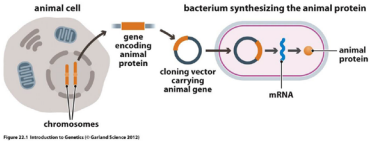
CHAPTER 22

Genes in Industry and Agriculture

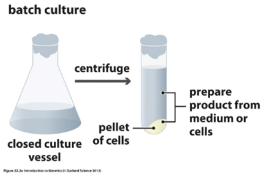
Production of Recombinant Protein by Microorganisms

Recombinant Protein: a protein that is produced from a clone.



Microorganisms are ideal hosts for producing recombinant proteins because they can be grown at high density and therefore produce large amounts of recombinant proteins.

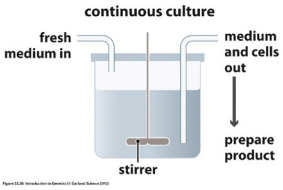
Production of Recombinant Protein by Microorganisms



- **The Batch Method:** Engineered microbes are grown in large culture vessels and the product is later purified by removal of the microbial cells
  - **Positive:** Gives high yields.
  - **Negative:** Yields are limited by the size of the culture vessel.

Production of Recombinant Protein by Microorganisms

- **Continuous Culture**: Microbes are grown in a fermenter.



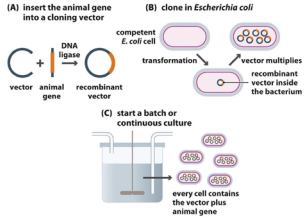
- New medium is continuously pumped in to the large drum and old medium and cells are pumped out. The product is then separated from the cells and purified.

Special Cloning Vectors are needed for the Synthesis of Recombinant Protein

- High yields of product are possible using Batch Cultures and Continuous Cultures, but first microbes most be engineered to synthesize the desired recombinant protein.

Special Cloning Vectors are needed for the Synthesis of Recombinant Protein

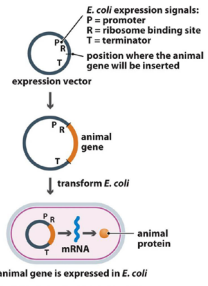
- *Escherichia Coli* was the first microorganism to be used for the synthesis of recombinant proteins.
- Animal genes that code for the desired recombinant protein are transferred into *E. coli* by DNA cloning.



Will the Animal Gene be Expressed so that the Recombinant Protein is Produced?

- An animal protein does not possess correct signals for expression in *E. coli* .
- In order for the animal protein to be transcribed and translated, the gene must be preceded by an *E. coli* promoter and followed by a termination sequence.

Expression Vectors

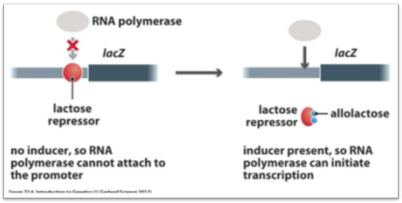


- Cloning vectors used in recombinant protein production must contain a set of *E. coli* expression signals.
- Plasmids that provide these signals are called **Expression Vectors**.
- The **promoter** is the most important.
  1.) determines how rapidly a gene is expressed.
  2.)controls gene expression.

An ideal promoter directs a high rate of transcription and can be controlled by the addition of regulatory chemicals.

Example: The Lactose Promotor

- In *E. coli* the lactose promoter controls transcription of the lactose operon.

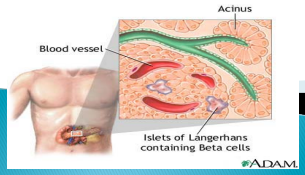


- An animal gene is inserted down stream of the lactose operon and is then expressed when an inducer is added to the culture medium.

Insulin was one of the first human proteins to be synthesized in *E. coli.*

What is insulin and its major role as a protein?

- A peptide hormone that is synthesized by the Beta cells of pancreatic islets of Langerhans.
- It controls the level of glucose in the blood.



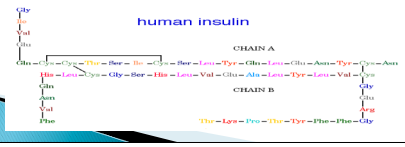
Why did it need to be synthesized in *E. coli?*

- Deficiency in insulin produced leads to diabetes and can cause fatal damages to the heart and blood vessels, eyes, kidneys, nerves, and the gums and teeth.
- Previously, the insulin from the pancreas of pigs and cows were used for their similarity in amino acid sequences to the human version. However, purification of insulin from pancreas tissue involved a complexity in removing the contaminants, which might lead to allergic reactions.
- "Recombinant" insulin was used to avoid this problem because purification from a bacterial culture was much simpler.

The production of Insulin is facilitated by two features with recombinant methods.

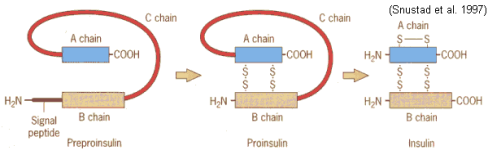
The recombinant insulin synthesized by *E. coli* should be identical to the ones made by the human pancreas because:

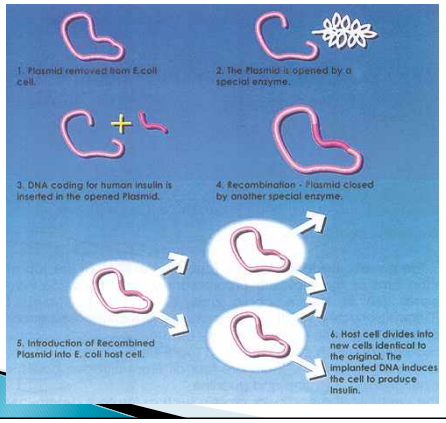
1. The human proteins does not undergo extensive post-translational modification.
2. The size of insulin molecule is small – two polypeptides made up of A and B chains.
   - A chain: 21 amino acids in length
   - B chain: 30 amino acids in length



Synthesis of insulin as a recombinant protein in *E. coli*

1. Construction of two recombinant vectors, containing the A chain and B chain.
2. Insertion of those recombinant vectors into *E. coli* to synthesize A and B purified fusion proteins.





***E. Coli* is not an ideal host for recombinant protein production.**

Recombinant protein synthesis in *E. coli* has two types of problem:

1. The differences of genes in animals and *E. coli*.
2. The biochemical and physiological limitations of bacteria.

1. The differences of genes in animals and *E. coli*.

The expression vector can be designed according to the specific need for the animal gene to be controlled with an *E. coli* promotor, ribosome binding site, and transcription termination sequence.

However the sequence of an animal gene may still be dismatched in its expression in *E. coli* because:

- the animal gene may contain introns.
- the animal gene may contain sequences that act as transcription signals in *E. coli.*
- the codon bias of the gene may not be ideal for translation.

Two possible ways to resolve the problems above:

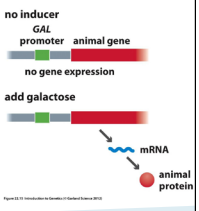
- By reverse transcriptase to create an intron-free version of the animal gene.
- By site-directed mutagenesis techniques to alter the nucleotide sequence.

2. The biochemical and physiological limitations of bacteria.

- Formation of an inclusion body may occur due to the differences in the protein-folding processes in bacterial cells and in eukaryotes. An incorrectly folded protein is inactive in the other type of organism.
- *E. coli's* inability to undergo some of the complex chemical modifications that animal proteins do lead to producing different glycosylated recombinant proteins.
  - Although glycosylation is not always essential for the function of the animal proteins, however Incorrect or deficient glycosylation may significantly reduce the stability of the protein when injected into a patient, and may also induce an allergic reaction.

Recombinant Protein can also be synthesized in Eukaryotic cells.

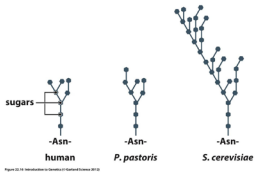
- Problems associated with obtaining high yields of active recombinant proteins from animal genes cloned in E. coli have led to the development of expression systems for Eukarytotic cells
- Saccharomyces cerevisiae is the most popular microbial eukaryote for protein synthesis
- Accepted as a safe organism for the production of proteins for use in medicine or in foods



- **Its' biochemistry and genetics have been known over the years.**
- **Yeast expression vectors carry the GAL promoter, the gene coding galactose epimerase, which is an enzyme involved in the metabolism of galactose.**
- **Gal promoter is induced by galactose, providing a convenient system for regulating the expression of a cloned animal gene**

Deficiencies of the S. cerevisiae as microbial eukaryote for recombinant protein synthesis

- **Hyperglycosylating**- adding too many sugar units
- Lacks an efficient system for secreting proteins into the growth medium which leads to recombinant proteins remaining in the cell and are less easy to purify
- Use of another specie of yeast: *Pichia pastoris*

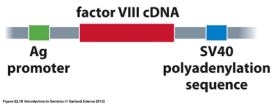


Main advantage of the *Pichia pastoris*

- Can synthesis large amounts of recombinant protein
- Does not glycosylate proteins in exactly the same way as animal cells
- There is no allergic reaction when injected into the bloodstream.
- Both *S.cerevisiae* and *P. pastoris* have there other short comings
- They do not allow the efficient production all animal proteins

Factor VIII as an example of a protein that is difficult to produce by recombinant means

- Factor VIII is the protein that is defective in the commonest form of hemophilia
- Leads to the breakdown in the blood clotting pathway
- Treatment is by regular injections of purified factor VIII.
- Until recently, the only source of the protein was human blood provided by donors

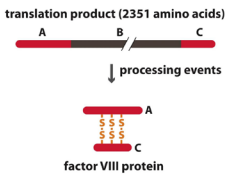


Shortfalls in this recombination process

- The purification of this protein directly from the blood is a complex with many difficulties
- Example is the removal of virus that may be in the donated blood since there has been cases of AIDs and hepatitis transfer through this method
- Recombinant factor VIII free from contamination will be great achievement for biotechnology.

Difficulty in obtaining factor VIII by recombinant methods

- The complexity of the pathway that converts the initial translation product of the factor VIII gene into active protein
- This translation product is a polypeptide of 2351 amino acids, cut into three segments
- The central segment is discarded and the two outer ones are joined by disulphide bonds
- This leads to the protein been extensively glycosylated
- This leads to impossibilities of the synthesis of an active version of recombinant factor VIII in E.coli.



Vaccines

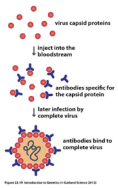
Recombinant techniques are not only used to synthesize proteins, but also to synthesize **VACCINES**

Vaccine - stimulates the synthesis of antibodies by the immune system to protect the body from infection.
- targets can be viruses, bacterium or any other pathogen organism

- The majority of vaccines are developed using inactivated forms of the infectious organism
  - Ex. Heat Killed Virus Particles
- This method requires a large amount of virus particles generally grown in infected tissue cultures

Problems with this method
1. Some viruses can't be grown in tissue cultures
   - Obtaining enough virus is then very difficult
2. Possible for viruses to survive the heat treatment
   - May cause the disease instead of provide protection

Vaccines can also be produced from recombinant technology



Two new strategies for using recombinant technologies for vaccine productions are being developed:
1. Using isolated components (Ex. Capsid Proteins) of a virus
   - Free of intact virus particles
   - Easily obtained in large particles

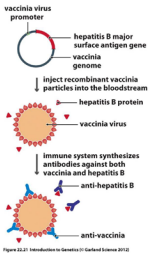
Ex. Hepatitis B Virus
- Causes Liver Disease and potentially Liver Cancer
- Immunity is developed by antibodies in the blood against the capsid protein, hepatitis B surface antigen (HBsAg)

Recombinant HBsAg
- Developed in Hamster and *S. cerevisiae* Cells
- Injections into live animals lead to protection against Hepatitis B

Has Been Approved for use in humans

Vaccines can also be produced from recombinant technology continued

2. Using engineered vaccinia virus
   - **Vaccinia = Cowpox virus**
     - Harmless to humans
     - Stimulates an immunity to Smallpox in humans
   - **Recombinant Vaccinia**
     - Developed by inserting new genes (ex. HBsAg) into the genome of Vaccinia
     - When introduced in the blood, replication of the recombinant virus results in vaccinia particles and HBsAg



Has not been approved for human use yet
- Shown to work in animals
- Currently used to control rabies in Europe and North America

Genetic Engineering

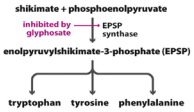
Definition: Introduction of new genes into plants and animals

Objective: Change the phenotype of a plant or animal in a way that is considered beneficial to the use of the organism.
- Ex. Domesticated plants and farm animals

A plant or animal with a new gene introduced is called **transgenic** or **genetically modified**

Crops can be engineered to become resistant to herbicides

Most common herbicide that resistance is obtained is **glyphosate**
- Widely Used
- Non-toxic to animals and insects
- Short residence in the soil (Few days)
- Kills plants by inhibiting EPSP Synthase, leading to a deficiency in amino acids



Problem: It is broad spectrum
- Kills all plants, not just weeds
- Has to be applied carefully to fields (Time and money consuming)

Solution: Genetically engineered crops with resistant to glyphosate
- Entire fields can be sprayed without concern for the crops
- Saves time and money

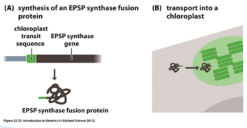
Engineering Process

Original Approach: Increase the amount of EPSP synthase produced by a crop
- Expectation= Plants would overproduce EPSP synthase enough to counteract the effects of glyphosate
- Result= Unsuccessful
  - Plants made up to 80% of the amount of EPSP synthase, however that was still not sufficient enough

New Approach: Transfer EPSP synthase genes from organism to naturally resistant organisms to crops
- *Agrobacterium* strain CP4
- Inserted into a cloning vector that fused with codons specific to a chloroplast transit sequence.
- Recombinant plasmid are introduced in soybean cells by biolistics.
- Plant cells are totipotent, therefore a single transformed cell gives rises to a mature plant with the new gene

These plants were three times as resistant

- This method is now being used on maize



Various GM Crops have been Produced by Gene Addition

- **Gene Addition:** introduction of genes into plants to create insect resistance.
- This technique is more environmentally friendly than techniques that were previously used.
- Gene Addition focused on δ-endotoxins of *Bacillus thuringiensis.*
- δ-endotoxins prevent bacteria from being ingested by insects.

Example: GM maize plants with resistance to the European Corn Borer.

- The caterpillar which tunnels into the plants after hatching from their eggs.
- The tunnels of the European Corn Borer were shortened greatly.

Various GM Crops have been Produced by Gene Addition

- Gene Addition has also been used to improve the nutritional quality of certain foods.

Plant genes can also be silenced by genetic modification.

In addition to the gene addition approach, a plant genetic engineering can be used to suppress a targetted gene via gene subtraction, the approach to suppress targetted genes partly or completely suppressed.

One of the most successful application of gene subtraction in plant genetic engineering is to inactivate ethylene synthesis with the antisense RNA technology, which has been used as a potential means of silencing disease genes by gene therapy.

This technology has been developed to mainly reduce crop spoilage.

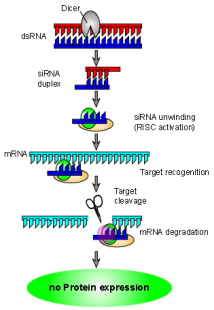
Inactivation of ethylene synthesis in plants

Ethylene acts as a hormone, a regulatory compound, involving in the later stage of fruit ripening in plants such as tomato.

synthesis of ethylene

S-adenosylmethionine (SAM)
↓
(ACC synthase)
1-aminocyclopropane-1-carboxylic acid (ACC)
↓
Ethylene

The strategy for delaying fruit ripening in tomato involved inactivation of ACC synthase enzyme, unabling the modified plants to complete the ripening process until treated with ethylene.



Use of engineered plants for the synthesis of recombinant proteins including vaccines

- Use of plants for this study since they have similar protein processing activities to those of animals
- By inserting the new gene downstream of a promoter that is active only in developing seeds, large amounts of recombinant protein can be obtained in a form that is easily harvested and processed.
- Usage of this process have been shown in crops like maize, tobacco, rice, sugarcane and potato tubers

Mass production of Recombinant proteins

- In general, plants offer a low technology means of mass producing recombinant proteins
- Possibility of plants used to synthesize vaccines, providing the basis for a cheap and efficient vaccination program
- If the recombinant vaccine is effective, after oral administration, acquire immunity simply by eating part or all of the transgenic plant
- Usage has been demonstrated in vaccines like HBsAg and capsid proteins of missile virus and respiratory syncytial virus.
- Immunity conferred by feeding the transgenic plant to test animals

Difficulties in the use of recombinants from plants as Vaccine

- Ensuring that the amount of recombinant protein synthesized by the plant is sufficient to stimulate complete immunity against the target disease
- Requires vaccine yields of 8-10% of the soluble protein content of the part of the plant that is eaten.
- In practice, yields achieved so far are much less that this range, usually not more than 0.5%.

Recombinant proteins can be produced in animals by pharming

Pharming: The process of producing recombinant proteins in transgenic animals

- Most successful in producing recombinant proteins in the mil of farm animals
  - This is accomplished by attaching the gene to an active promoter in the mammary tissue
  - The protein ten undergoes post-translational modifications and become fully activated
- The first recombinant protein obtained using this method was **antithrombin III**
  - Approved for medical use in humans to prevent blood clotting during heart surgery
  - Obtained from goat's milk
  - Led to various other blood proteins useful in the medical field to be synthesized this way.
    - Ex. Blood factor VIII