, hygiene, protection, sport, and industrial markets. However, antimicrobial fabrics differ in biocidal performance and durability. For example, such fabrics are used in wound dressings or bio-hazard protective clothings, and are nowadays compared in terms of 'zone of inhibition' and 'kill rate' of bacteria which are both related to the antibacterial activity. Metallic silver, silver oxides, and silver salts are known to have antimicrobial properties; unfortunately, slow-release systems, such as metallic silver, do not confer high zone of inhibition neither high kill rate because of limited availability of silver ions in such metallic systems. Therefore, it is highly desirable to obtain antimicrobial fabrics which possess high antibacterial activity while maintaining wide-range biocidal properties, such as those provided by slightly soluble silver salts. A colloidal solution of a silver salt was applied as a coating to different fabrics. Nano-sized crystals were deposited, as observed by electron microscopy, and presented a uniform surface distribution. The silver salt nanocrystals were obtained with the use of a suitable surfactant which prevented coagulation problems and large crystal precipitation. Moreover, the use of an antimicrobial surfactant improved the antimicrobial activity of the fabric, as demonstrated by antimicrobial test methods for antibacterial activity assessment

THE FUTURE OF BIONANOTECHNOLOGY

The discovery of microscopic life and the subsequent international effort in antisepsis have doubled the length of our lives. Computers have made entire worlds of inquiry possible and raised important questions about our own minds. The world wide web has expanded our ideas of information and communication. In each case, a scientific or engineering advance opened a previously unimagined world.

First of all, we can expect a solution to the protein folding problem. This will allow prediction of structure and function for a protein of arbitrary sequence, allowing the design of novel bionanomachines. This is a key step in bionanotechnology—extrapolating from existing machinery can only take us so far. By current expectations, effective computational methods for protein structure prediction should be expected in the next decade or so. And once natural proteins

are understood, we can move on to the larger problem of improving and expanding the natural building materials for increasingly nonbiological applications.

Cellular engineering

Cellular engineering is another probability. Given the rapidly growing number of genomes and proteomes, we will have a full parts list of living cells in the near future. The coming decades will yield an understanding of how these parts are arranged and how they interact to perform the processes of life. With this understanding will come the ability to modify, tailoring new cells for custom applications. Already, bacterial cells have been engineered to create specific products, such as growth hormone and insulin. In the future, we will see cells that clean pollution, that make plastic and other raw materials, that fight disease, and that are used for countless other applications.

Organismic engineering

Organismic engineering will open many doors, but so far only natural methods are available. We have a long history of artificially accelerating evolution. People have bred organisms for centuries, creating organisms better fit to human welfare. With understanding of the molecular processes of development, the exciting possibility of engineering organisms from scratch will become possible. By directly modifying the genome of organisms, all manner of changes might be made. Already, genetic engineering is improving the properties of agricultural plants and animals, although raising important questions about safety. We may also move on to engineering our own bodies, for improved health and welfare.

CONCLUSION

As an emerging field, the increasing focus of research in bionanotechnology and relating fields is in obtaining reliable structural information of biological matter in different functional states and at different scales towards a mechanistic understanding of biological function. The potential of bionanotechnology for feeding the world, for improving our health and for

providing rapid and cheap methods for manufacturing biomolecules is immense. But we must temper this excitement with careful thought. The challenge lies in our ability to interact with, comprehend and to communicate the beauty and intricacy of nanoworld. It would serve us well to look to Nature—to her world-spanning interconnectedness, to her unassuming creativity, to the sheer wonder of her accomplish-ments—for guidance as we proceed, tempering the strong cultural forces of novelty and capital gain.

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DISCUSSION

- Can bionanotechnology applied to food industries?

In future, the science may be used in food production, and to detect how fresh food is. Food can be designed by shaping molecules and atoms, that wiil be more diet specific, with more capability and precision.

- What is the prospect of nanomarket ?

A market research report " World Nanotechnology Market – An Industry Update 2005" predicts that nanotechnology would exceed 1000 billion dollar by the end of year 2010.The increase in demand for nanoscale materials, tools and devices would reach 29 billion dollar by 2008.

- What is the application of nanotechnology in agriculture?

Nanotechnology can be applied in agriculture in a way that fertilizers can be coated with nanoparticles and can be used for specific delivery, especially for studies taken under glass house conditions.

- What do you mean by organismic engineering?

Organismic engineering refers to genetically engineering organisms like modifying the genome of organisms, making changes for better fit to human life. With understanding of the molecular processes of development, the exciting possibility of engineering organisms from scratch will be possible.

- What is the advantage of bionanotechnology over chemotherapy?

Chemotherapy can cause significant problems in other non –diseased areas of the body too, leading to nasty side effects. Anticancer molecules are put them into tiny particles called nanoparticles, which help in targeted delivery of the drugs to specific cells. This will reduce the harmful effects caused due to chemotherapy.

- Why do you call these biological molecules as nanomachines?

Natural biomolecules have organic, visceral, and often unbelievable shapes, unlike the tidy designs of toasters and tractors. They perform their jobs in a foreign environment, where jittery thermal motion is constantly pushing and pulling on their component parts. They are held together by a complex collection of bonding and nonbonding forces. At their small scale, bionanomachines are almost immune to the laws of gravity and inertia that dominate our machines..

- What are the limitations of bionanotechnology?

Bionanotechnology is in its advancing stage ,hence the technology is very much expensive.

- Why immunotoxins are acting as targeted cell killers?

Immunotoxins hybrid molecule that links a specific antibody, such as a tumor-targeting antibody, to a cell-killing toxin. The result is an *immunotoxin* that will seek out cancer cells and kill them, while passing up healthy cells. Immunotoxins are suicide nanorobots, designed to , killing the target cell and being destroyed themselves in the process And antibodies are very specific

ABSTRACT

Nanotechnology literally means any technology performed on a nanoscale that has application in the real world. It is the willingful combination of atoms to produce a desired molecule. It is defined as the fabrication of functional materials, devices or systems through control of matter at a scale of 1- 100 nm (Foster, 2005).

Bionanotechnology combines nanotechnology with biotechnology to design and produce functionalized biological materials or devices that take the advantage of elements or effects that occur at the nanometer scale. It is the term coined for synthetic technology based on the principles and chemical pathways of living organisms, ranging from genetic engineered microbes to custom made organic molecules (Sammer *et al.*, 2005).

A biological system can be exceedingly small. Cells are very tiny, but they are very active, manufacture various substances; they walk around, wiggle and do all kinds of marvellous things on a very small scale. Cells build thousands of biomolecular nanomachines like proteins, nucleic acids, ribosomes, polysaccharides and lipids, which are built to atomic specifications, performing nanoscale tasks. Bionanomachines operate at the smallest level in the range of 10nm in diameter and require a water environment. ATP synthase and flagellar motor are the rotary motors present in the cell. Sarcomere- the unit of muscle contraction is an example of machine phase nanotechnology (Goodshell, 2004).

Biology provides a plethora of basic methods for construction of functional nanomachinery. Biotechnology has harnessed the natural methods of information driven nanoassembly with recombinant DNA to produce biomolecules of desired function. Advancements in technology and devices like atomic force microscopy along with molecular modelling and computer graphics will aid in the determination of biomolecular structure. Structural principle of bionanotechnology is the ability to build a stable nanostructure (Whitesides *et al.*, 2004). Functional principle of bionanotechnology lies on the fact that energy is the product of metabolic pathway triggering a specific molecule for a desired level of energy.

Bionanotechnology, materials science on a nanoscale, is an enabling technology with a wide range of applications across industries. It includes DNA molecule on an electronic chip as a means of data storage, optical tweezers to manipulate individual molecules of DNA for a particular purpose, two dimensional

and three dimensional scaffolds with a particular topography to promote growth and survival of cells and tissues for grafting, drug delivery vehicles targeted to specific cells, nanocomposites for bone replacement, nanoscale array for post genomic science and nanoscale fabrication of protein molecules.

As an emerging field, the increasing focus of research in bionanotechnology and relating fields is in obtaining reliable structural information of biological matter in different functional states and at different scales towards a mechanistic understanding of biological function. The potential of bionanotechnology for feeding the world, for improving our health and for providing rapid and cheap methods for manufacturing biomolecules is immense. The challenge lies in our ability to interact with, comprehend and to communicate the beauty and intricacy of nanoworld.

DIRECT GENE TRANSFER IN CROP IMPROVEMENT

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SEMINAR REPORT

Submitted in partial fulfillment for the requirement of the course PBT 651- Seminar

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COLLEGE OF HORTICULTURE
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CERTIFICATE

Certified that the seminar report entitled **"DIRECT GENE TRANSFER IN CROP IMPROVEMENT"** for the course PBT 651 has been prepared by Liffey Zachariah Antony (2005-11-142) after going through the various references cited here under my guidance and supervision, and he has not borrowed from any of his fellow students.

Vellanikkara
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DECLARATION

I, Liffey Zachariah Antony (2005-11-142) hereby declare that this seminar report entitled **"DIRECT GENE TRANSFER IN CROP IMOROVEMENT"** has been prepared by me, after going through the various references cited at the end of the report and has not been borrowed from any of my fellow students.

Vellanikkara
12– 07 – 06

Liffey Zahariah Antony
(2005- 11- 142)

INDEX

1. Introduction

In 1983, the era of plant transformation was initiated when *Agrobacterium*- mediated gene delivery was used for producing transgenic plants. Initially the success was limited to the *Solanaceae*, tobacco (*Nicotiana tabaccum* L.) in particular. This dramatically changed throughout the 1980's and into 1990's where now it is possible to transform a wide range of plants, many of which are agronomic importance (Songstad *et al.*, 1995).

An obvious limitation of *Agrobacterium* was the inability to transform monocots. Recent research has demonstrated that delivering DNA into plant cells can be achieved by innovative methods such as Electroporation, Particle bombardment method, Microinjection, Macroinjection, Silicon carbide fibers, Ultrasound mediated gene transfer and Chemical methods.

Vectorless or direct DNA transfer

Direct gene transfer has proved to be a simple and effective technique for the introduction of foreign DNA into plant genomes. It has been further subdivided into two

1. Physical gene transfer methods
2. Chemical gene transfer methods

Physical gene transfer methods

Species-and genotype-independent transformation methods wherein no natural vector is involved but which are based on the direct delivery of naked DNA to the plant cells have been grouped under this category. This is also referred to as DNA-mediated gene transfer (DMGT). The various methods are described in detail.

2. Electroporation

Electroporation is the process where electrical impulses of high field strength are used to reversibly permeabilize cell membranes to facilitate uptake of large molecules, including DNA. The electroporation method is based on Neumann *et al.* (1982) for

animal cells. It uses relatively high, initial field strength (1-1.5 kV) with a low capacitance and therefore a short decay time. Other methods with low initial field strength and long decay time have been described. In this procedure, a sample of protoplasts is pulsed with high/low voltage pulses in the chamber of an electroporator. The chamber is cylindrical in form with a distance of 1 cm between parallel steel electrodes. The pulse is applied by discharge of a capacitor across the cell. It has been reported that by using linear DNA rather than circular DNA, field strength of 1.25 kV/cm and employing polyethylene glycol (PEG) can increase protoplast transformation frequency. PEG is believed to assist the association of the DNA with the membrane.

Electroporation has been used for a long time for transient and integrative transformation off protoplasts. Only recently, conditions under which DNA molecules can be delivered into intact plant cells of sugarbeet and rice that are still surrounded by a cell wall have been standardized. Further, transformability of intact plant cells or tissues depends on pretreatment of the cells or tissues to be transformed, either by mechanical wounding or by treating the cells or tissues with hypertonic or enzyme-containing (e.g. 0.3% macerozyme) solutions.

The range of tissues that can be transformed by electroporation seems to be narrower. For tissues that are susceptible to DNA uptake by electroporation, this method is convenient, simple, fast, has low cell toxicity, and it is inexpensive to obtain transient and stable transformation in differentiated tissues. The disadvantage of the technique is the difficulty in regenerating plants from protoplasts.

3. Particle bombardment/microprojectile/ biolistics

The technique of particle bombardment, also known as biolistics, microprojectile bombardment, particle acceleration etc., has been shown to be the most versatile and effective way for the creation of many transgenic organisms, including microorganisms, mammalian cells, and plant species. The procedure in which high-velocity microprojectiles were used to deliver nucleic acids into living cells was described by Klein *et al.* (1987) and Sanford *et al.* (1987).

The tremendous advantage of particle gun bombardment over other technologies is the wide range of cellular compartments, cell types and plant species to which foreign DNA can be transferred (Vain *et al.*, 1995). The basic system that has received attention employs PDS 1000 (gun-powder-driven device) or the PDS-1000/He (helium driven particle gun). The DNA bearing tungsten or gold particles (1-3 µm in diameter), referred to as microprojectiles, is carried by a macroprojectile or macrocarrier and is accelerated into living plant cells. The DNA-bearing particles (microprojectiles) are placed on the leading surface of the macrocarrier and released from the macrocarrier upon impact with a stopping plate or screen. The stopping plate is designed to halt the forward motion of the microprojectile, while permitting the passage of the microprojectiles. In this procedure, when helium gas is released from the tank, a disc known as rupture disc blocks its entry into the chamber. These discs are available with various strengths to resist the pressure of gas, which varies from 500 to 1700 p.s.i. When the disc ruptures, compressed helium gas is suddenly released, which accelerates a thin plastic sheet carrying microprojectiles into a metal screen. Upon impact with a stopping plate or screen the macroprojectile movement is stopped, but this permits the passage of microprojectiles through the mesh screen. The microprojectiles then travel through a partial vacuum until they reach the target tissue. The partial vacuum is used to reduce the aerodynamic drag on the microprojectiles and decrease the force of the shock wave created when the macrocarrier impacts the stopping plate. The schematic diagram of the PDS-1000/He is given below.

Gene Gun

The use of particle bombardment requires careful consideration of a number of parameters. These can be classified into three general categories:

Physical parameters

Nature, chemical, and physical properties of the metal particles utilized to carry the foreign DNA:

Particles should be of high mass in order to possess adequate momentum to penetrate into the appropriate tissue. A variety of metals such as tungsten, gold, platinum, palladium, rhodium, iridium, and possibly other second-and third-row transition metals can be used. Size range of the particles is 1 µm. Tungsten particles of diameter 1.2 µm and gold particles of diameter 1.0-1.6 µm have been most commonly used metals should be chemically inert to prevent adverse reactions with the DNA or cell components.

Nature, preparation and binding of DNA on to the particles:

The nature of DNA, that is, as single versus double stranded, may be important under some conditions, but this may not be a significant variable in specific cases. In the process of coating metal particles with DNA, certain additives such as spermidine and calcium chloride appear to be useful.

Target tissue: It is very important to target the appropriate cells that are capable of cell division and are competent for transformation. It is apparent that different tissues have different requirements, thus extensive studies need to be performed in order to ascertain the origin of regenerating tissue in a particular transformation study. Penetration of microprojectile (DNA coated to inert metal of gold or tungsten) is one of the most important variables, and the ability to tune a system to achieve particle delivery to specific layers may result in success or failure in recovering transgenic plants from a given tissue. Generally the cells near the center of the target are injured and thus cannot proliferate.

Environmental parameters:

These include variables such as temperature, photoperiod and humidity of donor plants, explants, and bombarded tissues. These parameters affect the physiology of the tissues, influence receptiveness of target tissue to foreign DNA delivery, and also

affect its susceptibility to damage, injury that may adversely affect the outcome of the transformation process. Some explants may require a healing period after bombardment under special regimes of light, temperature, and humidity.

Biological parameters:

Choice and nature of explants, and pre- and post-bombardment, culture conditions determine whether experiments utilizing particle bombardments are successful. The explants derived from plants that are under stress or infected with bacteria or fungi, over- or under-watered will be inferior material for bombardment. Osmotic pre- and post-treatment of explant with mannitol has been shown to be important in transformation. Experiments performed with synchronized cultured cells indicate that the transformation frequencies may be also influenced by cell cycle stage.

Advantages

Several advantages make this technique a method of choice for engineering crop species.

1. It is clean and safe.
2. *Transformation of organized tissue:* The ability to engineer organized and potentially regenerable tissue permits introduction of foreign genes into elite germplasm.
3. *Universal delivery system:* Transient gene expression has been demonstrated in numerous tissues representing many different species.
4. *Transformation of recalcitrant species:* Engineering of important agronomic crops such as rice, maize, wheat, cotton, soybean, .etc. has been restricted to a few noncommercial varieties when conventional methods are used. Particle bombardment technology allowed recovery of transgenic plants from many commercial cultivars.
5. *Study of basic plant development processes:* It is possible to study developmental processes and also clarify the origin of germline in regenerated plants by utilizing chromogenic markers.

Disadvantages

1. In plants, gene transfer lead to nonhomologous integration into chromosome, and is characterized by multiple copies and some degree of rearrangement.

2. The emergence of chimaeral plants.
3. Lack of control over the velocity of bombardment, which often leads to substantial - damage to the target cells.

4. Macroinjection

It is the injection of DNA solution (5-10 µl) by micropipettes into the developing floral side shoots (tillers) of plants. Within the floral tillers are archesporial cells that give rise to pollen in the developing sac by two meiotic cell divisions. It was suggested that such cells might also be able to take up large molecules such as DNA. Thus a plasmid encoding kanamycin resistance and under the control of a promoter was injected into the tillers of rye plants. It resulted two plants: showing resistance to kanamycin. However, these experiments could not be repeated.

5. Microinjection

Microinjection is the direct mechanical introduction of DNA under microscopical control into a specific target. A. target can be a defined cell within a multicellular structure such as embryo, ovule, and meristem or protoplasts, cells, or a defined compartment of a single cell. As a direct physical method, microinjection is able to: penetrate intact cell walls. It is host-range independent and does not necessarily require a protoplast regeneration system. This method has been proposed for the transfer of cell organelles and for the manipulation of isolated chromosomes. When cells or protoplasts are used as targets in this technique, glass micropipettes of 0.5-10.0 µm diameter tips are used for transfer of micromolecules into the cytoplasm or the nucleus of a recipient cell or protoplast. Recipient cells can be immobilized using methods such as agarose embedding, agar embedding, poly-lysine treated glass surfaces, and suction holding pipettes. Once injection has been achieved, the injected cell must be cultured properly to ensure its continued growth and development. With this method the operator has the ability to manoeuver the cell and thus more accurately target the nucleus. Transgenic chimaeras have actually been obtained in tobacco and *Brassica napus* by this approach. The main disadvantage of this technique is the

production of chimaeric plants with only a part of the plant transformed. The microinjection process is slow, expensive, and requires highly skilled and experienced personnel (Veluthampi *et al.*, 2003).

6. Silicon carbide fiber-mediated transformation

Silicon carbide fibers (SCF) average 0.6 µm in diameter and 10-80 µm in length. It has been demonstrated that these fibers have the capability to deliver DNA into plant cells. The method involves vortexing a mixture of plasmid DNA encoding a selectable or screenable marker gene, silicone carbide fibers, and the explant tissue in an eppendorf tube. Silicon carbide fibers have great intrinsic hardness with sharp cutting edges. DNA delivery in this system is presumably due to cell wall penetration by DNA-coated silicon carbide fibers during vortexing of SCF with explant and DNA adhering to fibers might enter the cells. It is possible that SCF function as numerous needles facilitating DNA delivery into the cells. During the mixing process, DNA penetrates the cell to become stably integrated into the nuclear genome. Advantages of the silicon carbide fiber-mediated transformation method over other procedures include the ability to transform walled cells thus avoiding protoplast isolation relative ease of the procedure, and very low equipment costs. The disadvantage is that silicon carbide has some carcinogenic properties.The feasibility of this technique has been demonstrated in fertile transgenic plants of maize and tobacco.

7. Ultrasound-*mediated DNA transformation*

Ultrasound is used described for stimulating uptake of foreign DNA by plant protoplasts and leaf segments of tobacco. The procedure involves immersion of explant (Leaves/protoplasts) in sonication buffer containing plasmid DNA and sonication with an ultrasonic pulse generator at an acoustic intensity of 0.5 w/cm^2 for 30 min. Samples are rinsed in a buffer solution and then cultured for growth arid differentiation. This technique has the advantages of being simple, inexpensive, and multifunctional. One can use standardized conditions and there is no requirement for tissue culture expertise. Little success has been achieved by this technique.

8. DNA transfer via pollen

In this approach, stigma of a flower is cut off some time after pollination, and DNA solution is applied on to the cut surface. The time of stigma excision would depend on the rate of pollen tube growth and may range from 5-20 min to 2-3 hrs. In case of rice plasmid DNA containing *npt11* gene was applied to the cut surface of stigma; up to 20% of the seeds so produced contained the *npt11* gene in copy numbers ranging from 1-300. But in a similar work with barley, transformation frequency was only 10^{-3} to 10^{-4} of the seedling so obtained, the expression level of *npt11* was low, and the mature plants and their progeny failed to show any *npt11* expression. This method is simple, easy and very promising provided consistent results and stable transformations are achieved. This would necessitate a much better understanding of the mechanism of DNA transfer into the zygotes and the factors affecting it (Singh, 2003).

9. Chemical gene transfer methods

This involves plasma membrane destabilizing and/or precipitating agents. Protoplasts are mainly used being incubated with DNA in buffers containing PEG, poly L-ornithine, polyvinyl alcohol, or divalent ions. The chemical transformation techniques work for a broad spectrum of plants.

PEG-mediated gene transfer

Plant protoplasts can be transformed with naked DNA by treatment with polyethylene glycol (PEG) in the presence of divalent cations like calcium (Slater *et al*., 2004). The first conclusive demonstration of uptake and integration of isolated Ti plasmid DNA into plant protoplasts was reported in *Petunia* and tobacco in the presence of poly L-ornithine or polyethylene glycol (PEG). The presence of Ti DNA in the plant genome was demonstrated both by the phenotype of hormone auxotrophic growth, the production of the expected opine and by Southern blot analysis of DNA from the transformants.

In a general procedure, protoplasts are isolated and a particular concentration of protoplast suspension is taken in a tube followed by addition of

plasmid DNA (donor and carrier). To this 40% PEG 4000 (w/v) dissolved in mannitol and calcium nitrate solution is slowly added because of high viscosity, and this mixture is incubated for few minutes (5 min). As per the requirements of the experiment, transient or stable transformation studies are conducted.

Among the most important parameters that affect the efficiency of PEG-mediated gene transfer are the concentration of magnesium or calcium ions in the incubation mixture, and the presence of carrier DNA. The linearized double stranded plasmid DNA molecules are more efficiently expressed and integrated into the genome than supercoiled forms. Integration of foreign DNA into the nuclear genome occurs predominantly at random sites.

The main application of the technique, apart from analyzing the transformation process itself, is in introducing foreign genes to plant cells. This can be accomplished by constructing a molecule containing a selectable marker and the gene of interest, or more easily by simply mixing DNA of the gene of interest with the selectable marker plasmid in a ,molar ratio of approximately 3:1 to 10:1, transforming, selecting for the marker, and analyzing transformants for the presence of the second gene. The method can also be applied with DNA from different sources. Manipulation of nucleic acids prior to transformation is also possible and there are no host range limitations.

The advantage of the method is that the form of the DNA applied to the protoplasts is controlled entirely by the experimenter and not by an intermediate biological vector. The main disadvantage is that the system requires protoplasts and a functional system for regeneration of these protoplasts to calluses and whole plants. It is therefore not applicable to many plant systems. In addition, the relatively random way in which the DNA is integrated into the genome means that, for the introduction of nonselactable genes, a thorough characterization of the transformants by Southern blot analysis is necessary to confirm the nature of the integration event.

Calcium phosphate coprecipitation

In this method, DNA is mixed with calcium chloride solution and isotonic phosphate buffer to form a DNA-$CaPO_4$ precipitate. The precipitate is allowed to react

with actively dividing cells for several hours, washed, and then incubated in fresh culture medium. Giving them a physiological shock with DMSO can increase the efficiency of transformation to a certain extent. Relative success depends on high DNA concentration and its apparent protection in the precipitate.

Advantages of Direct gene transfer

Direct gene transfer for cell transformation circumvents the host range limitation. It is possible to introduce DNA sequences directly into cells and organs by biolistic or electroporation approaches. Cereals, the world's most important crops, have been transformed by direct gene transfer methods.

Disadvantages

i. Direct gene transfer usually depends on a culture system that allows regeneration of mature plants from protoplasts/cells/organs, which is not usually available for every desired plant species. The problem may be overcome by further improvement of plant tissue culture methods.

ii. The methods show unpredictable pattern of foreign DNA integration. During their passage into the nucleus, the DNA is subjected to nucleolytic cleavage resulting in truncation, recombination, and rearrangement or silencing.

10. STATUS AND EXPRESSION OF TRANSFERRED GENES

DNA is introduced into plant cells and these cells are then grown *in vitro* to regenerate plants. Selection and growth of plant cells on selective media provide initial phenotypic evidence for transformation. This includes resistance to antibiotics, herbicides, etc. Several studies have analyzed the inheritance and expression of transferred DNA. The location of the genes and temporal and spatial aspects of their expression may also be important. In general, when transgenic plants are produced by a direct DNA transformation system, more screening has to be done to select lines that contain a limited number of integrated copies. In most cases it will not be possible to separate these copies by crossing because they will often be tightly linked. The transgenic plants so obtained are self-fertilized. DNA introduced by methods other

than *Agrobacterium-mediated* transformation integrates into the genome by as *yet* unknown mechanisms. Generally the DNA integrates at a single locus, but it usually consists of rearranged multimers of the donor DNA. Molecular evidences (e.g. PCR analysis, Southern hybridization) are essential to confirm integration of transferred genes. Seeds are analyzed in the T1 (transgenic first) generation for segregation data. The simple pattern of inheritance (3:1) indicates a single site of insertion and this can be confirmed by Southern blotting. The copy number is usually low, between one and five copies per cell. The DNA is transmitted through meiosis as a simple Mendelian trait. Occasionally, however, more complex patterns of integration are seen.

In order to apply gene technology successfully to modern agriculture, it is essential to understand transgene expression. Sometimes when a transgene is introduced into an organism it may not show its expression. This is known as gene silencing.

11. Gene silencing

Partial or complete inactivation of gene(s). is known as gene silencing. Presence of multiple copies of a transgene in a plant nucleus can also lead to silencing of some or all copies of the gene(s) including the endogenous gene having homology with the transgene. It has also been established that in all such cases of transgene silencing, loss of expression of the transgenes is not due to the loss of these genes but due to their inactivation. Before considering the real causes of gene silencing it is must to know how a foreign DNA sequence is recognized by cell?

Detection of intrusive DNA: The ability to recognize "self" from "non-self" is a characteristic feature that occurs at cellular level. The observations from genetically engineered plants suggest that an ability to recognize "self" from "nonself" exists at the nucleic acid level. The possible mechanisms by which intrusive DNA is detected by cell are (i) Foreign sequence has different base sequence composition from that of endogenous chromosomal environment, (ii) Gene transfer via direct DNA delivery method has no control on copy number. These copies may form independent domains in the cell and work independently, with the result that they get recognized by genome scanning machinery, (iii) Every cell has its own modification and restriction

system that shows xenophobic effect, which does not allow the cell to contaminate its chromosomal environment by any foreign sequence.

Genomes are made of isochores that are very long stretches of DNA with high compositional homogeneity, On this basis, a concept of gene space has been given, According to this concept, if a GC-rich transgene is integrated in to a GC-rich gene space (GC isochore) or an AT-rich transgene is integrated into the AT-rich gene space (AT isochore), it is normally transcribed.

But if the GC-rich transgene gets integrated into the AT-rich gene space or vice versa, it is inactivated, as there is no compositional homogeneity with neighboring sequence A powerful initial response by the cell for the presence of foreign DNA may be fragmentation of invasive DNA via the action of cytoplasmic nucleases.

Causes of gene silencing: Silencing of a gene can be complete or partial. It may occur at transcriptional or translational level. After the entry of transgene into a receptive genome, there is an increase in methylation pattern. This is a major cause of gene silencing, leading to inactivation of gene(s). There are various cause of gene silencing.

1. *DNA methylation:* The inactivation of transgene is often accompanied by an increase in DNA methylation, and inactivation very frequently correlates with number of copies of integrated transgenes. When foreign DNA is introduced in plants, the modification and restriction system of cell causes methylation of foreign sequence to make it inactivated: DNA methylation is imposed on cytosine residues within symmetrical target sequences. In plants an inverse correlation between gene' transcription and cytosine methylation has been observed for certain controlling elements as methylated DNA prevents transcription by directly hindering. Transacting factors and basal transcription machinery accessibility. The presence of methylated cytosine residue in the opposite strand provides the information to methylate C-residue in newly synthesized complementary strand with the help of the enzyme methyl transferase and S-adenosyl methionine as a cofactor. On the basis of DNA methylation, gene silencing may be (i) transcriptional silencing, which is linked to

methylation of promoter region; (ii) post-transcriptional silencing, which involves inactivation of the coding sequence by methylation.

Induction of *de novo* methylation of transgenes involves different modes:

i) *DNA methylation* Via *DNA-DNA pairing:* When multiple transgene copies integrate as a concatamer and DNA coiling occurs, then these copies come in front of each other just like homologous sequences. Due to their mutual suppressing effect, there is increase in methylation pattern that leads to inactivation of genes.

ii) *Transgene recognition:* The transgene gets integrated properly, but due to older age of transgene(s) or certain environmental stresses the transgene get hypermethylated and inactivated.

iii) *Insertion into hypermethylated genomic regions:* When a transgenegets integrated into a hypermethylated region, then there may be spreading of hypermethylation pattern that leads to inactivation of transgene.

2. *Homology-dependent gene silencing.* Not only did homologous sequences affect the stability of transgene expression but also the activity of endogenous genes could be altered after insertion of transgene into genome. This homology-dependent inactivation may involve different modes:

i) *Inactivation of homologous transgenes:* When transgenic plants were retransformed with constructs that are partly homologous with the integrated transgene. In the presence of second construct, the primary transgene becomes inactivated and hypermethylated within the promoter region. Silencing is influenced by length of homology, Minimum length of homology reported between two homologous sequences for inactivation is 90 bp. It is also influenced by position of interacting sequences (linked copies are more efficiently silenced).

ii) *Paramutation:* It is interaction of homologous plant alleles that leads to heritable epigenetic effects, Thus, combination of two homologous alleles that differed in their state of methylation results in paramutation phenomenon.

iii) *Cosuppression:* Expression of endogenous genes can be inhibited by the introduction of a homologous sense construct that is capable of transcribing mRNA of same strandedness as transcript of the gene. In co-suppression, there is suppression of both the transgene and homologous resident gene or inactivation of either of two. This

phenomenon was first described in Petunia when a chalcone synthase gene *(chs)* involved in floral pigment biosynthesis was introduced in a deep violet flowering line of *P. hybrida*. Forty-two percent of the transgenic plants had white or variegated flowers. It was shown that cosuppression was due to a post-transcriptional event, and was independent of promoter, which was necessary for many different transgenes, and a single transgene can cause cosuppression of two or more endogenous genes.

3. *Suppression by antisense genes:* It is post transcriptional inhibition of gene expression. Introduction of antisense and sense transgenes into plants has been widely used to generate mutant phenotype. Since 1980, a large number of transgenic plants containing antisense and sense genes have been generated. One of the first successful applications of antisense gene approach was the inhibition of fruit softening by antisense polygalacturonase gene, which lead to first commercial transgenic tomato "Flavr Savr" variety.

However, it is of major concern that antisense RNA may block RNA production along different pathways. Antisense gene often leads to reduced levels of target mRNA and can potentially form a double-stranded structure with complementary mRNAs. Antisense RNA may interfere with the following processes:

i) Antisense transcripts affect the target gene directly in the nucleus, thereby preventing synthesis of mRNA. i.e. transcription.

ii) Antisense RNA may block processing of mRNAs by masking the sequences recognized by splicing and the polyadenylation apparatus. Antisense RNA may disturb the normal transport of mRNAs out of the nucleus by forming a hybrid with their target mRNAs and disturbs the regular flow of transcripts.

iii) Many antisense RNAs complementary to ribosome binding site have been shown to inhibit translation initiation.

iv) Antisense RNA prevents the accumulation of target mRNA. sense and antisense RNAs form. a double RNA intermediate that is rapidly degraded by ds RNA specific ribonucleases.

4. *Position effect* Whenever a transgene gets integrated into an improper region (hypermethylated, heterochromatic, telomeric, compositionally different genomic region), due to suppressing effect of the adjacent region or environment, it gets

inactivated.

5. *Increased copy number.* A correlation' between number of integrated copies and the frequency of inactivation is well documented for copies arranged in *cis* position. A reduction of copy number inside a locus was shown to increase gene expression or decrease the suppressing effect. The multiplicity does not need to include the whole gene. It may be either duplication of promoter or addition of a truncated transgene-coding region that can cause decreased expression. Increased copy number may be due to direct gene transfer methods as there is no control on copy number.

Strategies for avoiding gene silencing:

To obtain stable expression and inheritance of transgenes in genetically transformed plants, the following criteria should be considered.

i) Gene silencing is frequently observed upon integration of complex inserts. Thus it is better to opt for integration of single of inserts of transferred gene without duplication in the form of tandem or inverted repeats and consisting of single unique elements. This can be achieved by using the vector mediated gene transfer method, which generally introduces one copy (rarely two copies) in the cell.

ii) Irrespective of exact mechanism, homology at DNA or RNA levels seems to stimulate silencing events. If homology can be avoided or length and degree of homology should be controlled by interrupting perfect homology with mismatch or intron sequences.

iii) Integration of single-copy, unmethylated sequences of plant genome may increase the probability for continuous stable expression as the structure of integrated DNA itself, the environment of insert, may influence the stability of gene expression.

iv) Gene silencing occasionally becomes evident only after transmission of the gene to next generation. In some cases transgene expression decreased progressively over subsequent generations. Therefore continuous monitoring of expression levels in progeny of even well-established transgenic lines might be a precaution against unexpected epigenetic modifications (Chawla, 2004).

It is also necessary to arrange for the gene product to appear in the correct subcellular location. Also, it may be acceptable to have nuclear-encoded' genes that

are involved in functions such as detoxification or resistance switched on all the time in some situations. Sometimes, however, it is desirable to ensure the expression of a foreign gene at a particular developmental stage such as flowering, grain filling, during seed development, tuber formation, fruit ripening, or in response to environmental signals. Once the gene is integrated into the genome, DNA is usually stably maintained.

12. Conclusion

Improvements in the DNA delivery systems will assist in increasing the frequency of stable transformation and promote utility of such systems in the industry. One clear example of this is the recent production of stable transgenic maize. Direct gene transfer methods are most commonly used to transform monocotyledonous crops, such as cereals. Direct gene transfer methods and *Agrobacterium* mediated methods have their own advantages and disadvantages. Despite any problems, improvement in plant transformation technologies, especially when coupled to an efficient plant regeneration protocol, have seen crop species that can be routinely transformed. Crops that were once considered difficult to transform (cereals may fall in this category) are now routinely transformed in many laboratories around the world. These plant transformation technologies provide the basis for the advances in plant biotechnology.

13. DISCUSSION

1. Is there any problem in using silicon carbide fibers?

Yes, they are found to be carcinogenic and so it is replaced by other materials like ground glass wool fibres.

2. What do you mean by multiple copies?

In plants, Gene transfer leads to nonhomologus integration into the chromosome, and is characterized by multiple copies and some degree of rearrangement. Thus a single cell contains more than one copy of same gene.

3. Is there any substitute for gold in biolistic method?

A variety of metal particles like tungsten, platinum, palladium, rhodium, iridium can be used with diameter of 1.0-1.5µm. These are chemically inert materials.

4. Any difference in function of stationary and other type of gene gun?

The latest version of Helios gene gun is portable and is easy to handle. It also works with the help of helium gas.

5. What is the fate of transferred gene?

Instability of transgene expression is a problem encountered in many experiments involving transgenic plants, and is often referred to as 'gene silencing'.

6. How can we avoid gene silencing?

Gene silencing cannot be predicted. However we can reduce it by avoid using promoters and transgenes with high degree of similarity. Also, avoid the use of multiple copies of same promoter or terminator.

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14. ABSTRACT

Introduction of DNA into plant cells without the involvement of a biological agent and leading to stable transformation is known as direct gene transfer. Development of a genetic transformation system for crop plants has become an important tool in the study of basic plant processes and crop improvement. Plant transformation depends on the stable introduction of a foreign gene into the genome of the plant (Slater *et al.*, 2003). Various methods (direct and indirect) have been developed to achieve gene transfer. The major direct gene transfer method includes Biolistics and Electroporation.

The spontaneous uptake of DNA by plant cells is quite low. Therefore, different physical and chemical treatments are employed to facilitate the entry of DNA into plant cells. Monocotyledonous plants are generally more recalcitrant to genetic transformation than dicotyledonous species (Vain *et al.*, 1995). The absence of reliable *Agrobacterium*-mediated (indirect) transformation methods and the difficulties associated with the culture of monocotyledonous tissues *in vitro* are mainly responsible for the adoption of direct gene transfer techniques.

The traditional strength of many laboratories in plant tissue culture has facilitated a successful transition to plant genetic engineering. Crops that were once considered impossible to transform are now routinely transformed in many laboratories around the world (Veluthambi *et al.*, 2003). Through direct gene transfer methods stable transgenics were produced in major food crops such as maize, rice and wheat.

CHLOROPLAST ENGINEERING

By

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(2005-11-143)
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SEMINAR REPORT

Submitted in partial fulfillment for the requirement of the course
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CENTRE FOR PLANT BIOTECHINOLOGY AND MOLECULAR BIOLOGY
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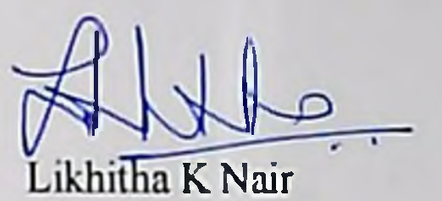
DECLARATION

I, **Likhitha K Nair** (2005-11-143), hereby declare that this seminar report entitled **"CHLOROPLAST ENGINEERING"** has been prepared by me, after going through the various references cited at the end of the report and has not been borrowed from any of my fellow students.



Vellanikkara

Likhitha K Nair

(2005- 11- 143)

CERTIFICATE

Certified that the seminar report entitled **"CHLOROPLAST ENGINEERING"** for the course PBT 651 has been prepared by Likhitha K Nair (2005 – 11-143) after going through the various references cited here under my guidance and supervision, and she has not borrowed from any of her fellow students.

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INDEX

INDEX OF FIGURES

LIST OF TABLES

Introduction

Increasing production of cereal crops, oilseed crops, and horticultural crops is becoming more important than ever before owing to increasing population and changing food habits of the people. The modern tools of biotechnology are being exploited to the fullest throughout the world for developing transgenic crop plants with improved traits. Already about 20 countries in the world are growing transgenic crops, predominantly with traits such as herbicide resistance and resistance to insect pests. However there is a constant fear of pollen flow from these transgenics to the neighbouring non – transgenic fields or to their wild or weedy relatives. It has become a matter of great concern to all biotechnologists to develop alternative strategies to prevent the pollen flow (Bansal and Sharma, 2003)

The best alternative strategy available today to circumvent the nuclear transformation associated bottlenecks in developing tenable crop transgenics is the Chloroplast Engineering or Chloroplast mediated Genetic transformation technology. This technology allows the introduction and insertion of foreign genes into the chloroplast genome, called plastome. Chloroplast transformation in higher plants is an extremely attractive approach for the development of transgenic traits that may be difficult to achieve via nuclear transformation (Grevich and Daniell, 2005)

Definition

Chloroplast engineering is the process of incorporation of foreign DNA in the plastid genome by the plastid homologous recombination machinery (Gupta, 2004). Chloroplast transformation utilizes two targeting sequences that flank the foreign genes and insert them through homologous recombination at a precise, predetermined location in the chloroplast genome resulting in uniform transgene expression among transgenic lines and eliminates the position effect often observed in nuclear transgenic plants. Such transgenes are referred to as transplastomics or those carrying the transgenic plastids.

Chloroplasts

Chloroplasts are the site for photosynthesis in plants mostly present in leaves. In general, called plastids, they are also present in other parts of the plant

like chromoplasts in coloured leaves and fruits, amyloplasts in starch storing organs, leucoplasts in roots and etioplasts in dark – grown seedlings. The chloroplasts arise either by division of an existing plastid or from proplastids. Proplastids are the undifferentiated form of plastids present in most plant cells. Plant cells contain DNA in three sub cellular compartments. Approximately 80% of the DNA is located in the nucleus as chromosomes, 10-20% in chloroplasts and around 1% in mitochondria. There are approximately 10-100 chloroplasts per cell. The DNA present in chloroplasts is double stranded, circular and contains about 100 genes. Around 500-10,000 copies of these chloroplast DNA are present per cell. The size of the genome varies from 120-200 Kbp depending on species (Ignacimuthu, S.1997)

The chloroplast is a suitable location for a wide range of foreign genes including those involved in photosynthesis, starch synthesis, fatty acid synthesis, oxidative stress tolerance and those conferring tolerance to herbicides. Moreover chloroplasts are useful compartments for storing polymers and pharmaceuticals (Bogorad, 2000)

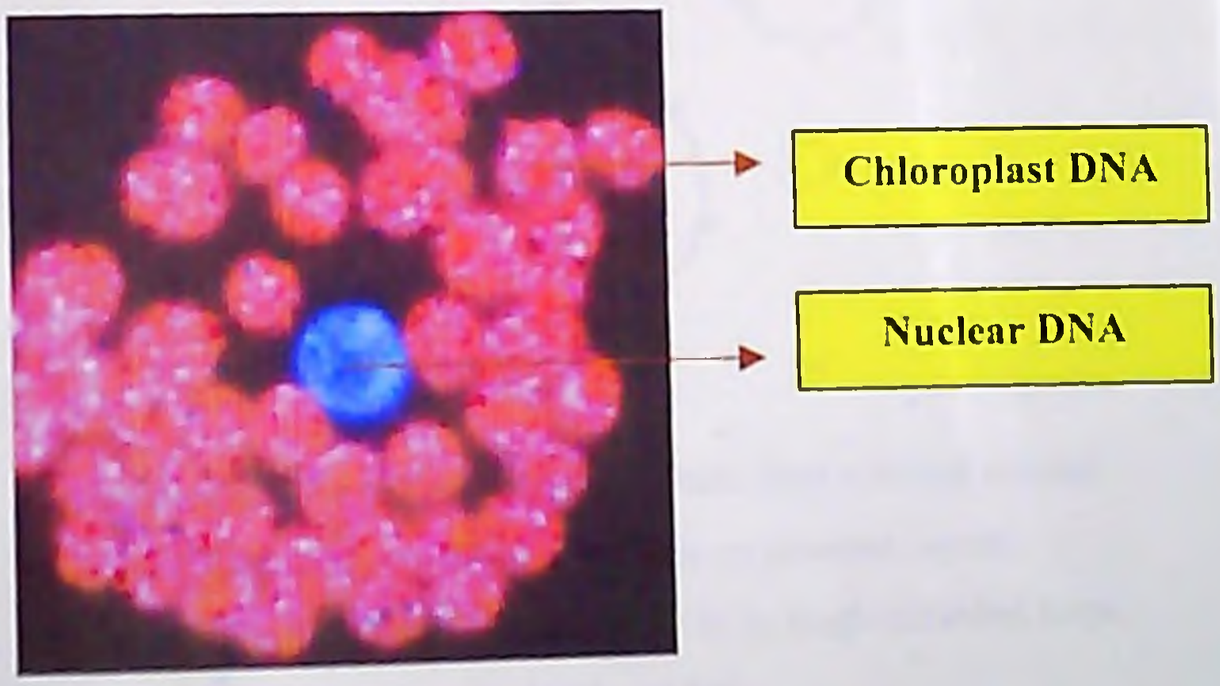

Fig 1. Chloroplast and nuclear DNA under a microscope

Origin of Chloroplasts

Chloroplasts in plants are actually second hand chloroplasts as they are derived from algae. Euglena was engulfed by a eukaryotic phagotroph and the engulfed cell underwent drastic reduction leaving behind residues called

chloroplast and other extra membranes created by engulfment. The bacteria become the chloroplast. Gene loss occurs during the transfer between the chloroplast and the nucleus. Lateral transfer of chloroplasts into non-photosynthetic lineages took place through secondary endosymbiosis leading to the present day green plants and red algae.

Structural features of Chloroplast

Chloroplast is enclosed by two concentric membranes – the inner and the outer. The inner membrane together with the thylakoids is the photosynthetic site. Thylakoids are developed by invagination and subsequent pinching of the inner membrane. The most notable feature of chloroplast is the presence of an inverted repeat that imparts physical stability to the genome.

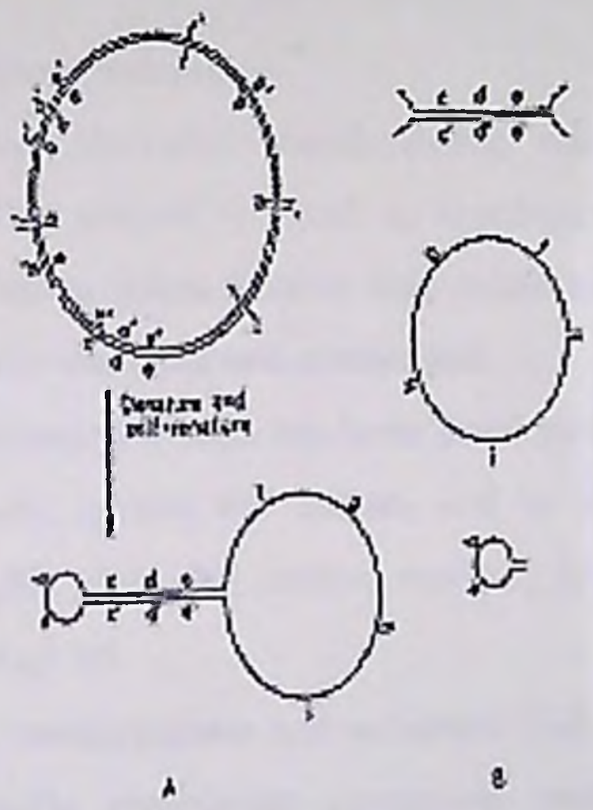

Fig. 2: (A) Illustration of a self-renatured molecule from a nicked circular chloroplast DNA molecule containing an inverted repeat.
(B) Duplex region (Top), and small and large single stranded loops (bottom) from broken molecules in (A).

Inverted Repeats

One unit is composed of two sequences, usually about 20 – 25 Kb in size, and contains the genes for chloroplast ribosomal RNA. An inverted copy

occurs in most chloroplast genomes, with the exception of genomes of certain legumes, including peas, broad beans and alfalfa. This portion in the plastid genome has been duplicated and is present in an inverted orientation in the plastid DNA molecule. This region separates a small single-copy region from a large single-copy DNA region

Chloroplast genome exhibits most of the prokaryotic features like,

1) Absence of introns
2) Genome organization, transcription and translation machinery similar to that of prokaryotes
3) mRNA produced from chloroplast genes are not usually polyadenylated and no transport required as mRNA produced in the same compartment of ribosomes on which it is to be translated.

History of Chloroplast transformation

A method of stable chloroplast transformation was developed first in tobacco (Maliga, 2002). This method is based on bombardment by the particle delivery system of whole leaves (taken from *in vitro* raised tobacco plants) by the plasmid DNA that is specially designed and constructed.

Chloroplast transformation system has been developed only in a few dicot solanaceous species, tobacco, potato and tomato, and in *Arabidopsis thaliana*. Prior to this report in a higher plant, this method was only available in unicellular alga, *Chlamydomonas reinhardtii*.

Stable chloroplast transformation was achieved (Sidorov *et al.*, 1999) in potato. Two tobacco-specific chloroplast expression vectors were used for integration of the transgene into the large single copy and inverted repeat region of the plastid genome of potato.

This was followed by tomato (Ruf *et al.*, 2001). Stable genetic transformation of tomato led to an unprecedented high-level accumulation of a foreign protein.

Introduction of a native Bt gene, Cry 1ac into tobacco chloroplasts (McBride *et al.*, 1995). Instant amplification of the unmodified bacterial gene resulted in the production of high level (3-5%) of the protoxin in tobacco leaves.

Overexpression of Bt cry2Aa2 operon in tobacco chloroplasts (DeCosa *et al.*, 2001) and demonstrated Bt protoxin accumulation to a level of 45.3% of the total soluble protein in leaves.

Herbicide resistant transplastomic tobacco developed (Daniell *et al.*, 1998) by integrating into the chloroplast genome petunia EPSPS gene encoding the enzyme 5-enol-pyruvyl shikimate-3-phosphate synthase.

Why Engineering Chloroplasts?

1) Chloroplast genomes are inherited maternally in most angiosperms Pollen does not contain plastids of any sort therefore chloroplast genes are only transmitted through the egg to the embryo. Maternal inheritance of novel genes is highly desirable in situations where out-crossing between crops and weeds or among crops is a concern. The risk of transgene escape will be rare if the gene of interest is inserted into the chloroplast genome (Daniell *et al.*, 1998).

2) Very high levels of transgene expression have been observed. Higher levels of expression of insect and herbicide resistance were observed in transplastomic plants than in plants containing transformed nuclei. Chloroplasts are ideal expression factories for high-yield protein production with up to 40% of the soluble protein in the cell being the target protein (De Cosa *et al.*, 2001). Chloroplasts can express bacterial genes better than plant nuclei as transcription and translation in chloroplasts is prokaryotic in nature. The high level of transgenic products is attributed to the fact that there are many chloroplasts within a plant cell whereas there is only one nucleus. DNA inserted into chloroplasts is copied 5,000 to 10,000 times and is only copied 1-4 times in the nucleus.

3) Transgene expression is more stable in transplastomic plants than in nuclear transformants. Nuclear transformation in plants occurs by the random integration of transgenes into unpredictable locations in the genome by non-homologous recombination and can result in varying levels of expression and in some cases, gene silencing. Transgenes are

integrated into chloroplast genomes by homologous recombination and are not affected by gene silencing. All transplastomic plants in an experiment should be genetically and phenotypically identical (Daniell *et al.*, 2005).

4) Chloroplasts have the capacity to express multiple genes from a polycistronic mRNA. This allows the pyramiding of genes to decrease the risk of promoting resistance in pest organisms.

Factors for Successful transformation

A. Introduction of transgenes by the biolistic process, followed by insertion of foreign genes by two homologous recombination events via flanking plastid "targeting" sequences (Daniell, 1999).

B. Efficient homologous recombination to incorporate the transforming DNA, success in selective amplification of the transformed plastid genome copies, and attainment of a homoplastomic state in calli before plant regeneration in culture.

Methods for Chloroplast Transformation

Biolistics

Chloroplast transformation was considered to be virtually impossible because the double membrane is a physical barrier and there are no viruses or bacteria known to infect chloroplasts that could be used as a vector for gene transfer. The invention of gene gun and biolistic technology provided the opportunity to introduce foreign DNA into living cells (Klein *et al.*, 1987). Tungsten or gold particles coated with plasmid DNA are applied onto the surface of a plastic disk. The DNA-loaded particles are forced through a metal grid and penetrate the target tissue. After biolistic bombardment, the leaf is cut into small pieces which are placed on a selective regeneration medium. Selection is carried out for 4-6 weeks and the remaining cells with transformed and thus spectinomycin resistant chloroplasts will form small green calli which eventually will give rise to antibiotic-resistant shoots.

The first successful chloroplast transformation by this method was reported by Boynton and Gillham (1988) in *Chlamydomonas reinhardtii* and the first successful chloroplast transformation in a higher plant was carried out by Svab *et al.* (1989) to introduce spectinomycin resistance into tobacco. Reciprocal crosses between the transplastomic and wild type plants showed that the introduced antibiotic resistance was maternally inherited.

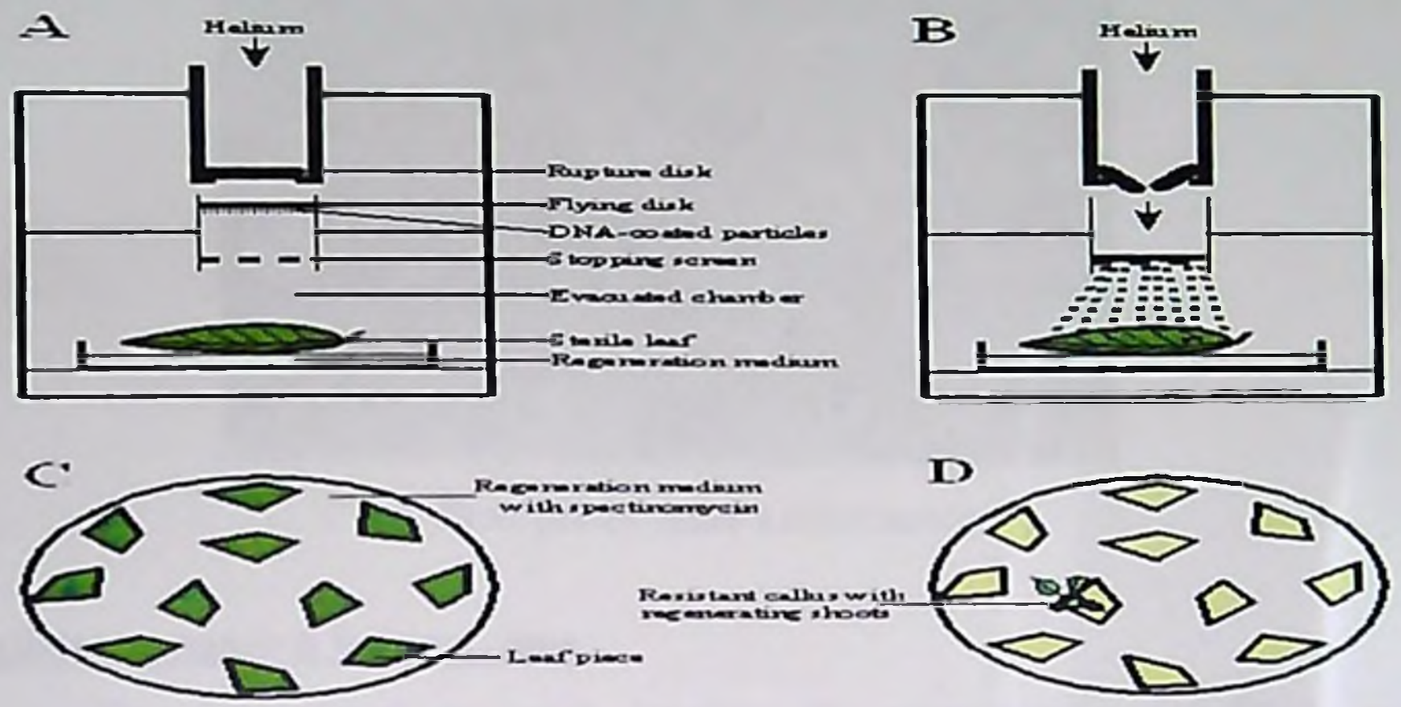

Fig 2. Schematic representation of biolistic transformation

Advantages:

(a) High efficiency rate.

(b) Rapid regeneration of transformed tissue.

(c) Allows the use of a variety of explants.

Disadvantages:

(a) Very expensive to perform.

(b) The target tissue must be regenerable.

PEG - mediated transformation

This approach requires the preparation of protoplasts and the target species used must be regenerable from protoplasts. Protoplasts take up DNA in the presence of PEG (Polyethylene glycol) and changes in the plasma membrane allow

DNA to penetrate and move into the cytoplasm. The foreign DNA is transported by some unknown means from the cytoplasm into the chloroplast where it may be integrated into the genome. It is less efficient than biolistic transformation.

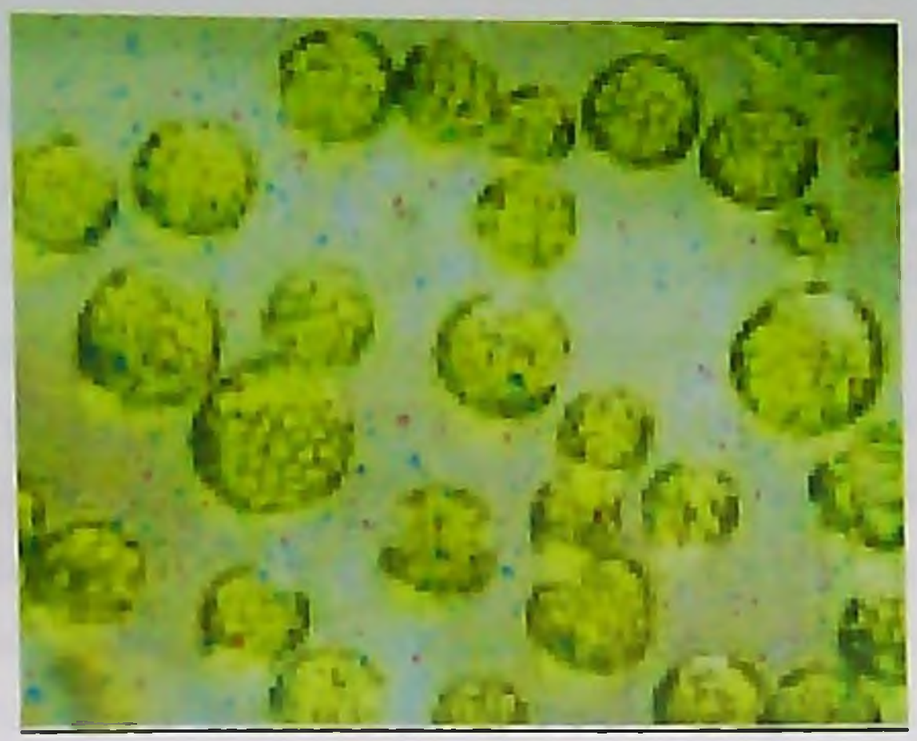

Fig 3. Protoplasts under a microscope

Galistan expansion femtosyringe

This is a novel approach involves the microinjection of DNA into chloroplast (Knoblauch *et al.*, 1999) and is not widely used. The heat induced expansion of a liquid metal, Galistan, within a glass syringe forces the transformation plasmid DNA through a capillary tip with a diameter of approximately 0.1 mm.

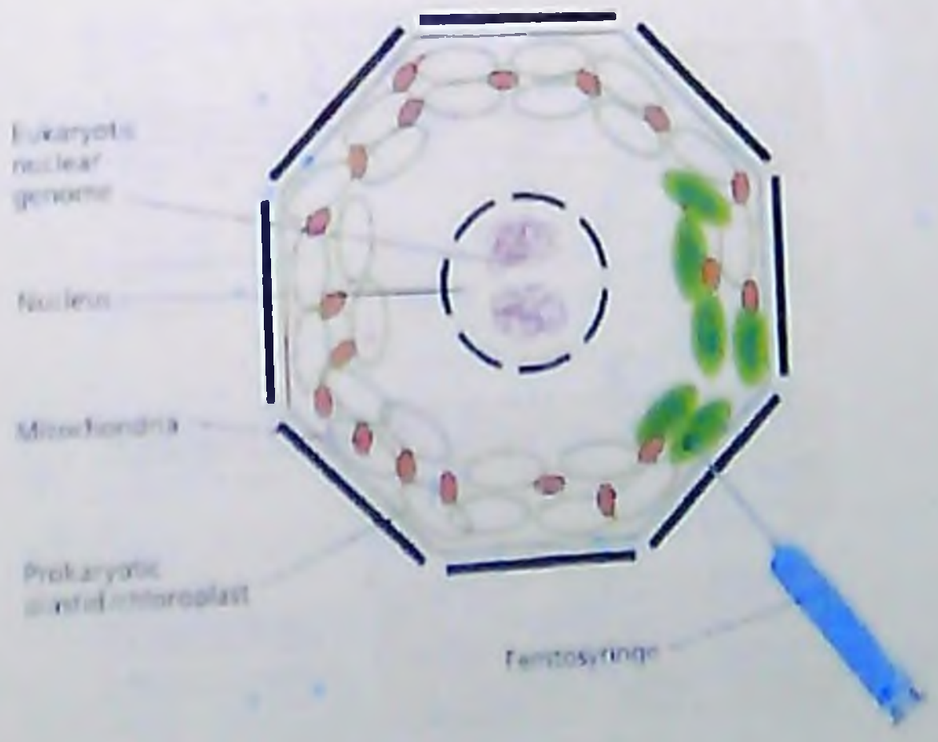

Fig 4. Galistan expansion femtosyringe approach

Incorporation of Transforming DNA into the Chloroplast Genome

Primary chloroplast transformation events involve the transformation of only one or a few genome copies within a single plant cell resulting in cells that contain a mix of transformed and wild type chloroplast genomes. The cells are referred to as heteroplastomic and are genetically unstable. Heteroplasmy falls into two categories, interplastidic and intraplastidic. Interplastidic Heteroplasmy is where a cell contains chloroplasts with wild type genomes and chloroplasts with transformed genomes. Intraplastidic Heteroplasmy is where wild type and transformed genomes are located within the same chloroplast. Usually heteroplastomic cells are resolved spontaneously to a homoplastomic condition where the chloroplasts are all transgenic or all wild type. This is achieved by random genome segregation during chloroplast division and random chloroplast segregation during cell division. Homoplasmy can be achieved in chloroplast transformation studies by allowing for a sufficient number of cell divisions under high concentrations of the selection agent, usually spectinomycin. Plantlets go through a series of regeneration and selection steps with spectinomycin. Interplastidic Heteroplasmy is more likely to disappear rapidly as chloroplasts containing only wild type genomes are sensitive to the selection agent and will not survive. Intraplastidic Heteroplasmy is more difficult to eliminate as the spectinomycin resistance gene functions as a dominant selectable marker and only few copies are sufficient to confer resistance. Homoplastomic transgenic shoots are typically obtained after 2-4 regeneration cycles under high selection pressure.

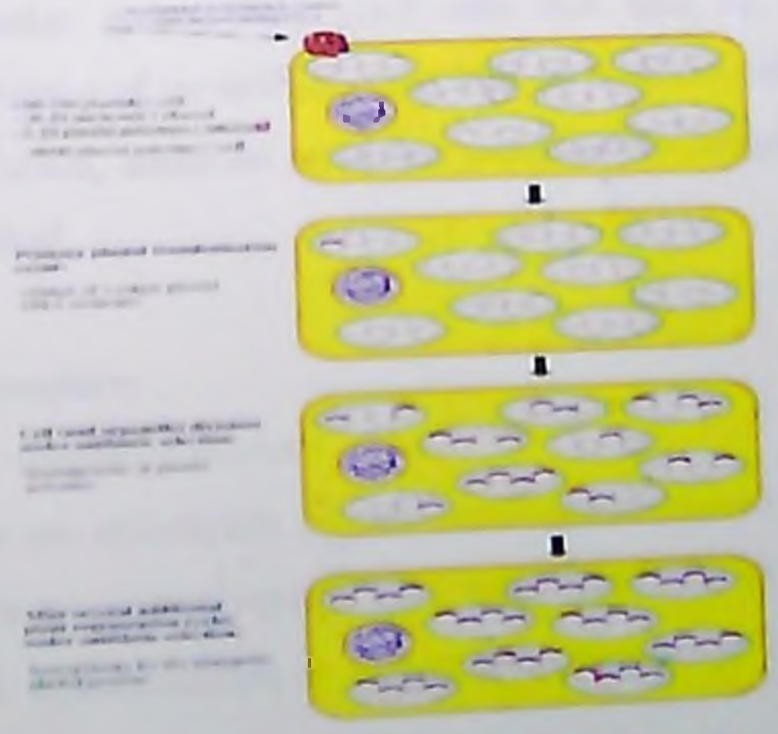

Fig. 5: Production of Homoplastomic cells

Vectors for Chloroplast Transformation

Stable chloroplast transformation depends on the integration of the foreign DNA into the chloroplast genome by homologous recombination, therefore the foreign gene that is being introduced must be flanked by sequences homologous to the chloroplast genome (Staub and Maliga, 1992). Greater than 400 bp of homologous sequence on each side of the construct is generally used to obtain chloroplast transformation at a reasonable frequency. Chloroplast genes are transcribed by chloroplast-specific promoters and use chloroplast-specific termination signals. Most chloroplast genes are transcribed as operons. This allows two open reading frames to be inserted into a vector in sequence under the same promoter. The selectable marker and the gene of interest are placed between the promoter and the terminator which are flanked by the 5' and 3' untranslated regions.

Screening of transformants

Antibiotic resistance genes are common components of gene transfer technologies. Transgenic plants are usually made by transferring one or two genes, commonly from another plant or bacterium, into chromosomes located in the nucleus of plant cells. Once transferred, these foreign genes are inherited along with the 25,000 to 50,000 native genes present on plant chromosomes. Gene transfer methods are inefficient and only a tiny proportion of cells usually take up foreign genes. To identify cells that take up foreign DNA, a foreign marker gene that confers a selectable property is required. These selectable marker genes are added along with genes of interest. Antibiotic resistance genes are one of the most commonly used marker genes. Only plant cells that take up foreign genes proliferate in the presence of an antibiotic that kills unmodified cells. Once these plants have been selected, antibiotic resistance genes are no longer required, but they are usually retained.

Types of Selection markers

1. Antibiotic resistance markers

These are chloroplast specific antibiotic resistance marker, aadA-conferring resistance to aminoglycoside type antibiotics such as spectinomycin (Svab *et al.*, 1990). Spectinomycin is mainly used because it is a prokaryotic

translational inhibitor and has little effect on plant cells. Spectinomycin is the most effective selectable marker used in chloroplast transformation. The neomycin phosphotransferase (nptII) gene, which confers kanamycin resistance, may also be used for chloroplast transformation.

2. Others

Other selectable markers are being studied as an alternative to antibiotic resistance genes as there is a risk of transferring antibiotic resistance to microbes in the soil or in the gut. The betaine aldehyde dehydrogenase (BADH) gene produces an enzyme that converts toxic betaine aldehyde to non-toxic glycine betaine and was an effective selectable marker in tobacco. Green fluorescent protein isolated from jellyfish has also been reported to function as a marker in chloroplast transformation.

Marker free transgenics

A consequence of placing a transgene in the chloroplast genome is that the antibiotic resistance genes used as selectable markers are highly amplified. Engineering genetically modified (GM) crops without the use of antibiotic reistance genes should eliminate the potential risk of their transfer to the environment or gut microbes. The betaine aldehyde dehydrogenase (BADH) gene from spinach was used as a selectable marker. The selection process involves conversion of toxic betaine aldehyde (BA) by the chloroplast BADH enzyme to non-toxic glycine betaine, which also serves as an osmoprotectant. Chloroplast transformation efficiency was 25-fold higher in BA selection than with spectinomycin. In addition rapid regeneration was obtained. Transgenic shoots appeared within 12 days in 80% of leaf disks under BA selection compared to 45 days in 15% of disks under spectinomycin selection (Daniell *et al.*, 2001). Southern blots confirmed stable integration of foreign genes into all of the chloroplast genomes resulting in Homoplasmy. Transgenic plants were morphologically indistinguishable from untransformed plants and the introduced trait was inherited stably in the subsequent generation.

Plant	Selection marker	Reference
Tobacco	Spectinomycin	Svab and Maliga, 1990
Potato	Spectinomycin	Sidorov *et al.*, 1999
Tomato	Spectinomycin	Ruf *et al.*,2001
Arabidopsis	Spectinomycin	Sikdar *et al.*,1998
Rice	Streptomycin	Khan and Maliga, 1999
Brassica napus	Spectinomycin	Hou *et al.*, 2003
Soybean	Spectinomycin	Dufourmantel *et al.*, 2004
Carrot	Betaine aldehyde	Kumar *et al.*, 2004
Cotton	Kanamycin	Kumar *et al.*, 2004
Petunia	Spectinomycin/ gusA	Zubko *et al.*,2004

Table 1. Plastid transformation in plants

Engineering of Chloroplast Genome in *Chlamydomonas*

Chlamydomonas has been a particularly favourable organism for chloroplast transformation for the following reasons:

(i) It has a single large chloroplast.

(ii) Photosynthetic mutants are viable, if reduced carbon is provided in the form of acetate.

Two different methods have been successfully utilized:

(a) bombardment of cells with DNA-coated tungsten particles.

(b) agitation of cells in the presence of glass beads and DNA.

Chloroplast transformation requires suitable selectable markers (Blowers *et* al., 1989). The markers include:

(1) Spectinomycin resistance ($spec^R$) and streptomycin resistance (str^R), encoded by 16S rDNA.

(2) Erythromycin resistance encoded by 23S rDNA.

(3) atpB gene (coding for a subunit of chloroplast ATPase) essential for photosynthesis.

Applications of Chloroplast Engineering

Herbicide resistance
Insect pest resistance
Improved plant productivity (CMS)
Production of vaccines and recombinant proteins
Phytoremediation
Tolerance to abiotic stress (drought, salt).

1. Herbicide resistance

Glyphosate resistance

Glyphosate is a potent broad-spectrum herbicide that is effective in the control of most grasses and broadleaf weeds. This chemical affects the aromatic amino acid biosynthetic pathway in plants and microorganisms and is, therefore, not toxic to animals (Robert and Baumann, 1998). Glyphosate is inactivated rapidly in the soil leaving no harmful residues. Roundup Ready products contain a glyphosate resistance gene in the nuclear genome, but the gene can be transmitted via pollen to weedy relatives and non-transgenic crops of the same species. Daniell et al. (1998) successfully transformed a gene

conferring glyphosate resistance into the tobacco chloroplast genome. The transplastomic tobacco plants were able to survive on ten times the normally lethal concentration of glyphosate. The gene is contained within the chloroplast and will not be transmitted via pollen to other plants.

2. Insect resistance via Bt toxin

Bacillus thuringensis (Bt) toxins are harmful to insects when ingested. Hyper-expression of Bt toxins in transformed tobacco chloroplasts that resulted in a high insect mortality rate. The advantage of this application is that the toxins are located in green leaf tissue, which is the most likely part of the plant to be consumed by insects. There is an added advantage that insecticidal proteins are not produced in fruit or pollen hence the proteins will not likely be consumed by humans or animals and the transgene will not be transmitted to other plants via pollen.

3. Engineering Cytoplasmic Male Sterility via the Chloroplast Genome

Cytoplasmic male sterility systems are used to produce commercial F1 hybrid lines. Ruiz and Daniell (2005) reported the first engineered cytoplasmic male sterility system in plants. They studied the effect of light regulation of the phaA gene coding for β-ketothiolase engineered via the chloroplast genome. The phaA gene was efficiently expressed in all tissue types examined, including leaves, flowers and anthers. The transgenic lines were normal except for the male sterile phenotype, lacking pollen. Transgenic lines show an accelerated pattern of anther development, affecting their maturation, and result in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium, and vacuolation decrease the inner space of the locules, affect pollen grain, and result in the irregular shape or collapsed phenotype.

Novel mechanism of Cytoplasmic Male Sterility

Polyhydroxybutyrate (PHB) synthesis takes place by the consecutive metabolic action of β-ketothiolase (phaA gene), acetoacetyl-CoA reductase (phaB) and PHB synthase (phaC). The phbA, phbB and phbC genes were introduced in individual nuclear *Arbidopsis* transgenic lines and reconstructed the entire pathway, targeting all enzymes to the plastids. This resulted in PHB expression up

to 14% leaf dry weight. With expression of optimized gene constructs, PHB yield increased up to 40% of leaf dry weight, but this was accompanied by severe growth reduction and chlorosis (Lossl *et al.*, 2003) indicating that targeting the PHB pathway to the chloroplast can result in pleiotropic effects, at higher concentrations of polymer synthesis. Continuous expression of the phbA gene led to a significant decrease in transformation efficiency, inhibiting the recovery of transgenic lines. The toxic effect exerted by phbA expression was the result of PHB biosynthesis intermediates or its derivatives, the depletion of the acetyl-CoA pool or of the interaction of β-ketothiolase with other proteins or substrates.

4. Production of Plantibodies

The efficient method of recombinant Human Serum Albumin (HAS) production is used as a model system to enrich or purify biopharmaceutical proteins from transgenic plants that are highly susceptible to proteolytic degradation. Expression of HSA in transgenic chloroplasts using Shine-Dalgarno sequence (SD), that usually facilitates hyper-expression of transgenes, resulted only in 0.02% HSA in total protein. Modification of HSA regulatory sequences using chloroplast untranslated regions result in hyper-expression of HSA (up to 11.1% total protein), compensating for excessive proteolytic degradation (Staub *et al.*, 2000).HSA is translated so rapidly using UTRs that aggregates are formed within transgenic chloroplasts and all such aggregates are in the pellet rather than in the supernatant when crude plant extracts are centrifuged.

5. Phytoremediation using Chloroplast Engineering

Mercury is highly toxic and bacteria biomagnify its toxicity through its conversion to methyl-Hg. Organomercurials are neurotoxins easily absorbed into blood and known to harm human beings and over 90% of it is absorbed into blood. In plants, the primary target of Hg damage is the chloroplast as it inhibits electron transport and photosynthesis. Novel phytoremediation approach via chloroplast genetic engineering was achieved by integrating the native bacterial merA and merB genes, which code for mercuric ion reductase and organomercurial lyase, respectively, into the chloroplast genome in a single transformation event. Stable integration of the merAB operon into the chloroplast genome was confirmed by

PCR and Southern blot analyses. Expression of the mer operon resulted in high levels of tolerance to phenylmercuric acetate (PMA) when grown in soil containing up to 400 μM PMA. Transgenic lines are able to accumulate very high concentrations of Hg in roots up to 3-fold higher than in the wild type (Ruiz *et al.*, 2001)

6. Recombinant protein production

Bock *et al* reported successful plastid transformation in tomato, achieving notable levels of protein accumulation from a plastid transgene in the tomato fruit. The transplastomic tomato fruit served as a useful system for production of edible vaccines. The approach used to generate transplastomic tomato plants was by altering the conditions for chloroplast transformation, using low-light conditions during the selection phase, extending the selection phase, focusing on smaller leaf pieces, and optimizing the selection and plant regeneration systems. In plants other than tomato and tobacco, this method has been only partly successful. A significant aspect of the transplastomic tomato is the level of protein accumulation in the fruit up to nearly 0.5% of the total soluble cellular protein. The protein is the marker gene product, aminoglycoside-3'-adenyltransferase (AAD), which confers spectinomycin reistance (Bock, R. and Khan, M.S.2004).Although plastid genes have a relatively low GC content, expression of a bacterial barnase gene (bar) with a relatively high GC content was not a problem. High level protein accumulation from the marker gene in the tomato fruit is good for those interested in protein expression, but bad for those who are planning to produce transplastomic crops.

Fig 8. Transplastomic tomato plants regenerated from homoplasmic callus tissue transferred on the surface of agar-solidified medium.

The selective marker may be removed after selection of the lines. The transformation and marker gene elimination is done separately using the Cre-*lox* plastid marker elimination system. In this approach, the marker gene (flanked by two directly oriented *lox* sites) and the gene of interest are introduced into the plastid genome in the absence of Cre activity. When elimination of the marker gene is required, a Cre gene is introduced into the nucleus that encodes a plastid targeted Cre site-specific recombinase, which then excises sequences between the lox sites (Iamtham and Day, 2000). Cre may be introduced by a second *Agrobacterium*-mediated transformation or by crossing. The nuclear Cre is subsequently removed by segregation in the seed progeny. This is the first report on the generation of fertile transplastomic plants in a food crop with an edible fruit (Ruf *et al.*, 2001)

7. Abiotic Stress tolerance

Drought tolerance

Trehalose is a non-reducing disaccharide of glucose and is found in diverse organisms including algae, bacteria, insects, yeast, fungi, animal and plants. It accumulates under various stress conditions such as freezing, heat, salt or drought. Trehalose protects against damages imposed by these stresses. Trehalose also accumulates in anhydrobiotic organisms that survive complete dehydration, the resurrection plant and some desiccation tolerant angiosperms (Lee *et al.*, 2003). Trehalose accumulation in nuclear transgenic plants resulted in the loss of apical dominance, stunted growth, lancet shaped leaves and sterility. In order to minimize the pleiotropic effects observed in nuclear transgenic plants accumulating Trehalose, the Chloroplast engineering compartmentalizes Trehalose accumulation within chloroplasts indicating that this organelle can be used as a repository like vacuole. Inhibition of trehalase activity is known to enhance Trehalose accumulation in plants.

Salt tolerance

Salt stress is a major abiotic stress in agriculture. The problem of salinity has been compounded by irrigation and excessive use of fertilizers. Carrot is classified as a salt sensitive plant and there is 7% growth reduction for every 10mM increment in salinity above 20mM salt. Plastid transformation carried out in

carrot using the BADH gene and they were able to grow in very high saline conditions (up to 400mM sodium chloride) which only halophytes could tolerate. Also high levels of foreign gene expression were achieved in the edible part (carrot roots).

Henry Daniell

Henry Daniell is Pegasus Professor and Trustee chair at the University of Central Florida. Daniell is recognized for his pioneering inventions on chloroplast genetic engineering.

Daniell Laboratory

1. Accumulation of Bt Cry2Aa2 protein at 46.1`% in transgenic chloroplasts and complete bacterial operon was successfully expressed for the first time.
2. Developed chloroplast genome derived CMS system layers destroyed in developing pollen.
3. Expression and accumulation of certain proteins or their biosynthetic products that would otherwise be harmful to plant if expressed in cytoplasm
4. Demonstrated expression and assembly of several vaccine antigens including Cholera toxin B subunit (CTB). F1-V fusion antigen for plague, anthrax protective antigen (PA) and NS3 protein as a vaccine antigen for hepatitis C.

New Company to make drugs from plants

A new biotechnology company has come up with a way to manufacture human proteins in plants by adding DNA to plant chloroplasts (Daniell *et al.*, 2002). Chlorogen, Inc., of Missouri produces drugs and vaccines using the chloroplast technology. The company's first commercial product is a human protein often used in blood transfusions, called human serum albumin. Chloroplast technology, pioneered by one of the company founders, Henry Daniell of the University of Central Florida in Orlando, is potentially a cheap, efficient and environmentally friendly way to produce proteins. An anthrax vaccine was developed. One plant can produce 400 million doses of anthrax vaccine and the

vaccine is free of any contaminants and human pathogens. The company uses tobacco plants for most proteins including interferon, insulin and human serum albumin.

Tobacco: An ideal crop for therapeutic proteins

Tobacco is a non-food and non-feed crop and an ideal choice for the production of therapeutic proteins because of its relative tractability to genetic manipulation. Tobacco is an excellent biomass producer (40 tons of leaf fresh weight/acre, based on multiple cuttings per season) and a prolific seed producer (up to one million seeds produced per plant), thus hastening the time in which a product can be scaled up and brought to market. Tobacco is widely used as a model system to test the suitability of plant based expression systems for production of therapeutic proteins and other transgenes.

Steroid controlled plant regeneration in *Arabidopsis thaliana*

Plastid transformation is tissue culture based, as gradual replacement of the thousands of wild-type plastid genome copies with transgenic ones can be best accomplished in the tissue culture environment. One bottleneck of plastid transformation in *Arabidopsis thaliana* is the difficulty to maintain the normal diploid state in a cell culture environment. Although most leaf cells become polyploidy, the diploid state is maintained in plants in the germline cells of embryos and the shoot apex. A tissue culture system was developed in which the embryogenic state of *Arabidopsis thaliana* cells is maintained by expressing Babyboom under inducible control. Babyboom is an *Arabidopsis* transcription factor regulating embryogenesis. The embryogenic cells form when the steroid hormone inducer is incorporated in the culture medium, but regeneration of normal plants can be readily obtained when the inducer is removed (Sikdar *et al.*, 1998)

Gene	Gene Product	Function	Reference
cry1Ac	Crystal toxin	Insect resistance	McBride *et al.*1995
cry2Aa2 operon	Crystal toxin	Insect resistance	Kota *et al.*1999
hST	Human Somatotropin	Human growth hormone	Staub *et al.*2000
cry2Aa2	Crystal toxin	Insect resistance	DeCosa *et al.*2001

Gene	Gene Product	Function	Reference
bar	Phosphinothricin acetyl transferase	Herbicide tolerance	Lutz *et al.*2001
BADH	Betaine aldehyde dehydrogenase	Drought tolerance	Daniell *et al.* 2001
ctxB	Cholera toxin beta subunit	Edible vaccine	Daniell *et al.*2001
EPSPS	5-enol-pyruvyl shikimate 3-phosphate synthase	Herbicide tolerance	Chin et al.2003
tps1	Trehalose phosphate synthase	Drought tolerance	Lee et al 2003
cry3Aa2	Crystal toxin	Insect resistance	Rooz and Bansal 2003

mer B	Organo mercurial lyase	Phytoremediation	Rooz and Bansal, 2003
phb operon	Polyhydroxybutyrate	Thermo plastic synthesis	Lossl et al.2003
Cod A	Choline oxidase	Drought and salinity tolerance	Bansal et al.

Table 2. Expression of useful foreign genes in chloroplasts

Advantages of Chloroplast transformation

1. High level uniform transgene expression
2. Multi-gene engineering in a single transformation event
3. Transgene containment via maternal inheritance
4. Lack of gene silencing
5. Position effect due to site-specific transgene integration
6. Lack of pleiotropic effects due to sub-cellular compartmentalization

Disadvantages

1. <u>Concomitant nuclear conundrum</u>

Although transgene constructs can be tailored for homologous recombination in the chloroplast genome, shooting transgenic DNA by gene gun into the plant cells does not specifically target transgenic DNA to the chloroplast genome. Much of the DNA ends up in the cytoplasm or the nucleus, where it may be available for integration into the nuclear genome.

The best way to determine the frequency of concomitant nuclear integration is through molecular analysis. The absence of signal in Southern blots shows that nuclear integration has not also taken place. But the method used was not sensitive enough to detect low-copy number integration into the nuclear genome.

2. Transformation frequencies are much lower than those for nuclear genes.
3. Prolonged selection pressures under high selection pressure are required for the recovery of transformants.

4. The method of transgene transfer into chloroplasts are limited, and they are either expensive or require regeneration from protoplasts.
5. The products of transgenes ordinarily accumulate in green parts only. As with any fresh tissue protein stability will change over time even with refrigeration.
6. Extraction and purification must be performed at very specific times following harvest

and large volume products and edible vaccines would not appear too feasible using

this system.

Challenges

1. The reliable performance of the transgenes over time.
2. Maintenance of transgenic- homoplasmic plants (where all the chloroplasts are transformed).
3. Well – designed and transparent scientific studies on the stability and ecology of transgenes are needed before considering large-scale release into the environment, and especially so for transgenes inserted into the chloroplast genome.
4. The inherent hyper-expressivity of the transgenes and the complexity of the regulation of chloroplast gene expression greatly increase the risks to health and the environment
5. Extending this concept to important crops.
6. Lack of 100% homologous species-specific chloroplast transformation vectors containing suitable selectable markers
7. Ability to regulate transgene expression in developing plastids and inadequate tissue culture systems via somatic embryogenesis.

Summary

Although generation of chloroplast transgenics is not routine yet in majority of agriculturally important crop plants, enough data have been generated on the fitness of the chloroplast transformation technology for the production of agronomically useful transplastomic plants. Infact, plant biotechnologists prefer incorporating particularly the resistance genes or the genes of bacterial origin into chloroplasts rather than nucleus. Besides exhibiting high level of resistance to

herbicides, the chloroplast transgenic plants are environment friendly Development of herbicide resistant plants through chloroplast transformation is an improvement over the existing technology of generating transgenic plants by which herbicide resistant genes are introduced into the nuclear genome.

It may be mentioned that the plastid transformation technology has a great potential particularly to the improvement of horticultural crops. Development of next generation transgenic crops with various agronomic traits is likely to be relied mostly on this technology.

Discussion

1. Whether nuclear or chloroplast transformation is better in your point of view? Is there any limitation?

Chloroplast transformation is better due to many advantages. The most important is transgene containment. The ability to hyperexpress foreign proteins, single-step multigene engineering, lack of gene silencing, vector sequences and pleiotropic effects have resulted in several 100-fold more tolerance to the environmental stresses via chloroplast genetic engineering than nuclear genetic engineering.

The only limitation is the phenomenon of Concomitant nuclear conundrum whereby the target DNA will integrate with the nucleus or the cytoplasm instead of integrating with the chloroplast during transfer.

2. What is the problem from 1988?

Lack of complete chloroplast genome sequence was the major limitation in extending this technology to some of the useful crops

3. What is the main advantage of Chloroplast engineering?

The most important advantage of chloroplast engineering is the unique feature that the chloroplasts are maternally inherited thereby preventing the pollen flow from transgenic plants to other wild or weedy non transgenic plants thereby conferring transgene containment.

4. Is there any other bacterial gene to confer resistance to toxic metals?

Phytoremediation was achieved only by integrating the native bacterial merA and merB genes. So far no other gene reported.

5. Is there any other bacterial gene to confer resistance to toxic metals?

Phytoremediation was achieved only by integrating the native bacterial merA and merB genes. So far no other gene reported.

6. What is the problem of using antibiotic resistance genes?

Antibiotic resistance genes when used as the marker for transgene expression, it would be difficult to obtain regulatory approval to release transplastomic crops carrying the resistance gene. Though there is no hazard in using transgenic products, still the public is concerned about the environmental release of antibiotic resistance genes.

7. How the transformed tissues are identified?

Only the transformed tissues are able to produce the green calli under regeneration medium. Moreover when the molecular techniques like PCR and Southern blot analyses are carried out, the transformed tissues produce distinct bands showing that they are not transformed via nucleus.

8. What does Gene silencing mean?

Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic mutation. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell.

9. How can we know that a particular character is controlled by chloroplasts?

Chloroplasts confer only certain traits and the only those traits can be exploited using chloroplast engineering. So depending on what trait we need to transfer, we use this technology.

10. Why can't we use chloroplast engineering to transform plastids in cereals?

The cereals lack a proper plant regeneration system from green tissues. Moreover a suitable antibiotic marker system is not available in cereals.

11. What is the specificity of using Spectinomycin as an antibiotic selection marker?

Spectinomycin is a prokaryotic translational inhibitor. So it will have no effect on the eukaryotes when it is used as an antibiotic selection marker.

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ABSTRACT

Most hereditary traits in eukaryotes are determined by genes present in the nucleus while others are determined by organellar genes present in chloroplast and mitochondria. Modern biotechnological approach is to introduce genes into the chloroplast genome rather than into the nuclear genome of the plant. Already about 20 countries of the world are growing transgenic crops, predominantly with traits such as herbicide tolerance and resistance to insect pests. However, there is a constant fear of pollen flow from these transgenics to the neighbouring non-transgenic fields or to their weedy or wild relatives (Bansal and Sharma, 2003).

The best alternative strategy available today to circumvent the nuclear transformation associated bottlenecks in developing tenable crop transgenics is the chloroplast transformation technology. Chloroplasts are the site for photosynthesis in plants mostly present in leaves. In general called plastids, they are also present in other parts of the plant. Just like nucleus, these plastids contain their own genetic material on the chloroplast genome. The chloroplast genome is a self-replicating circular double-stranded DNA molecule with two inverted repeats. With up to 100 genomes per chloroplast and up to 100 chloroplasts per cell, the integration of a transgene through homologous recombination into the inverted repeat region can generate up to 20,000 copies of the transgene per cell (Ignacimuthu, 1997).

Various methods have been developed or attempted to transform chloroplasts, which use biolistic gun, *Agrobacterium*, PEG mediated uptake and microinjection of DNA for inserting a foreign gene into the chloroplast. Gene conferring resistance to Glyphosate was introduced into tobacco chloroplasts and these transgenics were able to survive on 10 times the normally lethal concentration of the herbicide (Daniell *et al.*, 2002).

The chloroplast genetic engineering approach offers a number of unique advantages, including high-level transgene expression, multi-gene engineering in a single transformation event, transgene containment via maternal inheritance, lack of gene silencing, position and pleiotropic effects and undesirable foreign DNA (Grevich and Daniell, 2005). In addition to introducing resistance genes against insect pests or herbicides, benefits of this technology can be harvested for the growth of pharmaceutical industries for the production of vaccines, recombinant proteins and plantibodies.

17.5 3/8/01

COMPUTATIONAL BIOLOGY IN MOLECULAR MAPPING AND DRUG DESIGNING

By
ABDULLA FAYAS T.
(2005-11-144)

SEMINAR REPORT
Submitted in partial fulfillment of the course
PBT 651: Seminar

CENTRE FOR PLANT BIOTECHINOLOGY AND MOLECULAR BIOLOGY
KERALA AGRICULTURAL UNIVERSITY
VELLANIKKARA, THRISSUR – 680656

CERTIFICATE

Certified that the seminar report entitled "Computational biology in molecular mapping and drug designing" for the course PBT 651 has been prepared by Abdulla Fayas T. (2005-11-144) after going through various references cited here under my guidance and he has not copied from any of fellow students.

Vellanikkara
26.07.06

Dr. P.C. Rajendran
Associate Professor (CPBMB)
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DECLARATION

I, Abdulla Fayas T. (2005-11-144) hereby declare that this seminar report entitled "Computational Biology in Molecular mapping and drug designing" has been prepared by me independently, after going through the various references cited herein and that I have not copied or adopted from any of the fellow students or previous seminar reports

Vellanikkara — Abdulla Fayas T.

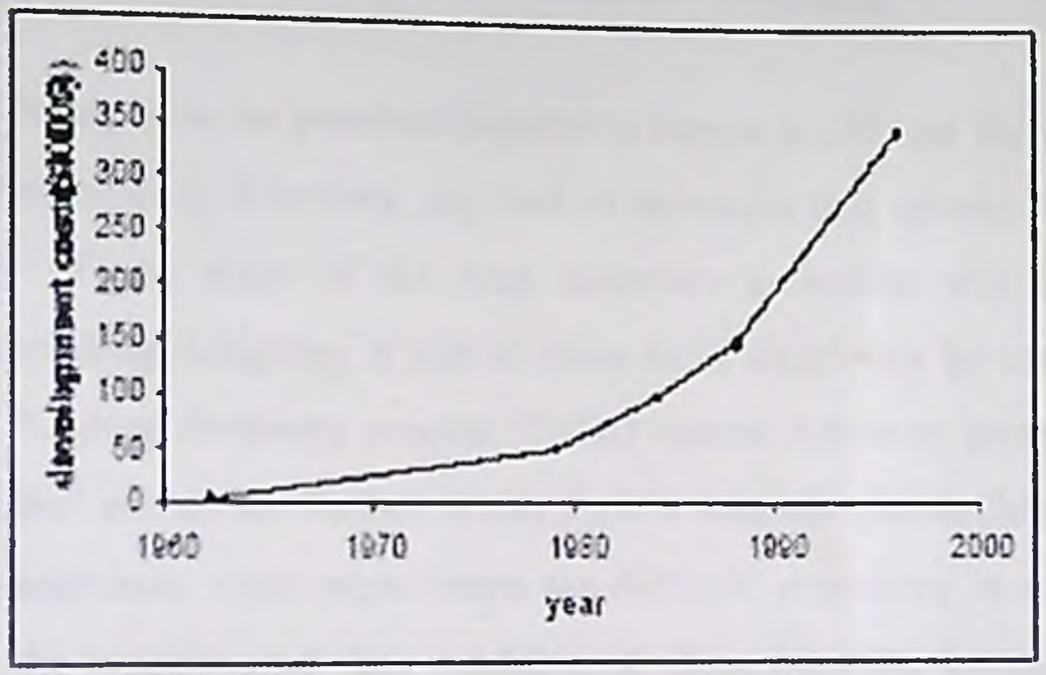
25.7.06 — (2005 - 11- 144)

Contents

1. Introduction

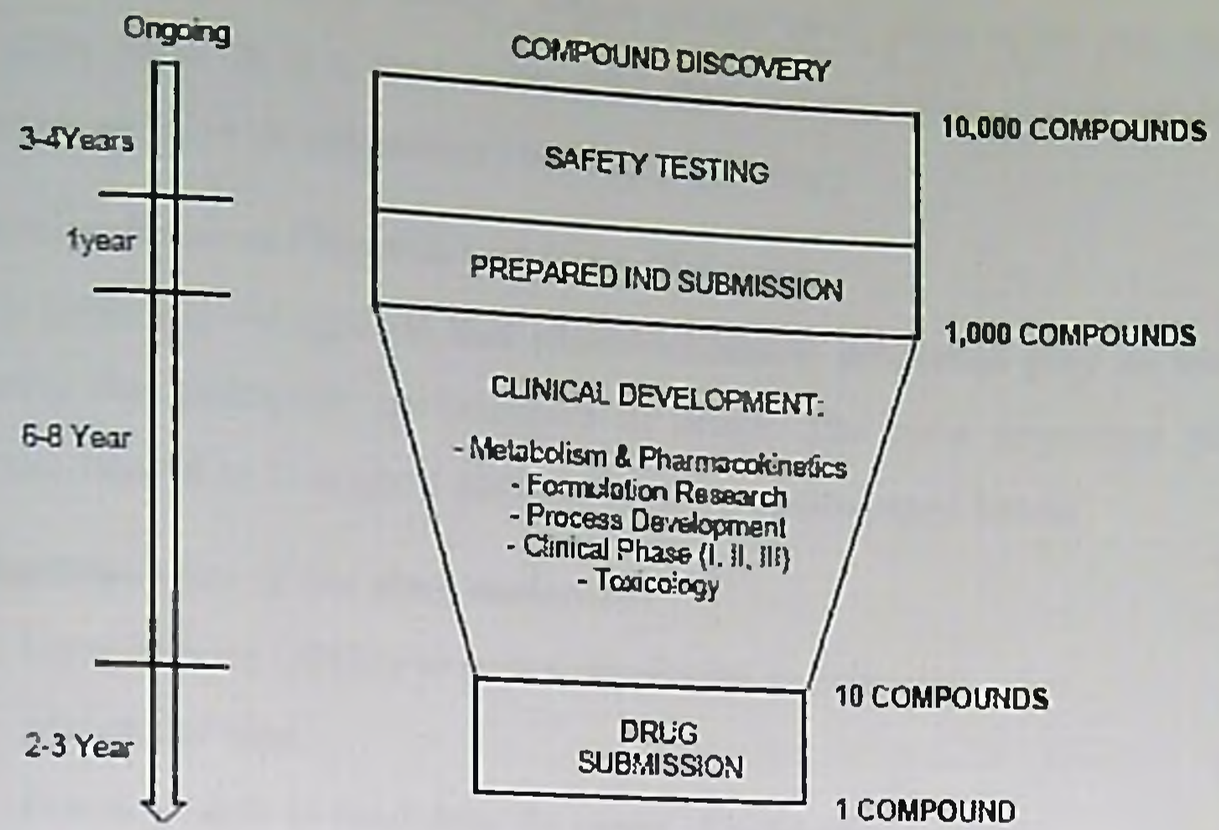
1.1 Conventional drug discovery

Design, development and commercialization of drug is a tedious, time consuming and cost intensive process. The cost of this process has increased significantly during the past thirty-four years. Industry averages reported to the pharmaceutical Manufacturer's Association, have shown that the cost of drug development has increased from $4 million in 1962 to over $350 million in 1996. Between 1960 and 1980, the development time of a substance from the first synthesis to its introduction on the market, has almost quadrupled and has remained relatively unchanged since 1980 (Ooms, 2000) with a present time period of 9-13 years [2-5].



The cost of drug development from $4 million in 1962 to over $350 million in 1996.

Moreover, during this process, only a small amount of candidates will be examined in clinic and few will be marketed. In 1950, it was estimated that 7000 compounds had to be isolated or synthesized and then tested for therapeutic activity for each one that become a pharmaceutical product. The challenge is becoming more difficult: 10,000 compounds had to be evaluated in 1979, and this number could be as high as 20,000 today. The reason for this is several folds. The market for so called high value-added compounds is very competitive. The new compound must offer improved characteristics in order to be worthwhile for commercialization. Also there are serious hurdles regarding ease and cost of synthesis, patentability, safety, and social need for the new compound.



Considering both the potential benefits to human health and the enormous costs in time and money of drug discovery, any tool or technique that increases the efficiency of the efficiency of any stage of the drug discovery enterprise will be highly prized. Computer-aided drug designing is one of these tools which can be used to increase the efficiency of the drug discovery process. CADD cannot, however, maximize its utility in isolation and will not do so. Rather, it can form a valuable partnership with experiment by providing estimates when experiments are difficult, expensive or impossible, and by coordinating the experimental data available. A close coupling between computational chemists and experimentalists allows information to flow immediately and directly between the two. This helps CADD chemists to better understand the details of the problem and to refine their approach. It also provides valuable information for the experimentalist; it helps to guide further experimental planning and potentially makes this process more efficient. CADD is, however, not a direct route to new drugs, but it provides a somewhat more detailed map to the goal. The hope is that by providing bit and pieces of information and by helping to coordinate the information, CADD will help to save days and money for drug discovery projects.

2. Drug definition

A drug is any substance that can be used to treat an illness, relieve a symptom, or modify a chemical process or processes in the body. The word "drug" is derived from the

Dutch/Low German word "droog", which means "dry", since in the past; most drugs were dried plant parts. It is a lead compound that can modulate (block or open; inhibit or accelerate; agonize or antagonize) the target (disease).

2.1 Physicochemical Properties of Drug Molecules

It is generally recognized that physicochemical properties play an important role in governing the biological performance of drugs. The most important physicochemical properties related to biological performance are summarized below:

2.2 Characteristics of the drug molecule:-

- Lipophilicity (ability to penetrate plasma membrane).
- Molecular size.
- Potency (able to modulate the target effectively) .
- Chemical stability.
- Enzymatic stability .
- Specific molecular properties .
- Metabolic stability (should not get destroyed quickly inside the body. A long shelf life is also desirable).
- Solubility (as a drug it should be easily soluble in water for quicker action).
- Molecular interactions.
- Drug complexation (drug-drug, drug-medium, drug-metal).
- Adsorption.
- Melting point or boiling point.
- Bioavailability (quicker absorption into the body).
- Excretion (should be retained in the body sufficiently for sustained activity and excreted gradually).
- Protein binding (The specific protein binding is important. Non specific binding is undesirable).
- Toxicity (should be less toxic).

2.3. *Sites of Drug Action:*

2.3.1 *Enzyme Inhibition:*

Drugs act within the cell by modifying normal biochemical reactions. Enzyme inhibition may be reversible or non reversible; competitive or non-competitive. Antimetabolites may be used which mimic natural metabolites. Gene functions may be suppressed.

2.3.2 *Non-specific Interactions:*

Drugs act exclusively by physical means outside of cells. These sites include external surfaces of skin and gastrointestinal tract. Drugs also act outside of cell membranes by chemical interactions. Neutralization of stomach acid by antacids is a good example.

2.3.3 *Drug-Receptor Interaction:*

Drugs act on the cell membrane by physical and/or chemical interactions. This is usually through specific drug receptor sites known to be located on the membrane. A receptor is the specific chemical constituents of the cell with which a drug interacts to produce its pharmacological effects. Some receptor sites have been identified with specific parts of proteins and nucleic acids. In most cases, the chemical nature of the receptor site remains obscure.

2.4 *Drug Classification:*

Drugs can be classified according to various criteria including chemical structure or pharmacological action. The preferred classification is the latter one which may be divided into main groups as follows:

- Chemotherapeutic agents - used to cure infectious diseases and cancer. (Sulfa drugs, Antibiotics)
- Pharmaco-dynamic agents - used in non-infectious diseases (Cholinergic, Adrenergic, Hallucinogenic, Sedatives)
- Miscellaneous agents (Narcotic Analgesics, Local Anesthetics)

2.5 *Drug Receptor*

A macromolecular component of a cell with which a drug interacts to produce a response, usually a protein.

2.5.1 *Concept of specific drug receptors*

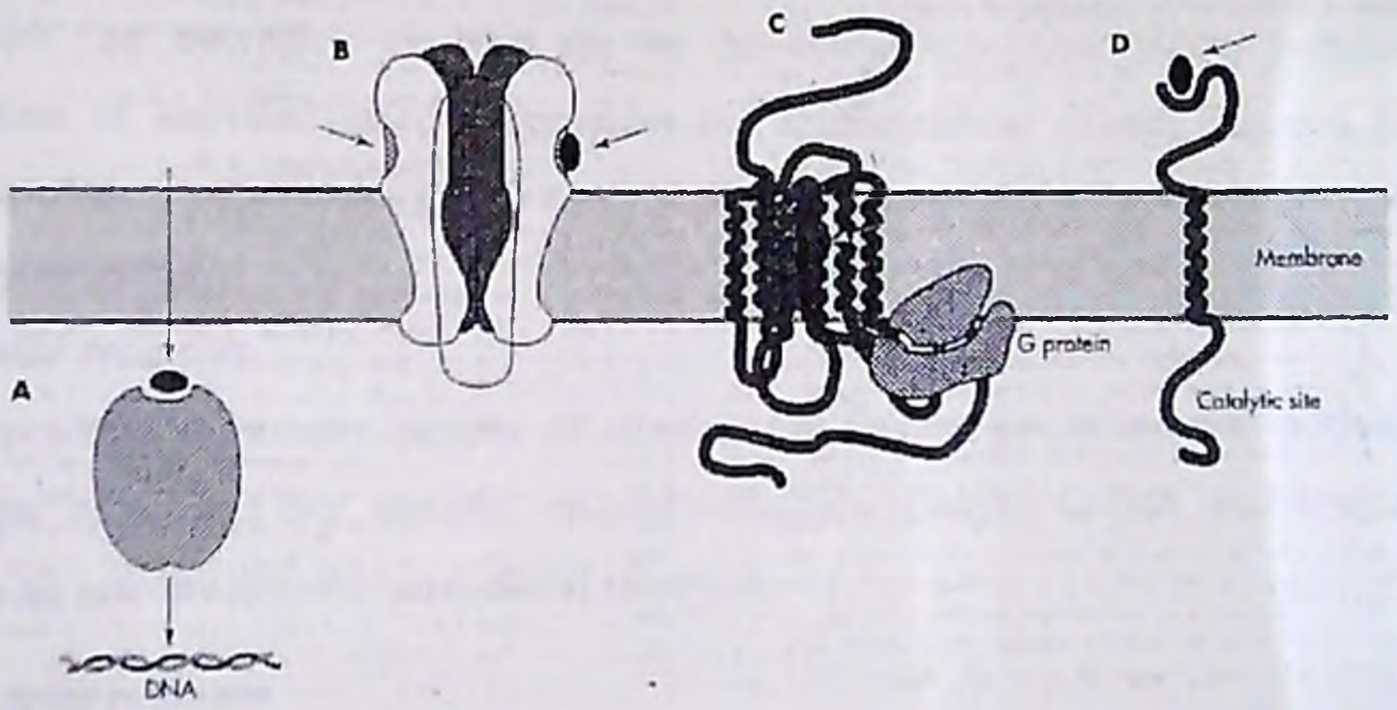
Drug receptors are any cellular macromolecule that a drug binds to initiate its effects. Most drugs combine with specific sites on macromolecules (e.g. cell membrane components, enzymes, proteins) by precise physiochemical and stearic interactions between specific chemical groups of the drug. These sites are termed **receptors.**

2.5.2 *Types of Protein Receptors*

First classification

- Regulatory – change the activity of cellular enzymes
- Enzymes – may be inhibited or activated
- Transport – e.g. Na+ /K+ ATP'ase
- Structural – these form cell parts

Receptor Types



Second classification

Ion-channel-linked receptors

There are two general classes of ion channels: voltage gated and ligand gated Voltage-gated ion channels are activated by alterations in membrane voltage. For example, voltage-gated sodium (Na+) channels open when the membrane is depolarized to a threshold potential and contribute to further membrane depolarization by allowing Na+ influx into the cell. Ligand-gated ion channels are activated after binding to specific

ligands or drugs. Many neurotransmitters and drugs activate membrane- bound ligand ion -gated channels, including several types of glutamate receptors

G-protein-linked receptors

G-protein-linked receptors compose a large class of membrane-bound receptors. The protein structure of these receptors includes a common seven- membered transmembrane domain. In general, receptors linked to G proteins greatly amplify the biologic signal because they activate G proteins, which in turn activate ion channels or, more commonly, other enzymes (e.g., adenylate cyclase), leading to stimulation of still other enzymes (e.g., protein kinase A)." This amplification system, which generally involves an extended duration of activation of the G protein relative to the binding of drug to the receptor, may explain why maximal pharmacologic effects are often observed when only a small proportion of receptors are activated.

Enzyme-linked receptors

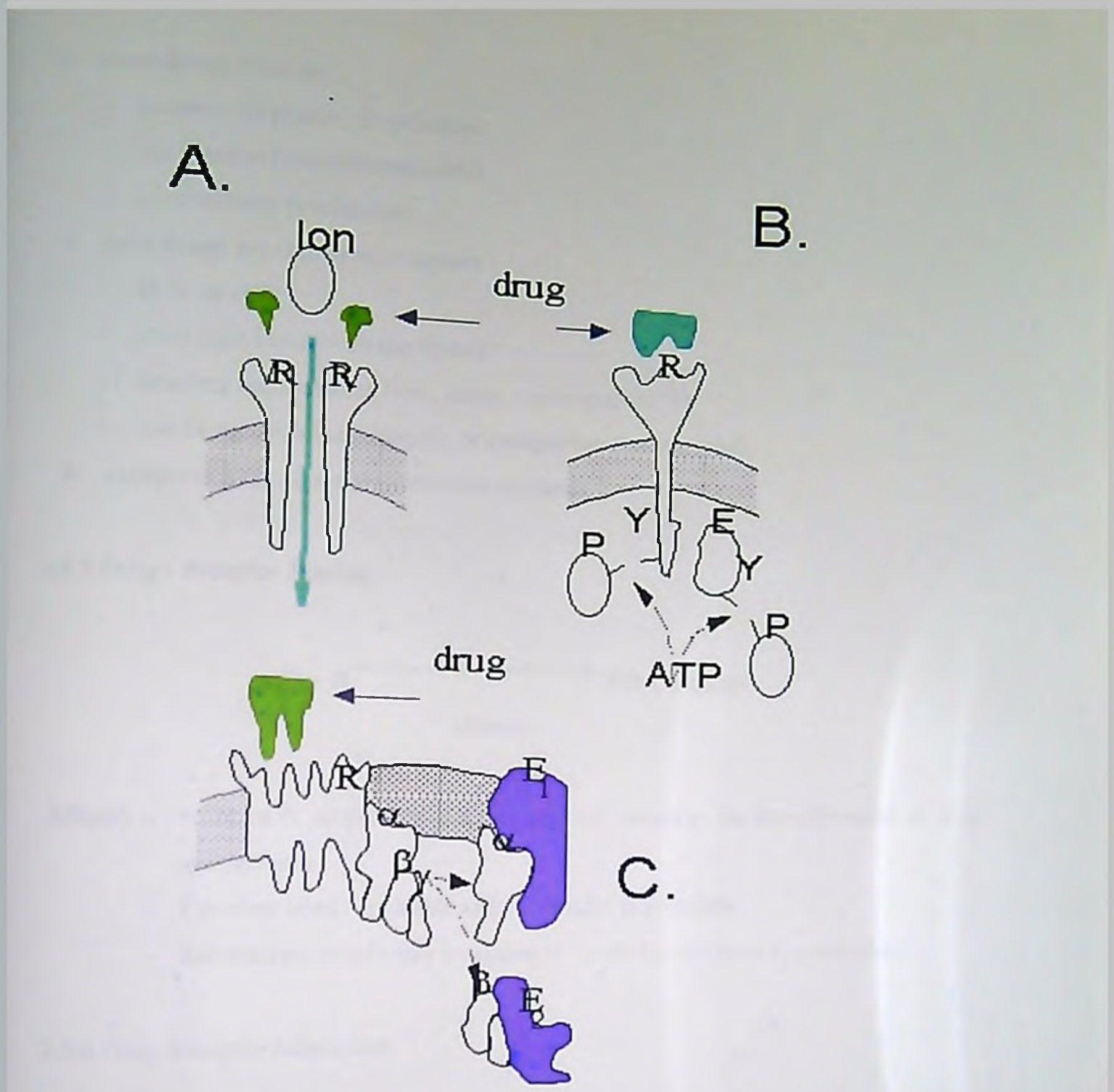
Enzyme-linked receptors have only one transmembrane domain per protein subunit,with "an enzymatic catalytic site on the cytoplasmic side of the receptor. Dimerization of activated receptors provides the confirmational change required for expression of enzymatic activity. The catalytic sites are commonly protein kinases than phosphorylate tyrosine.

Intracellular receptors

Lipophilic substances capable of crossing the plasma membrane may activate intracellular receptors: Sex steroids, mineralocorticoids, glucocorticoids, and thyroid hormones all activate specific intracellular receptors.

2.5.3 Receptor response

The response caused by an activated receptor can involve a variety of different mechanisms. Some receptors directly effect the response of interest (e.g., the ionotropic receptor in **A**.). Even in this case, other factors (including allosteric modulators, cofactors, etc.) that can influence the observed response. In other cases, like a tyrosine kinase (**B**.), the receptor may itself be modified (e.g., phosphorylated) in the process of catalyzing a reaction (phosphorylation in this example). In the case of G protein coupled receptors (**C**.), the receptor may start a cascade of biochemical events due to actions at several effectors, and in some cases, also may be phosphorylated itself.



2.5.4 ***Drug Receptors and Pharmacodynamics (how drugs work on the body)***

Pharmacodynamics

- **many drugs inhibit *enzymes***

  Enzymes control a number of metabolic processes. A very common mode of action of many drugs

  - in the patient (ACE inhibitors)
  - in microbes (sulfas, penicillins)
  - in cancer cells (5-FU, 6-MP)

- **some drugs bind to:**
  - proteins (in patient, or microbes)
  - the genome (cyclophosphamide)
  - microtubules (vincristine)
- **most drugs act (bind) on *receptors***
  - in or on cells
  - form tight bonds with the ***ligand***
  - exacting requirements (size, shape, stereospecificity)
  - can be ***agonists*** (salbutamol), or ***antagonists*** (propranolol)
- **receptors have *signal transduction* methods**

2.5.5 *Drug - Receptor Binding*

$$D + R \xrightarrow[\text{Affinity}]{} DR\ Complex$$

Affinity – measure of propensity of a drug to bind receptor; the attractiveness of drug and receptor

- Covalent bonds are stable and essentially irreversible
- Electrostatic bonds may be strong or weak, but are usually reversible

2.5.6 *Drug Receptor Interaction*

$$DR\ Complex \longrightarrow Effect$$

Efficacy (or Intrinsic Activity) – ability of a bound drug to change the receptor in a way that produces an effect; some drugs possess affinity but NOT efficacy.

$$\underset{D}{Drug} + \underset{(100-DR)}{Free\ Receptor} \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} \underset{Complex\ (DR)}{Drug\text{-}receptor\ Complex}$$

Where:

D = drug concentration

DR= concentration of drug-receptor complex

100 - DR = free receptor concentration

2.5.7 *Theory and assumptions of drug-receptor interaction*

- Combination or binding to receptor causes some event which leads to the response.
- Response to a drug is graded or dose-dependent.

  Drug receptor interaction follows simple mass-action relationships, i.e., only one drug molecule occupies each receptor site and binding is reversible.
- For a given drug, the magnitude of response is directly proportional to the fraction of total receptor sites occupied by drug molecules (i.e. the occupancy assumption).
- The number of drug molecules is assumed to be much greater than the number of receptor sites.
- Combination of drug with a receptor produces a specific response. "lock and key".
- Drug-receptor interactions are analogous to enzyme-substrate interactions.
- Endogenous ligands (e.g. enkephalin versus morphine).
- Drugs without specific receptors (e.g. gaseous anesthetics).

A drug receptor interaction can conveniently be considered in two stages:

- The physical interaction of the drug and the receptor;
- The production of the response by the drug receptor complex.

3. Computational Biology

Computational Biology represents the marriage of Information Technology (IT) and biology, and spans many disciplines, such as bioinformatics, molecular modeling, bioengineering, biosimulation, clinical informatics, medical imaging, and many others.

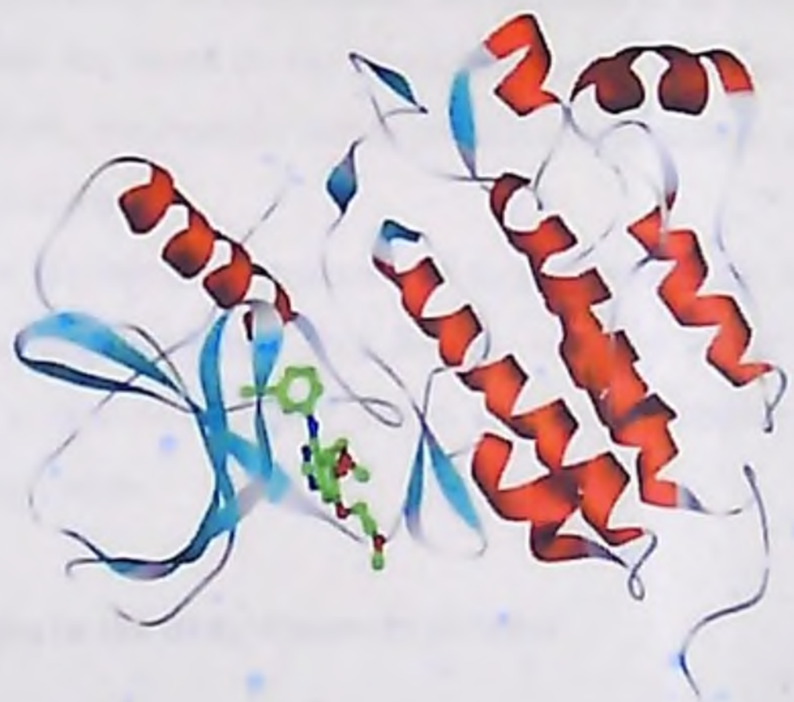
- ➢ It is a fast growing field of Computer Science, synthesizing both the information and biological sciences.
- ➢ Its emergence as an independent field was driven by late 1970s.

- Computational Biology also studies novel algorithmic approaches to efficient assay design, building on new chemical and molecular biology techniques.

Computational biology strategies in drug designing vary depending on the extent of structural and other information available regarding the target and ligand. Two distinct approaches are possible in the area of computer-aided drug design (Richards, 1994). Direct and indirect designs are two major modeling strategies currently used in drug design process. In the indirect approach the design is based on comparative analysis of the structural features of known active and inactive compounds. In the direct design the three dimensional features of the target are directly considered.

3.1 Computer aided drug design

Computer aided drug design is a specialized discipline that uses computational method to simulate drug – receptor interaction.

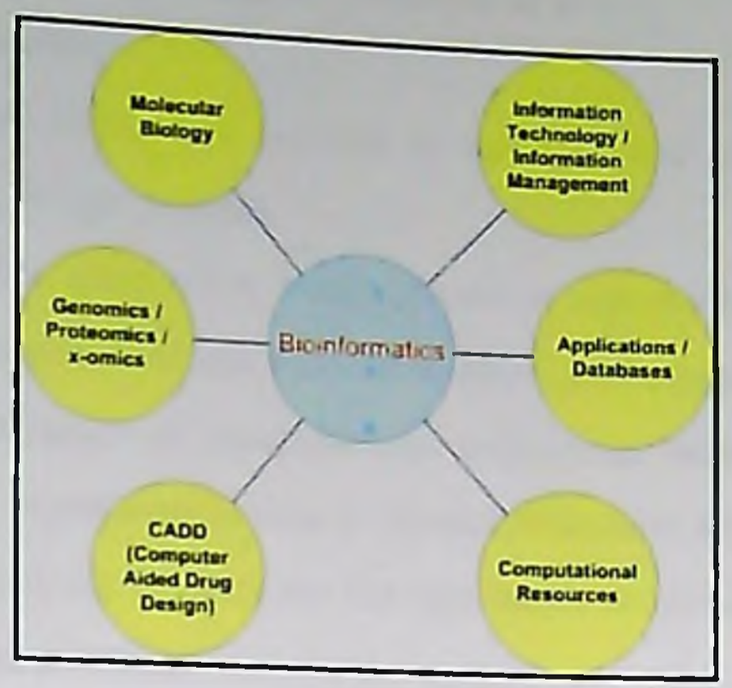


Computer aided drug design of EGFR inhibitor

CADD cannot, however, maximize its utility in isolation. Rather, it can form a valuable partnership with the experiment by providing the estimates when experiments are difficult, expensive, or impossible, and by coordinating the experimental data available.

3.2 Bioinformatics in computer aided drug design

Bioinformatics can be thought of as a central hub that unites several disciplines and methodologies.



3.3 Merits of computer aided drug designing

- Computer aided drug design methods are effective tools for drug discoveries.
- Only a small number of compounds are required to be synthesized.
- The methods are based on the interaction theory between drugs and their target proteins. Steric, electrostatic and hydrophobic interactions are there between drug and target proteins.
- When three dimensional structure of target protein are known, the method is called as a structure-based drug design, while in case that the structure are unknown , a ligand-based drug design approach is employed for computer aided rational drug design.

3.4CADD strategies in the drug discovery process

3.4.1 *QSAR*

Quantitative structure-activity relationships (QSAR) represent an attempt to correlate structural or property descriptors of compounds with activities. It is the quantitative correlation of the biological (ecological, toxicological and pharmacological) activity to structure of chemical compounds, which allows the prediction of the so called "drug efficacy" of a structurally related compound.

3.4.2 *Docking*

Docking is the process by which two molecules fit together in 3Dspace. Docking refers to interaction of a small molecules to active site of a macro molecule.Docking is a

term used for computational schemes that attempt to find the "best" matching between two molecules: a receptor and a ligand (Halperin et al.).

3.4.3 *Molecular modeling*

Molecular modeling has become a valuable and essential tool to medicinal chemists in the drug design process.

- It describes the generation, manipulation or representation of three- dimensional structures of molecules and associated physico-chemical properties.
- It involves a range of computerized techniques based on theoretical chemistry methods and experimental data to predict molecular and biological properties
- Direct and indirect designs are the two major modeling strategies currently used in drug design process.
- In the direct design the three-dimensional features of the target (enzyme/receptor) are directly considered
- In the indirect approach the design is based on comparative analysis of the structural features of known active and inactive compounds.

3.5 Drug Designing Process

I. Find what is known

- Find out all that is known about the disease and existing or traditional remedies.
- Look at very similar afflictions and their known treatments.

II Develop an assay

- Develop an assay technique to test drug effectiveness
- An ideal assay is one in which a compound can be added to tissue samples or micro-organism colonies and there will be a visible indication of an effective treatment.

III Find Lead compounds

- Lead compounds are compounds that have some activity against a disease.
- These may be only marginally useful and may have severe side effects.

- Lead compounds provide a starting point for refinement of the chemical structures.

IV Isolate the molecular basis for the disease

- If it is known that a drug must bind to a particular spot on a particular protein or nucleotide then the drug can be tailored to bind at that site

- This is often modeled computationally using any of several different techniques.

Various techniques employed are

- X-ray crystallography
- Distance geometry – internuclear distances are found using NMR (Nuclear Overhauser Effect) experiments and then find molecular geometries that have these distances.

V Refine Drug Activity

- Once a number of lead compounds have been found, computational and laboratory techniques have been very successful in refining the molecular structures to give a greater drug activity and fewer side effects.

- This is done both in the laboratory and computationally by examining the molecular structures
- Computationally the technique used is called as QSAR (Quantitative Structure Activity Relationships)
- Solubility

VI Drug testing

Once a drug has been shown to be effective by an initial assay, much more testing must be done before it can be given to human patients.

1. Pre-clinical testing in animals and test tubes.
2. Phase II clinical trials in a few hundred patients.
3. Phase III clinical trials in a few thousand patients.
4. An advisory panel of doctors reviews the data and makes reommendations to the FDA
5. FDA approval or rejection

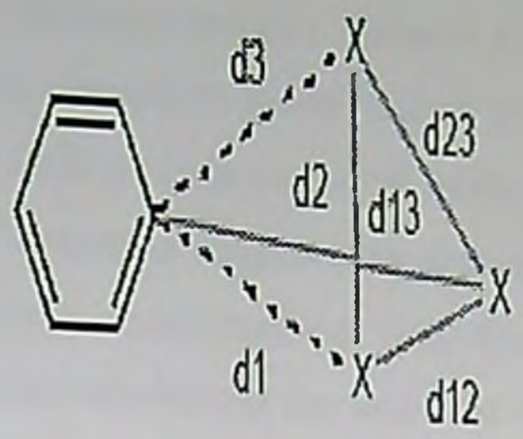
6. The FDA continues to monitor drug performance long after approval has been given
7. Formulation
8. Production
9. Non- Prescription sales
10. Generic production

3.6 CADD in lead Generation when 3D structure of protein unknown

Lead generation and optimization is two major part in computer aided drug designing. Lead compounds are compounds that have some activity against a disease. These may be only marginally useful and may have severe side effects. However, the lead compounds provide a starting point for refinement of the chemical structures. Lead compounds may come from many sources, including (Young, 2001).

In the early stage of a drug discovery process, researchers may be faced with little or no structure-activity relationship (SAR) information. At this point, assay development and screening should be undertaken immediately by high-throughput screening (HTS).The compounds screened could be commercially available, natural products (Kingston,1996.), collections of in-house synthesized compounds or emerge from combinatorial libraries (Hobbs,1996). Instead of performing random screening, a set of compounds presenting diversity in their physicochemical properties can be selected to find leads. If however, a lead is known, then more focused approach can be adopted by searching for compounds with similar structures to the lead candidate or by substructure searching. In the substructure searching the query will retrieve those structures from the database that contain groups present in the primary lead. These molecules can then be screened in a biological assay.

Once primary lead and their corresponding structural information are or become available the computational chemist can use these data to derive new lead classes and 'fine tune' the leads that the chemist have already been pursuing. The first step to derive a new lead, also called secondary lead, will be to study the stereo-electronic properties of selected primary leads



d1 = 2.3-3.0 Å d12 = 1.3-3.6 Å
d2 = 3.3-5.7 Å d23 = 2.2-3.7 Å
d3 = 2.3-2.7 Å d13 = 2.2-2.7 Å

Example of a pharmacophore model derived for the MAO-B inhibitors. With X = O, N, S.

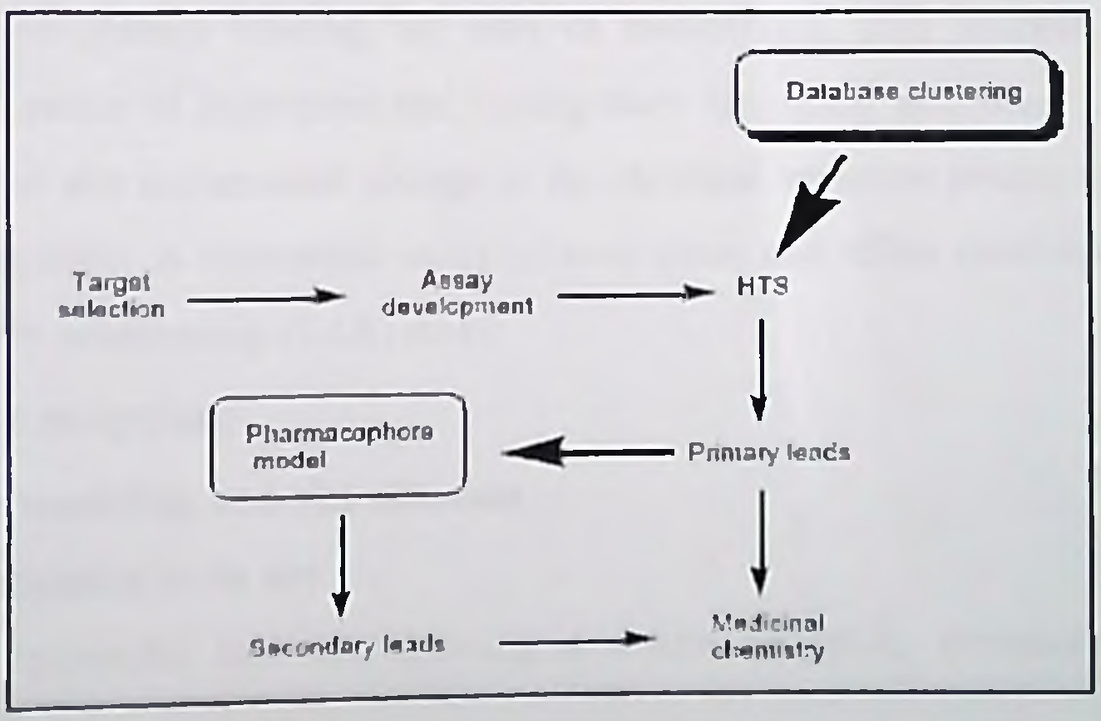
3.7 Main Stereo electronic properties used in CADD

Steric	L (Substituent length) B5 (Substituent width) MR (Molar refractivity) Volume Surface area ...
Electronic	σ (Hammet constant) F, R (Field and resonance parameters) pK_a (Ionization constants) q (atomic charges) MEP (Molecular Electrostatic Potential)...
Lipophilic	π (Hansch constant) f (Hydrophic fragmental constant) Log P (partition coefficients) Log k_W (capacity factor values from RP-HPLC) CLOGP (calculated log P values) MLP (Molecular Lipophilic Potential)...
H-bonding	HA (number of H-bond acceptors) HD (number of H-bond donors) Δ log P (oct-hex) (H-bond capability)...

The primary lead should be selected among a set of compounds showing a large variety in chemical structures. It should interact with the same target via the same binding mechanism. By comparison of the stereo-electronic properties of primary leads, a pharmacophore is defined. A pharmacophore model is a spatial arrangement of atoms or

functional groups believed to be responsible for biological activity (Ghose, 1998). In this model the rest of the molecule act as a skeleton to hold the groups in the right place. In the course of the pharmacophore identification process, two different steps have to be taken in succession. First a conformational analysis has to be carried out. Indeed, the biological activity of a drug is supposed to depend on one unique conformation hidden among all the low-energy conformations. Only the so called bioactive conformation can bind to the specific macromolecular environment at the active site of the target protein. The bioactive conformation is not necessarily identical with the lowest-energy conformation but on other hand, it cannot be a high energy conformation (Vieth, 1998). During the second step the bioactive conformations are used to calculate their stereo electronic properties. Based on this three dimensional description of the stereo electronic properties of the considered compounds, a pharmacophore can be obtained. Once the pharmacophore has been partially identified, the next step is to find compounds which contain it embedded in their structure by three dimension database searching (Martin, 1990). The classical database of three dimensional molecular structures is the Cambridge Structural Database (CSD) (Allen, 1993).

3.8 CADD in lead generation when no structural information about target protein are available



Case study

"Success stories" have been published in the design and discovery of Human Immunodeficiency Virus type 1 integrase inhibitor (Hong, 1997). HIV-1IN is among one of the most important enzyme responsible for HIV-1 replication cycle. It mediates the

integration of HIV-1 DNA into host chromosomal targets and is known to be essential for effective viral replication. Because of its essential nature in replicative cycle of HIV-1, it is an attractive target for the development of anti-AIDS drugs. Starting from a pharmacophore hypothesis derived from a known inhibitor of HIV-1 IN, caffeic acid phenethyl ester (CAPE, 1), a three dimensional search of NCI database was performed. 267 structures were found to match the pharmacophore, 60 of those were tested in an invitro assay against HIV-1 IN and 19 were found to inhibit both 3'processing and strand transfer. The relevance of the proposed pharmacophore was then tested using a small three dimensional validation database of known HIV-1 IN inhibitor. This search strongly supports for the existence of the postulated pharmacophore and in addition, it hinted at the existence of a possible second pharmacophore relevant in binding to IN[41]. Using the second pharmacophore in a three dimensional search of the NCI database, 10 novel structurally diverse HIV-1 IN inhibitors were found. Four of these 10 inhibitors were particularly potent.

3.9 CADD in Lead Optimization

When leads are available, the next step consists in their optimization. In medical chemistry the lead optimization process concerns many aspects such as the optimization of the affinity for biological target, the toxicity, the oral bioavailability, the cell permeability, the plasma binding, the ease of metabolism. This process requires the synthesis of a series of analogues and testing their biological activities. The principle employed is that any incremental change in the chemical structure produces incremental change in bioactivity. A systematic study of such cause and effect relationship is called structure activity relationship (SAR) study.

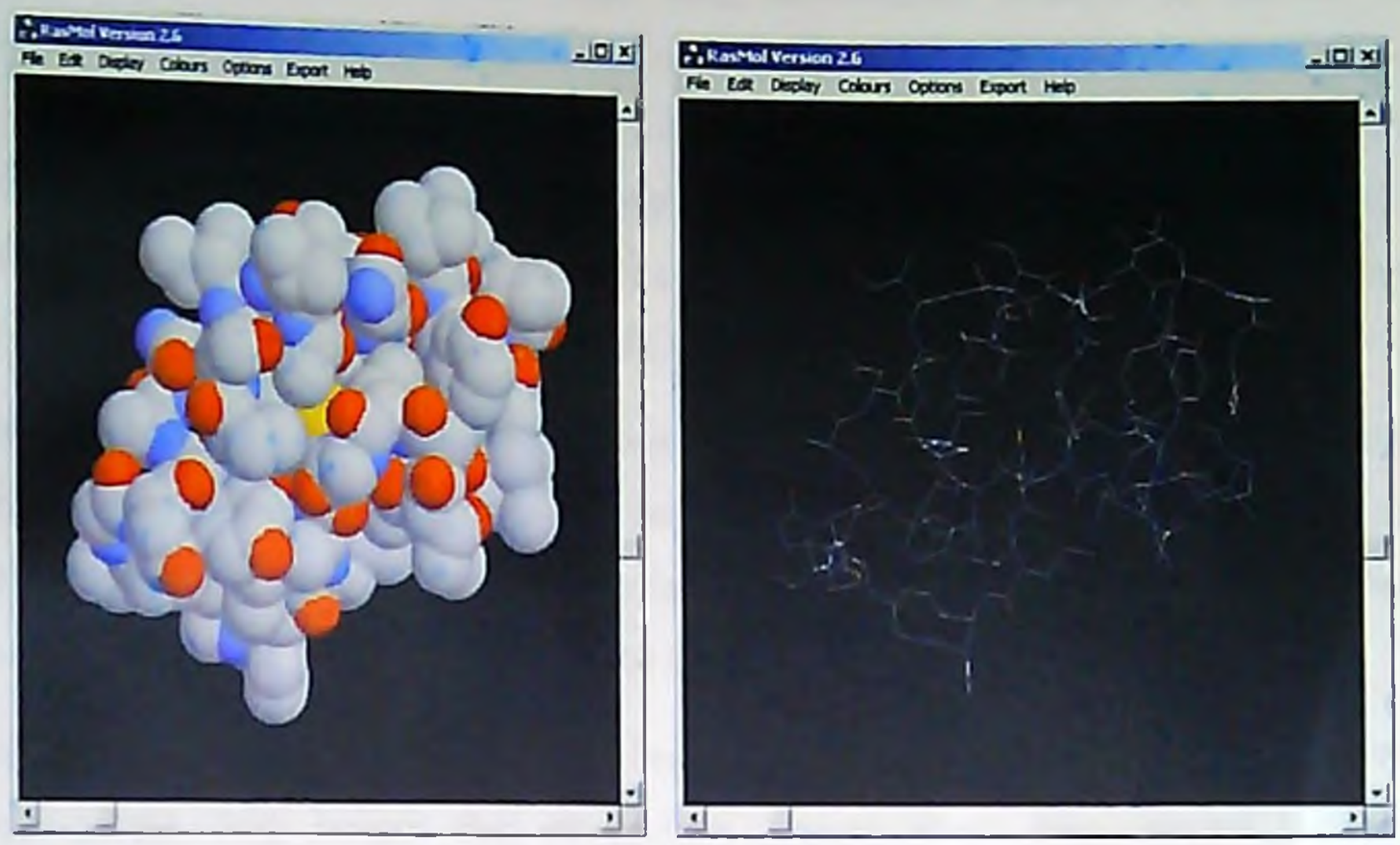
4. Molecular mapping

4.1 Molecular modeling and visualization

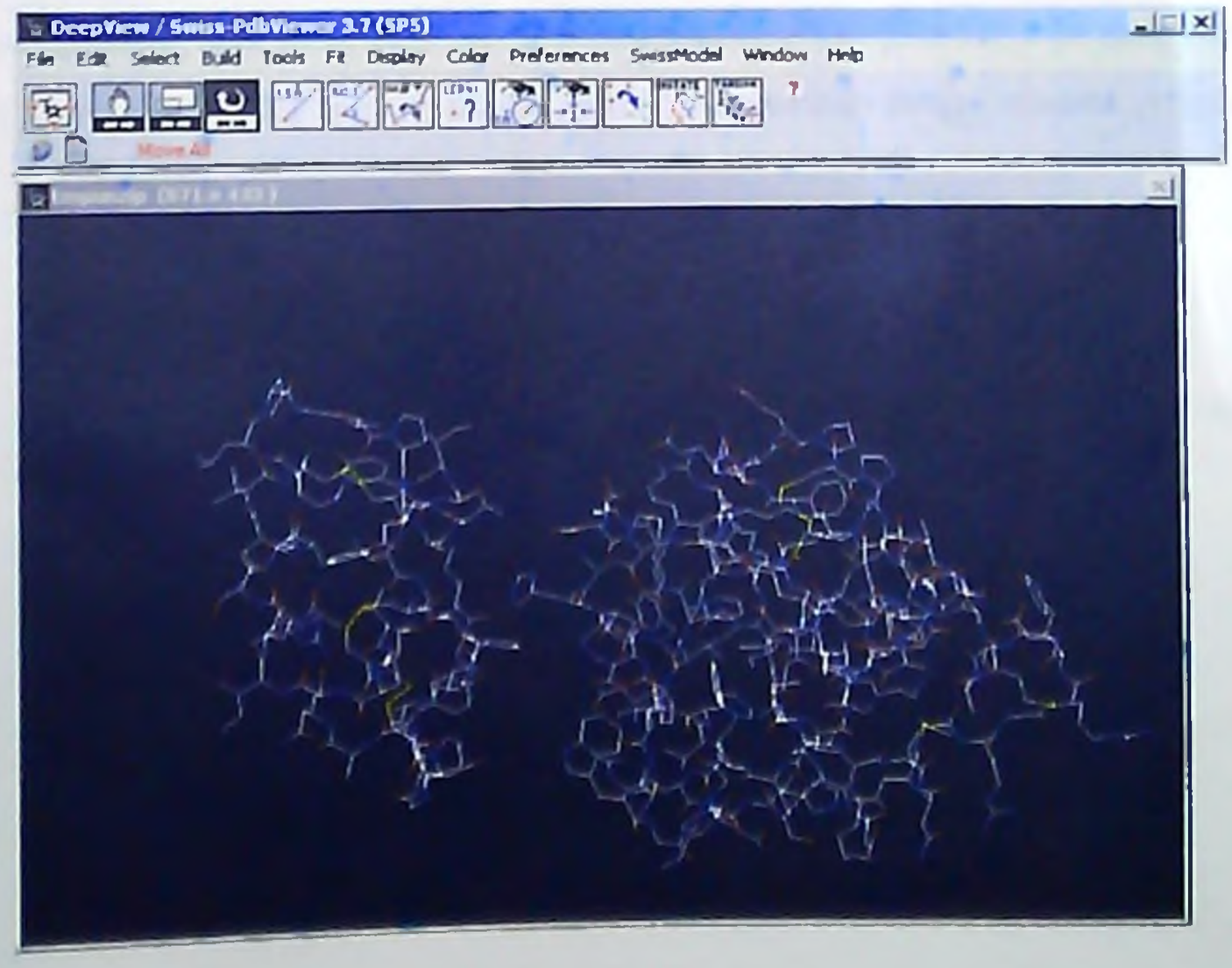
Various visualization tools are

1. Rasmol- powerful tool for showing structure of DNA, proteins and smaller molecules.
2. Chime – sits directly on a webpage and run inside a web browser as a plug-in.
3. Protein explorer
4. Swiss PDB viewer

Protein visualization using Rasmol



Protein visualization using Swiss PDB viewer



5. Benefits and limitation of Computational biology in drug designing

5.1 Benefits of Computational biology

Cost Savings. The Tufts Report suggests that the cost of drug discovery and development has reached $800 million for each drug successfully brought to market. Many biopharmaceutical companies now use computational methods and bioinformatics tools to reduce this cost burden. Virtual screening, lead optimization and predictions of bioavailability and bioactivity can help guide experimental research. Only the most promising experimental lines of inquiry can be followed and experimental dead-ends can be avoided early based on the results of CADD simulations.

Time-to-Market. The predictive power of CADD can help drug research programs choose only the most promising drug candidates. By focusing drug research on specific lead candidates and avoiding potential "dead-end" compounds, biopharmaceutical companies can get drugs to market more quickly.

Insight. One of the non-quantifiable benefits of CADD and the use of bioinformatics tools is the deep insight that researchers acquire about drug-receptor interactions. Molecular models of drug compounds can reveal intricate, atomic scale binding properties that are difficult to envision in any other way. When we show researchers new molecular models of their putative drug compounds, their protein targets and how the two bind together, they often come up with new ideas on how to modify the drug compounds for improved fit. This is an intangible benefit that can help design research programs.

5.2 Limitation of computational biology

- System simulated are large and nonlinear
- Simulation are computationally expensive
- There is often a barrier between biological scientists and computer or computational scientists

6. Conclusion

Computational biology in designing drug is no longer a promising technique. It is a practical and realistic way of helping the medicinal chemist. On its own it is unlikely to lead to pharmaceutical novelties but it has become a significant tool, an aid to thought and guide to synthesis. Still drugs must be synthesized and tested by the computational techniques can contribute a clear molecular rationale and above all provide a spur to the imagination

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Discussion

1) How drug analysis is carried out?

   Drug analysis is carried out using Lipinski's Rule of 5. The rule of 5 states that poor absorption or permeation is more likely when:

   - Molecular mass greater than 500Da.
   - High lipophilicity (expressed as clogP greater than 5).
   - More than 5 hydrogen bond donors
   - More than 10 hydrogen bond acceptors

   All of these are close to five or a multiple of five.

2) Is computer aided drug designing being utilized in India?

   Yes, computer aided drug designing is utilized in India.

3) Name the institutions where CADD is utilized?

   CDRI, Lucknow and CCMB, Hyderabad

4) Are all new generation drugs synthesized using CADD?

   All new generation drugs are not synthesized using CADD, but few like antiAIDS drug and some cancer curing drugs are synthesized using Computer aided drug design.

5) Explain the role of biologist in computer aided drug designing? In what way are they contributing?

   Computational biology which is the part of bioinformatics is a marriage between information technology and biology. So it requires the both computers software specialist and biologist. Biologist are required in the sense that it is biologists who can explain the questions regarding drug properties and drug receptor interaction where as computer software specialist are involved in designing biological databases and computer simulations for the drug action.

6) How can resistance to drug be explained?

   Many receptor-mediated events show the phenomenon of desensitization, which means that *continued or repeated administration of a drug, produces* progressively *smaller effect.* When desensitization occurs very rapidly, it is referred to as tachyphylaxis. This occurs when the receptor becomes phosphorylated and becomes less efficient and also exhibits lower affinity for agonists.

7) What is Rasmol?

Rasmol is a powerful tool for showing structure of DNA, proteins and smaller molecules.

8) Explain the differences between conventional and computer aided drug designing methods.

The drug was once made by screening natural and synthetic compounds, and it was an expensive and laborious process. Conventionally, optimization of the lead is achieved by random exploration of the chemical derivatives. Computational biology approaches aim to increase the speed and efficiency in the drug discovery process. It provides somewhat more detailed map to the goal. Providing bit and pieces of information about drug-receptor interaction and by helping to coordinate the information, CADD will help to make the drug design process more rational.

ABSTRACT

The development of new drugs with potential therapeutic application is one of the most complex and difficult process in the pharmaceutical industry. Millions of dollars and man-hours are devoted to discovery of new therapeutical agents. As, the activity of a drug is the result of a multitude of factors such as bioavailability, toxicity, metabolism, rational drug design has been utopias for centuries. Very recently, impressive technological advances in areas such as structural characterization of bio-macromolecules, computer sciences and molecular biology have made rational drug design feasible.

The drug was once made by screening natural and synthetic compounds, and it was an expensive and laborious process. Conventionally, optimization of the lead is achieved by random exploration of the chemical derivatives. The cost of this process has increased significantly during the past thirty-four years. Industry averages reported to the Pharmaceutical Manufacturer's Association, have shown that the cost of drug development has increased from $4 million in 1962 to over $350 million in 1996. Between 1960 and 1980 the development time of a substance from first synthesis to its introduction on the market, has almost quadrupled and has remained relatively unchanged since 1980 (Ooms, 2000).

Computer aided drug design (CADD) is one of these tools which can be used to increase the efficiency of drug discovery process. CADD cannot, however, maximize its utility in isolation and will not do so. Rather it can form a valuable partnership with experiment by providing estimates when experiments are difficult, expensive or impossible, and by coordinating the experimental data available.

Computational Biology represents the marriage of Information Technology (IT) and biology, and spans many disciplines, such as bioinformatics (both genomics and post-genomics), molecular modeling, bioengineering, bio-simulation, clinical informatics, medical imaging, and many others.

Computational biology strategies in drug designing vary depending on the extent of structural and other information available regarding the target and ligand. Two distinct approaches are possible in the area of computer-aided drug design (Richards, 1994). Direct and indirect designs are two major modeling strategies currently used in drug

design process. In the indirect approach the design is based on comparative analysis of the structural features of known active and inactive compounds. In the direct design the three dimensional features of the target are directly considered.

Lead generation and optimization is two major part in computer aided drug designing. Lead compounds are compounds that have some activity against a disease. These may be only marginally useful and may have severe side effects. However, the lead compounds provide a starting point for refinement of the chemical structures. Lead compounds may come from many sources, including (Young, 2001).

Computational biology approaches aim to increase the speed and efficiency in the drug discovery process. CADD is, however, not a direct route to new drugs, but it provides somewhat more detailed map to the goal. The hope is that providing bit and pieces of information and by helping to coordinate the information, CADD will help to make the drug design process more rational.