RECOMBINANT DNA TECHNOLOGY AND BIOTECHNOLOGY

Application of Biotechnology

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Introduction

Biotechnology essentially involves the industrial application of living organism to produce product or process for the betterment of humanity. Long before the new biology in the name of recombinant DNA technology came in to play; living organisms have been used for producing useful compounds. This involves production of alcohol using yeast and fermented food like curd. However the real exploitation of living organism started with the discovery of penicillin around 1930. Ever since the living organisms particularly bacteria, yeast, fungus and viruses have been exploited to produce many important biomolecules starting from simple chemicals like acetic acid, citric acid to antibiotics, vitamins hormones, enzymes, steroids and vaccines. The living organisms have also been used as a catalyst to carry out many enzymatic biotransformation reactions to produce complex organism molecules which otherwise are difficult to produce using chemical synthesis route. Apart from this, the metabolic activities of the living organism have been exploited to carry out new biotechnological process, notable among them have been extraction of minerals using *Thiobacillus* and waste water treatment process using methanogenic bacteria. The concept of exploiting living organism has been extended to plants and animal cell culture to produce special metabolites. Production of shikonin *in vitro* using plant cell culture and development of cell culture based viral vaccine are the important applications of biotechnology using higher classes of living cells. However with the introduction of recombinant DNA technology and better understanding of the genome organization, the application of biotechnology has crossed the species barrier. It has not only helped to improve the product yield of many old biopharmaceutical but has also made possible to produce human proteins in other living organisms like bacteria ,fungi and even in plants.

Biotechnology based on the principle of recombinant DNA technology started in early 1970 with Paul Berg of Stanford University producing the first recombinant DNA. This was followed by the generation of transformed *Escherichia coli* in 1973 by Herbert Boyer of University of California (San Francisco), which resulted in the production of recombinant human insulin by Eli Lilly in 1982. The practical reach of genetically modified organisms has grown considerably since then due to the possibilities to express virtually any kind of coding sequence from any possible source. Efforts have been made to genetically engineer most of the living systems such as bacteria, yeast, fungi, plant and animals to have novel gene product or characteristics. Thousands of genes have been cloned and expressed using recombinant DNA technology. The genetic manipulations using r-DNA technology are more precise and outcomes are more certain over other methods resulting in faster production of organisms with desired traits. Progress in molecular biology and genetic engineering techniques has made impact in two major areas (a) understanding the biology of the living system by manipulation of genome information and (b) production of useful metabolites or living organisms having desired metabolic characteristics. This has not only resulted in the production of specific biomolecules in different organism but also has helped to synthesize genetic material and its related product in the laboratory. In fact, the application of genetic engineering and recombinant DNA technology has led to the generation of new classes of organism called genetically modified organisms (GMO) or live modified organism (LMO). More so, the ability of genetic manipulation of almost all living organism has led to the genomics evolution with far reaching applications of the modern biology system. The most notable applications of the recombinant technology having direct impact on humanity have been:

1. Large scale production of therapeutic protein such as insulin, hormones, vaccine and interleukins using recombinant microorganisms.

2. Production of humanized monoclonal antibodies for therapeutic application
3. Production of insect resistant cotton plant by incorporation of insecticidal toxin of *Bacillus thuringiensis* (Bt cotton plant).
4. Production of golden rice (rice having vitamin A) by incorporating three genes required for its synthesis in rice plant.
5. Bioremediation by the use of recombinant organisms and
6. Use of genetic engineering techniques in forensic medicine.

The applications of the recombinant DNA technology had major impact on biopharmaceutical production and agriculture followed by controlling environmental pollution.

Application in industrial production of biomolecules

Human therapeutics from recombinant DNA technology

One of the greatest benefit of the recombinant DNA technology has been the production of human therapeutics such as hormones, growth factors and antibodies which are not only scarcely available but also are very costly for human use. Ever since the recombinant insulin was produced by Eli Lilly in 1982, considerable efforts has been made world wide to clone and express many therapeutically important proteins, which are otherwise difficult to produce either by extraction from the natural sources or by chemical synthesis. Therapeutic proteins are preferred over conventional drugs because of their higher specificity and absence of side effects. Therapeutic proteins are less toxic than chemical drugs and are neither carcinogenic nor teratogenic. Further, once the biologically active form of a protein is identified for medical application, its further development into a medicinal product involves fewer risks than chemical drugs. Notable diseases for which recombinant therapeutics have been produced include diabetes, hemophilia, hepatitis, myocardial infarction and various cancers. Recombinant therapeutics include proteins that help the body to fight infection or to carry out specific functions such as blood factors, hormones, growth factors, interferons and interleukins. Starting with simple protein such as insulin and then growth hormone, recombinant biopharmaceuticals has increased considerably in recent years. Till today, around 165 biopharmaceuticals (recombinant proteins, antibodies, and nucleic acid based drugs) have been approved. Table 1 lists the type of biomolecules that have been produced by the recombinant DNA technology. This includes hormones, growth factors, blood products, monoclonal antibody, enzymes and many others. Production using recombinant DNA technology has made these molecules available for the treatment of human diseases at a relatively lower cost. Availability of large amount of pure molecules has helped in development of its different modified form to have improved pharmacokinetic parameters. Pegylated proteins and controlled release formulation of biomolecules have become reality with improved characteristics. This has not only helped in cheap availability of the biomolecules for health care but also has led to development of new molecules having improved performances. The best example being different varieties of insulin analogs (long acting , slow release, acid stable etc). Others are being pegylated proteins such as peg-interferon and peg-antibodies and growth factors.

Table 1: Types of biomolecules produced through recombinant DNA technology

Recombinant Hormones
Insulin (and its analogs), growth hormone, follicle stimulating hormone, salmon calcitonin.

Blood products
Albumin, thrombolytics, fibrinolytics, and clotting factors (Factor VII, Factor IX, tissue plasminogen activator, recombinant hirudin)

Cytokines and growth factors
Interferons, interleukins and colony stimulating factors (Interferon, α, β and γ, erythropoietin, interlukin-2, GM-CSF, GCSF)

Monoclonal antibodies and related products
Mouse, chimeric or humanized; whole molecule or fragment; single chain or bispecific; and conjugated (rituximab, trastuzmab, infliximab, bevacizumab)

Recombinant Vaccines
Recombinant protein or peptides, DNA plasmid and anti-idiotype (HBsAg vaccine, HPV vaccine)

Recombinant Enzymes
Dornase– α (Pulomozyme), Acid glucosidase (Myozyme), α –L-iduronidase (Aldurazyme) and Urate Oxidase

Miscellaneous products
Bone morphogenic protein, conjugate antibody, pegylated recombinant proteins, antagonist

The market for recombinant therapeutics has considerably improved with generation of new molecules and new expression systems. Even though the overall world market has been around $30-40 billion it is expected to reach around $75 billion by end of this decade. In fact the major money producer has been few biomolecules such as insulin, erythropoietin, interferon and hormones. These few molecules take a major share of biopharmaceutical market (Table 2). Among these biomolecules, erythropoietin is the most valuable product followed by insulin, growth factors and interleukin. It is projected that erythropoietin market will be around $10 billion by next five years.

Success story of Insulin

Insulin is the most important molecule both from human health care and recombinant DNA technology point of view. It has a key role in regulation of glucose metabolism and thus is important for diabetes mellitus. Particularly for type 1 diabetes, which involves the loss of insulin producing β cells in the pancreas, injection of insulin is the primary therapy. Ever since its discovery and introduction as drug almost 85 years ago (Banting FG and Best CH *et al.* isolated the insulin around 1921), the lives of millions of people with diabetes have been saved, prolonged and improved. Initially, pig insulin and human insulin extracted from

whole body was used as the source of insulin, but the scenario changed after the successful expression of human insulin in 1980 in *E .coli*.

Table 2: Top ten recombinant therapeutic proteins and their global sales for the year 2003

Product (generic) / marketing company	**$ million**
Procrit (epoetin alfa, erythhopoetin)/ Johnson & Johnson	3,986
Epogen (epoetin alfa, erythhopoetin)/Amgen	2,435
Neupogen (filgrastim, GMCSF)/ Amgen	1.268
Neulasta (pegfilgrastim. Peg-GMCSF)/ Amgen	1,255
Novolin (Insulin systemic)/ Novo Nordisk	2,235
Avonex(Interferon beta-1a) /Biogen IDEC	1,170
PEG-Intron A franchise (pegylated interferon alpha) Schering Plough	1,851
Enbrel (Etanercept)/Amgen	1,300
Aranesp (darbepoetin alfa)/ Amgen	1,544
NeoRecormon (epoetin-beta)/ Roche	1,318
Top ten product sales	**18,362**
Total market value of recombinant product	**32,065**

Approximately, 194 million people worldwide have diabetes and it is estimated that the number of people with diabetes will be more than double by 2030 . The current healthcare costs could be as much as US $ 286 billion worldwide, with the majority of these costs linked to treating diabetes-related complications. The current market for injectable insulin is worth more than US$ 7.2 billion globally (17% growth, 2005 versus 2004). Four products represent 50% of the world's injectable insulin market; long-acting insulin glargine (Lantus; Sanofi-Aventis); ultra-short-acting insulin Lispro (Humalog; Lilly); biphasic insulin base / insulin isophane (Actraphane HM; Novo Nordisk). Lantus and Novorapid showed phenomenal annual growth rates in 2005; 47 % and 52 % respectively. In addition, two other fast growers are predicted to increase their market share; biphasic insulin aspart /insulin aspart potamine (Novomix; Novo Nordisk, 72% growth) and long – acting detemir (Levemir; Novo nordisk, 510% growth).

Insulin preparation

During its first 50 years of use, insulin was extracted from animal sources (bovine or porcine pancreas). Concerns about purity led to the production of highly purified, mono component insulin in the 1970s. In the 1980s, recombinant-DNA technology and the development of protein-engineering techniques led to the production of human insulin and modified insulin analogues with improved pharmacokinetic properties. Insulin is expressed in recombinant *E. coli* and then subsequently purified and refolded in to bioactive form. Cloning of insulin, expression in different host, fermentation process optimization, refolding and purification of

active protein from inclusion bodies and finally formulation of insulin having better pharmacokinetics has provided model system for production of most of the therapeutics using r-DNA technology. New methods of producing insulin were accompanied by advances in formulation, which led to the development of rapid-acting insulins for use at meal times and long-acting insulins for basal insulin requirements. In addition, a series of pre-mixed insulins that combine the various forms are in use. At present, about 180 individual insulin preparations are available worldwide.

Soluble insulin: This form of insulin is unmodified. An initial lag phase after injection is followed by a rise in plasma insulin levels that peaks after 1–3 hours and returns to basal levels within 6–8 hours.

Basal insulins: These insulins have a longer duration of action due to slower rates of absorption from subcutaneous tissue. This is achieved by reducing solubility at physiological pH by mixing with protamine (NPH insulin) or zinc (Lente insulins), by engineering insulin variants with increased isoelectric points (insulin glargine) or by covalent acylation (insulin detemir), which promotes reversible binding with albumin to create an insulin depot**.**

Rapid-acting insulins: These are produced by engineering insulin variants with amino-acid changes that reduce the tendency of the insulin molecules to self-associate. This facilitates more rapid absorption from the subcutaneous tissue. Examples include insulin lispro and insulin as part.

Pre-mixed insulins: Mixing soluble and NPH insulin does not alter the pharmacokinetic properties of the components. Several NPH-insulin–soluble-insulin pre-mixtures have been developed, and, more recently, pre-mixed formulations that include the rapid-acting insulins have been created. These preparations have gained widespread acceptance as a means of reducing the number of daily injections that are required. There are marked differences in prescribing habits within and between countries. Globally, pre-mixed insulins represent the largest sector (39%), followed by basal insulins (35%) and soluble insulin (26%).

Insulin analogues

In the early 1980s, human insulin produced by recombinant-DNA technology became available for the treatment of diabetes. Subsequently, protein engineering has been used to produce variant forms of insulin, known as insulin analogues, with modified amino-acid sequences and improved pharmacokinetic properties. A number of insulin analogues have now been licensed for treatment, including rapid-acting forms for use at meal times and long-acting insulins for basal requirements.

Rapid-acting analogues: The absorption of insulin after subcutaneous injection can be improved by increasing the rate of dissociation of insulin molecules into monomers. Insulin lispro and insulin aspart are rapid-acting analogues that have reduced self-association as a result of protein engineering. In insulin lispro, a lysine-proline (Lys-Pro) sequence at the end of the insulin-B chain is reversed, which creates steric hindrance and a reduced ability to self-associate. Insulin aspart incorporates an amino-acid change (Pro B28 to aspartic acid (Asp)) that also creates charge repulsion and steric hindrance due to a local conformational change at the carboxyl terminus of the B chain. Both are absorbed more rapidly than regular insulin and reduce post-prandial glucose excursions more efficiently. Because of their short-lived action,

adjustments in basal insulin levels are required to achieve improvements in overall glycaemic control.

Long-acting analogues: A constant low level of plasma insulin in the fasting and interprandial state is essential to maintain overall glycaemic control and to complement the rapid-acting insulins given at meal time. Long-acting insulin analogues to fulfil basal insulin requirements have been produced, either by introducing amino-acid changes that increase the isoelectric point of insulin and reduce its solubility at physiological pH, or by covalent acylation. Insulin glargine is long-acting insulin that contains two extra arginine molecules at the end of the B chain (Arg B31 and Arg B32) to alter the isoelectric point. A glycine substitution at A21 (A chain) was made to stabilize the molecule. After subcutaneous injection, insulin levels rise slowly to a plateau within 6–8 hours and remain essentially unchanged for up to 24 hours, suitable for once-daily administration. Acylation of the ε-amino group of Lys B29, as in insulin detemir ($N^{\varepsilon B29}$–myristoyl des (B30) human insulin), promotes reversible binding of insulin to albumin, thereby delaying its absorption from the subcutaneous tissue and transport across the capillary endothelium of skeletal muscle. This form of insulin is under development. C14-FA, myristoylic acid.

Insulin in many ways remains as the lead biopharmaceutical molecule. It was first commercialized in1982 using recombinant DNA technology. It was the first recombinant DNA molecule which was reengineered to have improved characteristics (fast acting and long acting insulin anlalogs are in industry). It has become the first recombinant DNA derived molecules which has been approved to be delivered by pulmonary route (Exubera, : nasal spray recombinant insulin by Fizer inc.).The world health organization (WHO) estimated around 170 million diabetic globally which will be almost doubled in next 25 years. Considering that around 10 % of these patients are type I diabetic and need insulin treatment, it is estimated that more than 5 tons of insulin will be needed globally with a sales target of $8- 10 billion. This necessitates the easy production and purification of these molecules.

The improvement in quality of life offered by an inhaled insulin option gives Exubera potential to take a significant share of the insulin market, competing mainly against short acting insulins such as Novorapid. This convenient delivery system could also increase the size of potential market by competing with oral anti-diabetics (a market value at US$10.6 billion in 2005), with patients able to switch earlier from oral anti-diabetics to insulin. Exubera is the first non-injectable insulin therapy to be approved; this fact, combined with the growing incidence of diabetes, has led some analysts to predict that Exubera will reach sales of US$0.6-1 billion by 2008, although estimates are divided given uncertainties around the drug's side effects. There are three other inhaled insulin products in phase III trials that could be available by 2010.Two of these are dry-powder products – AIR insulin (Alkermes/Lilly) and the Technosphere insulin system (Mankind) - and the third is a liquid formulation, the AERx Insulin system (Novo Nordisk).

Hosts used for recombinant protein production

Wide varieties of living organism have been tried for the production of recombinant proteins (Table 3). The selection of a particular organism depend on the expression level, quality of biomolecules, safety, manipulation of genetic system and finally the cost of production. The two most important recombinant biomolecules, insulin and erythropoietin were produced using *E. coli* and mammalian (CHO cells) respectively. Most of the recombinant

biomolecules are produced industrially using these two organism followed by yeast. For production of simple biomolecules which do not require posttranslational modification (insulin, growth hormone and proteins), *E. coli* or bacterial expression system works good. It is easy to manipulate and can be grown in relatively cheap medium. In *E .coli,* expression level of around 10g/L of the protein can be achieved both using strong promoter and high cell density fermentation process. Expression of recombinant protein in *E. coli* most of the time results in accumulation of the protein in denatured form as inclusion bodies. Protein recovery from inclusion body aggregate is cumbersome, empirical and is an expensive process. However due to very high level of expression of recombinant protein in *E. coli,* the loss during is recovery is compensated to some extent. For a recombinant protein whose refolding procedures are easy and straight forward, expression as inclusion body provided advantages of easy purification. Many proteins where gycosylation is not necessary for biological function have been expressed as inclusion bodies in *E. coli* and subsequently refolded in to bioactive conformation.

Table 3: Hosts for Recombinant Protein Production

S.No.	Hosts
1.	*Escherichia coli*
2.	*Bacillus subtilis*
3.	*Streptomyces*
4.	*Saccharomyces cerevisiae*
5.	*Pichia pastoris*
6.	*Aspergillus*
7.	Animal cells (CHO, SP20/NSO)
8.	Insect cells: Baculovirus system
9.	Transgenic Animals
10.	Transgenic Plants

For production of complex protein such as glycoprotein, mammalian expression system is the best host. Mammalian cell culture is complex process, time consuming and expensive in term of medium requirements. In spite of these disadvantages in comparison to the microbial system, mammalian cells have been most extensively used for the production of glycoprotein and monoclonal antibodies. CHO cell have been most extensively used for the production of glycoprotein followed by yeast, insect cell and plant based expression system. Protein molecules requiring both O-linked and N linked glycosylation can be produced using CHO cell lines as expression system. Glycosylation influences, immunogenicity, ligand binding, stability, serum half life and to some extent efficacy of the recombinant molecules thus is very important in the case of hormones, blood factors and monoclonal antibody production. With the advancement in expression construct, understanding of animal cell metabolism and physiology recombinant protein yield up to the extent of 5 g/L has been achieved. It is expected that in spite of the complex nature of growth of animal cells will be more frequently used as host for the production of recombinant proteins. In fact, more than 20 recombinant biomolecules have been produced using CHO cell lines.

Yeast and filamentous fungi have also been extensively used for the production of recombinant proteins. Among them, yeast has been the most widely used fungus for production of proteins and vaccines (because of its easy growth and glycosylation capacity). Plant cells and transgenic animals have been extensively explored to produce complex biological molecules with little success so far. Plants produce protein with complex pattern where as in transgenic animals there is variability in protein structure during production. Recently monoclonal antibody has been expressed in egg up to an yield of 1-3 mg/single egg. The only success story so far using transgenic system is the recombinant antithrombin produced in goat by GTC Biotherapeutics.

Safety of the recombinant biomolecules

Irrespective of the expression system used for the production of recombinant molecules, all biotherapeutics share major safety concern. This is due to inherent nature of the biological molecules, where the conformation of the molecule is more important along with the chemical nature. Thus, any biomolecules manufactured using a living system need complete characterization and safety evaluation. The most important criteria during the manufacturing of recombinant molecules using live organism are the quality, safety and efficacy. All these three parameters influence the toxicity and immunogenicity of the final product and thus needs critical evaluation. Table 4 highlights some of the parameters associated with different types of product. It is most essential to verify this parameter so that purity, safety and efficacy is maintained not only during production but also during storage and application. It is also essential that all precautions are taken at each step of biological production taking care of the biosaftey aspects. The host organism and waste product coming out of the r-DNA process need to be processed according to the environmental protection act of the country so that biodiversity is not affected.

Table 4: Risk associated with recombinant biomolecules

S.No.	Biomolecule	Associated risk
1.	**Recombinant DNA (r DNA) protein**	Quality, stability, Species specificity, vector and transgene toxicity, including immunogenicity and species relevance for activity, toxicity related to the pharmcodynamic effects, biological toxicity, local tolerance, oncogenicity
2.	**Vaccines**	Frequency or duration of therapy, local reactogenicity, tissue cross reactivity with antigen of interest, induction of aberrant immune response and adjuvant toxicity, induction of tolerance
3.	**Monoclonal antibodies**	Cross reactivity with target and non-target tissues(in human and relevant animal species), immunogenicity, conjugate and/or linker toxicity (inherent toxicity of conjugate; bystander effect)
4.	**Modified proteins**	Above considerations for active ingredient, comparative kinetics and toxicity with unmodified protein and duration of effect/persistence, immunogenicity, bioavailability

Human monoclonal antibody

Monoclonal antibodies (Mab) are very specific immunoglobulin that exhibit a wide range of biological activities. In addition to use in diagnostics, antigen binding sites of antibody molecules have great potential for developing bioactive peptides Because of their specific ligand binding activity, they were considered as the magic bullets as hypothesized by Paul Ehrlich. Hybridoma technology, which used the fusion of myoeloma and B cells, helped in the *in vitro* production of monoclonal antibody. However, this technology developed by Kohler and Milestein was not very helpful as most of the antibodies were of murine origin and have the problems of their low immunogenicity. Application of recombinant DNA technology resulted in development of chimeric and humanized antibody with high efficiency and activity. Because of their efficacy in cancer, there have been tremendous activities in developing monoclonal antibodies for human therapy. In humans, antibodies are classified as member of five family or isotypes. These are named as immunoglobulin alpha, (IgA), delta (IgD), epsilon (IgE), gamma (IgG) and mu (IgM). Most of the isotypes have molecular weight around 160-190 kD except IgM whose molecular weight is around 1000kD due to its pentameric nature. The most prevalent antibody in human is IgG and majority of the therapeutic antibodies are of IgG types.

Even though antibodies act on wide varieties of pathways, therapeutic antibodies work on one of the following four ways (a) as immunotoxicotherapy where the antibody prevent or reverse toxic effect of venom, toxin, drug or ligand (b) destruction of target cell, where the antibodies are used to destruct the target cell such as lymphocytes, cancer cells etc. (c) alteration of the cell function and finally (d) antibody mediated drug delivery, where the drug is conjugated with the antibody for specific targeting. For the large-scale production of monoclonal antibodies, expression of monoclonal antibody genes is accomplished through recombinant DNA technology. More than 20 monoclonal antibodies have been approved for human uses. Table 5 lists some of the important monoclonal antibodies as a result of recombinant DNA technology. It is expected that in near future, due to production of humanized antibodies using recombinant DNA technology, there will be many more Mab in the market.

Application of Recombinant Technology in Agriculture

The genetic manipulation of plants has been going on since prehistoric times, when early farmers began carefully selecting and maintaining seeds from their best sow for the next season. Plant breeders have cross fertilized related plants to provide next generation plants with new characteristics such as higher yield, resistance to diseases and better nutrient content long before the science of genetics was developed. Recombinant DNA technology can be used for insertion of genes in plants not only from related plant species, but also from unrelated species such as microorganisms. This process of creation of transgenic plants is far more precise and selective than traditional breeding. Application of recombinant technology is primarily for the production of transgenic plants with higher yield and nutritional values, increased resistance to stress and pests. Several commercially important transgenic crops such as maize, soybean, tomato, cotton, potato, mustard, rice etc. have been genetically modified. During the last couple of decades, considerable progress has been made to understand the function of genes, isolation of novel genes and promoters as well as the utilization of these genes for the development of transgenic crops with improved and new characters. There are many potential application of plant genetic engineering. In fact, in 2002, more than 5.5 million farmers worldwide cultivated about 58.7 million hectares (about 148 million acres) crops that were genetically manipulated for herbicide tolerance, insect resistance, delayed fruit ripening and improved oil quality. Application of recombinant DNA

technology has primarily helped in producing three major types of transgenic plant having improved performances. These are:

(1) Development of stress tolerant plant
(2) Development of plant having improved yield
(3) Transgenic plant as a source of biopharmaceuticals

Table 5: Therapeutic monoclonal antibodies (Mab) approved for use in oncology

S.No.	Generic name (trade name)	Mab type	Indication	Sponsor company	Year of FDA approval
1.	Trastuzumab (Herceptin)	Humanized	Breast cancer	Genentech	1998
2.	Rituximab (Rituxan)	Chimeric	Lymphoma	Biogen/ IDEC	1997
3.	Alemtuzumab (Campath-1H)	Humanized	Chronic lymphatic leukemia	Millennium ILEX	2001
4.	Cetuximab (Erbitux)	Chimeric	Colorectal cancer	Imclone System	2004
5.	Bevacizumab (Avastin)	Humanized	Lung cancer	Genentech	2004
6.	Inflixmab (Remicade)	Chimeric	Crohn disease	Centocor	1998
7.	Muromonab-CD3 (Orthoclone OKT3)	Murine	Transplant rejection	Ortho Biotech	1986
8.	Abciximab (Reopro)	Chimeric	Prevention of blood clot	Centocor	1994
9.	Adalimumab (Humira)	Human	Rheumatoid arthritis	Abbot, USA	2002
10.	Daclizumab (Zenapex)	Humanized	Kidney transplant rejection	Hoffmann-la Roche	1997
11.	Omalizum (Xolair)	Humanized	Asthma	Gnentech	2003

Development of stress tolerant plant

(a) Plant resistant to environmental stress: Plants need to cope up with abiotic stresses such as drought, cold, heat and soils that are too acidic or salty to support plant growth. While plant breeders have successfully incorporated genetic resistance to biotic stresses into many crop plants through crossbreeding, their success at creating crops resistant to abiotic stresses has been more limited, largely because few crops have close relatives with genes for resistance to these stresses. Therefore rDNA technology is being increasingly used to develop crops that can tolerate difficult growing conditions. Genetically modified tomato and canola

plants that tolerate salt levels 300 percent greater than non-genetically modified varieties have been developed. Other researchers have identified many genes involved in cold, heat and drought tolerance found naturally in some plants and bacteria. Scientists in Mexico have produced maize and papaya that are tolerant to the high levels of aluminum that significantly impede crop plant productivity in many developing countries.

(b) Herbicide Resistant plant: Many effective broad spectrum herbicides do not distinguish between weeds and crops, but crop plants can be modified to make them resistant to herbicides, so as to eliminate weeds more selectively. For example, the herbicide Roundup^{TM} contains the active ingredient glyphosate, which kills plants by binding to the active site of enzymes called enolpyruvalshikimate phosphate synthase (EPSP synthase). This enzyme is critical for the synthesis of aromatic amino acids. Roundup is an extremely effective herbicide but it kills almost all species of plants, including most crop plants. On the other hand, it is very safe for humans and animals because they do not have EPSP synthase. By using rDNA technology, modified EPSP synthase gene (that produced enzymes that were still functional but were not inhibited by glyphosate) have been introduced into crop plants such as cotton and soyabean. These genetically modified plants were found to be highly resistant to treatment with Roundup. Genes that provide resistance to other herbicides such as sulfonyl ureas, gluphosinates etc. have also been developed and transferred to produce various transgenic plants.

(c) Insect resistant plant: To minimize crop damage by insects, mites and nematodes, farmers use synthetic pesticides extensively which cause severe effects on human health and environment. The transgenic technology provides an alternative and innovative method to improve pest control management which is eco friendly, effective, sustainable and beneficial in term of yield. This involves genetic incorporation of toxic gene (product of which is lethal to insect) in to the plant. This kill the insects without use if dangerous insecticide thus has double benefit in crop improvement. The first genes available for genetic engineering of crop plats for pest resistance were *Cry* genes (popularly known as Bt genes) from a bacterium *Bacillus thuringiensis.* These are specific to particular group of insect pests, and are not harmful to other useful insects like butter flies and silk worms. Transgenic crops (e.g. cotton, rice, maize, potato, brinjal, cauliflower, cabbage etc.) with Bt genes have been developed and such transgenic varieties proved effective in controlling the insect pests and it has been claimed worldwide that it has led to significant increase in yield along with dramatic reduction in pesticides use. The most notable example is Bt cotton (which contains *Cry/Ac* gene) that is resistant to a notorious insect pest Bollworm (*Hellicoperpa armigera*) and only last year (2002) Bt cotton was adopted in India.

Biotechnology has opened up new avenues for natural protection for plants by providing new biopesticides, such as microorganisms, that are toxic to targeted crop pests but do not harm humans, animals, fish, birds or beneficial insects. As biopesticides act in unique ways, they can even control pest population that have developed resistance to conventional pesticides. Using recombinant DNA technology, the gene that makes these microorganisms lethal to certain insects can be transplanted into the plants on which that insect feeds. The plant that once was a food source for the insect now kills it, lessening the need to spray crops with chemical pesticides to control infestation. One such microorganism is commonly found soil bacterium *Bacillus thuringiensis.* The spores of *Bacillus thuringiensis* (Bt) contain a crystalline protein (Cry) which breaks down to release a toxin, known as delta-endotoxin which is highly toxic to lepidopteran larvae. This toxin binds the intestinal lining and creates pores resulting in an ion imbalance, paralysis of the digestive system, and consequent death

of the insect. Bt toxin sprays and powders have been in use for many years. Different Cry genes, also known as Bt genes have been identified, cloned and characterized. Effective gene constructs have made it possible to deliver these genes into plant tissues so that they are expressed at levels high enough to kill the insects. The Bt genes are effective against different orders of insects. Bt cotton and maize which have increased resistance to boll worms have been developed and cultivated since 1996. Farmers get benefited by saving costs by using less of traditional pesticides. However, one of the major concerns about Bt based transgenics is the possibility of development of toxin resistant insects. Efforts are also underway to identify and transfer other genes from Bt, which can impart insecticidal properties to the plants. One example in this is transfer of vip gene i.e. vegetative insecticidal proteins, for which the trials are being conducted in some countries.

(d) Disease resistance plant: Plants are susceptible to viral, bacterial and fungal diseases. Much progress has been made in evolving transgenic plants resistant to viruses. For example, expression of a gene that encodes the coat protein of tobacco mosaic virus (TMV) in transgenic tobacco plants has been shown to cause the plants to resist TMV infection. A number of other viral resistant plants species have been developed including squash and potatoes. Genetic engineering of crop plants for resistance to fungal and bacterial infections has been more difficult. However, by studying the protective genes that are expressed in naturally disease-resistant plants, an encouraging progress has been made. The proteins encoded by these so called pathogenesis related proteins (PR proteins) can, in some cases, provide limited disease protection in transgenic plants. There are several strategies for engineering plants for viral resistance and these utilizes the genes from virus itself (e.g. the viral coat protein gene). The virus-derived resistance has given promising results in number of crop plants such as tobacco, tomato, potato, alfalfa, and papaya. Some viral resistant transgenic plants like papaya resistance to papaya ring spot virus have been commercialized in some countries. Plants respond to pathogens by inducing a variety of defense responses like pathogenesis-related proteins (PR proteins), enzymes that degrade/destroy fungal cell wall (chitinase), antifungal proteins and compounds, phytoalexins, etc. Several transgenic crop plants showing increased resistance to fungal pathogens are being raised with genes coding for the different compounds mentioned above.

Development of plant having improved yield

(a) Increasing yield

In addition to increase crop productivity by using built-in protection against diseases, pests, environmental stresses and weeds to minimize losses, attempts are being made to use biotechnology to improve crop yields directly. Researchers at Japan's National Institute of Agrobiological Resources added maize photosynthesis genes to rice to increase its efficiency of converting sunlight to plant starch and increased yields by 30 percent. Other scientists are altering plant metabolism by blocking gene action in order to shunt nutrients to certain plant parts. Yields increase as starch accumulates in potato tubers and not leaves, or oilseed crops, such as canola, allocate most fatty acids to the seeds. Crops that have better accessibility to the micronutrients they need are also being developed. Mexican scientists have genetically modified plants to secrete citric acid, a naturally occurring compound, from their roots. In response to the slight increase in acidity, minerals bound to soil particles, such as calcium, phosphorous and potassium, are released and made available to the plant. Nitrogen is the critical limiting element for plant growth and researchers from many scientific disciplines are tearing apart the details of the symbiotic relationship that allows nitrogen-fixing bacteria

to capture atmospheric nitrogen and provide it to the plants that harbor them in root nodules as given below:

(1) Plant geneticists in Hungary and England have identified the plant gene and protein that enable the plant to establish a relationship with nitrogen-fixing bacteria in the surrounding soil.
(2) Microbial geneticists at the University of Queensland have identified the bacterial gene that stimulates root nodule formation.
(3) Collaboration among molecular biologists in the European Union, United States and Canada yielded the complete genome sequence of one of the nitrogen-fixing bacteria species.
(4) Protein chemists have documented the precise structure of the bacterial enzyme that converts atmospheric nitrogen into a form the plant can use.

(b) Increase in quality of plant products

One of the most successful research efforts to change the characteristics of a plant produce was carried out with tomatoes. Tomatoes need to be picked while still green so that they are firm enough to withstand mechanical handling and transport. Unfortunately, they do not develop the same flavor and texture of vineripened tomatoes. Softening of tomatoes and many other fruits is caused by the enzyme pectinase or polygalacturonase (PGA). This enzyme digests the pectin polysaccharide that cements the plant cells together. Softening of the fruit is caused, in part by this breakdown of pectin. In order to reduce the levels of PGA in ripening tomatoes, researchers placed the PGA gene in reverse orientation relative to the CaMV 35S promoter. This results in transcription of an antisense RNA that is complementary to the normal sense PGA mRNA. Although the exact mechanism is unknown, antisense RNA is able to arrest the translation of the endogenous PGA mRNA in the tomato fruit. Transgenic tomato plants that express an antisense PGA gene only have about 5 to 10% of normal PGA levels. Fruits of these plants have normal color and flavor but they soften more slowly and can be picked and processed after they are ripe. They also have a higher content of soluble solids and are therefore better than normal tomatoes for processed tomato products. Transgenic lines of potato having increased levels of starch also have been developed by introducing a gene construct that expresses a gene from bacteria that produce an enzyme that enhances starch biosynthesis. A promoter from a potato gene that encodes the major protein in potato tubers has been used, so that the expression of the introduced gene is limited to the tuber. Tubers accumulate approximately 3 to 5% more starch than normal potatoes and when they are deep fried absorb less oil and yield chips having fewer calories. Some of the other value added transgenic crops include:

(a) Golden rice: containing beta carotene to overcome vitamin A deficiency in regions where rice is the staple food
(b) Canola containing high levels of oleic acids and laurate
(c) Barley containing feed enzymes
(d) tomatoes which does not rot in room temperature
(e) Other vegetables and fruits with delayed ripening as well as modified flavour characteristics

Transgenic crops with improved nutrition quality have already been produced by introducing genes involved in the metabolism of vitamins, minerals and amino acids. Few examples of genetic modification of nutritional quality are described below.

***Vitamin A*:** Vitamin A deficiency can lead to night blindness and skin disorders, among others. About 124 million children worldwide are deficient in vitamin A and a quarter of a million go blind each year due to vitamin A deficiency.

The staple food rice is extremely low in vitamin A, and therefore the improvement of vitamin A content is very important. In a remarkable example of genetic engineering, Prof. Ingo Potrykus and Dr. Peter Beyer developed genetically engineered rice (popularly known as 'Golden Rice'), which is enriched in pro-vitamin A by introducing three genes involved in the biosynthetic pathway for carotenoid, the precursor for vitamin A. The seeds of Golden Rice are yellow in colour because of pro-vitamin A is produced in the entire grain.

Seed Protein Quality: The Nutritional quality of cereals and legumes are limited because of deficiency of the essential amino acids, i.e. lysine in cereals, and methionine and tryptophan in pulses. Two genetic engineering approaches have been used to improve the seed protein quality. In the first case, a transgene (e.g. gene for protein containing sulphur rich amino acids) was introduced into pea plant (which is deficient in methionine and cysteine, but rich in lysine) under the control of seed specific promoter. In the second approach, the endogenous genes are modified so as to increase the essential amino acids like lysine in the seed proteins of cereals.

The gas hormone, ethylene is involved in the regulation of fruit ripening. Therefore, ripening can be slowed down by blocking or reducing ethylene production. This can be achieved by introducing ethylene forming gene(s) in a way that will suppress its own expression in the crop plants. Such fruits ripen very slowly (however, they can be ripen by ethylene application) and are very important for export to longer distances without spoilage as they show longer-self life due to slow ripening. A notable example of this kind is the '**Flavr Savr**' transgenic tomatoes, which were commercialized in U.S about 6 year ago.

Transgenic plant as a source of bio pharmaceuticals

Plants are among the most efficient bioreactors which produce quantities of material with sunlight and soil based nutrients as inputs. Attempts are being made to replace the traditional fermentation procedure for the production of biopharmaceuticals to plant based production. The benefits of using plants are the ability to increase production at low cost by planting more acres, rather than building fermentation capacity, lower capital and operating cost, simplified downstream processing etc. Therapeutic drugs to treat cancer, infectious diseases, autoimmune diseases, cardiovascular diseases and other conditions and several vaccines can potentially be grown in plants. Plant transgenic technology is being used to produce a *plant* that will generate a *seed* that expresses a desired *therapeutic protein*. This seed can *propagate* under the right growing conditions to yield plants and seed stock for producing the desired protein. The desired protein can be extracted from the seed to make a biopharmaceutical. Plant based therapeutics are expected to be much more cost effective. For example, Dow Plant Pharmaceuticals is using corn to grow pharmaceuticals by designing and selecting the plant which will contain the active pharmaceutical within the endosperm seed compartment. Benefits of producing the pharmaceuticals in the corn include long term storage advantage, easier purification in view of limited number of soluble seed proteins in a corn seeds, low microbial load, low proteolytic activity and specialized promoters to enable expression of the protein in specific parts of the plants.

Therapeutic proteins, enzymes and diagnostics

Transgenic plants can also produce a variety of proteins used in diagnostics for detecting human diseases and therapeutics for curing human and animal diseases in large-scale with low cost. The monoclonal antibodies, blood plasma proteins, peptide hormones and cytokinins are being produced in transgenic plants and their parts such as tobacco (in leaves), potato (in tubers), sugarcane (in stems) and maize (in seed endosperm). Plants are amazing and cheap chemical factories that need only water, minerals, sun light and carbon dioxide to produce thousands of sophisticated chemical molecules with different structures. Given the right genes, plants can serve as bioreactors to modified or new compounds such as amino acids, proteins, vitamins, plastics, pharmaceuticals (peptides and proteins), drugs, and enzymes for food industry and so on. Some of the potential and remarkable examples of this kind are described here.

Edible vaccines

Crop plants offer cost-effective bioreactors to express antigens which can be used as edible vaccines. The genes encoding antigenic proteins can be isolated from the pathogens and expressed in plants and such transgenic plants or their tissues producing antigens can be eaten for vaccination/immunization (edible vaccines). The expression of such antigenic proteins in crops like banana and tomato are useful for immunization of humans since banana and tomato fruits can be eaten raw. The edible vaccines that are produced in transgenic plants have great advantages like the alleviation of storage problems, easy delivery system by feeding and low cost as compared to recombinant vaccines produced by bacterial fermentation. Vaccinating people against dreadful diseases like cholera and hepatitis B by feeding them banana/ tomato, and vaccinating animals against important diseases such as foot and mouth disease by feeding them sugar beets could be a reality in the near future.

Metabolic engineering and Secondary products

Plant biotechnology will lead to improved plant sources for the production of valuable secondary metabolites mentioned in previous section on cell culture products. Over-expression of the gene which encode for the first enzyme in a pathway generally results in higher levels of the desired end product, and this has been successfully done in the enhancement of taxol production from the transformed tissue cultures of *Taxus* sp. Another strategy involves use of *Agrobaccterium rhizogenes* to induce the excessive formation of secondary roots in plants that normally produce useful secondary metabolites in this region. Transgenic plants can be used as factories to produce polyhydroxy butyrate (PHB, biodegradable plastic). Genetically engineered *Arabidopsis* plants produced PHB globules exclusively in their chloroplasts with out effecting plant growth and development The large scale production of PHB may be easily achieved in tree plants like populus, where PHB can be extracted from leaves. Industry has already begun to explore the production of biodegradable plastics from transgenic plants.

Application of Recombinant DNA in Environment

A vast majority of applications of environmental biotechnology use naturally occurring microorganisms (bacteria, fungi, etc.) to identify and filter manufacturing waste before it is introduced into the environment. Bioremediation program involving the use of microorganisms are currently in progress to clean up contaminated air, tracks of land, lakes and waterways. Recombinant technology helps in improving the efficacy of these processes so that their basic biological processes are more efficient and can degrade more complex

chemicals and higher volumes of waste materials. Recombinant DNA technology also is being used in development of bioindicators where bacteria have been genetically modified as 'bioluminescors' that give off light in response to several chemical pollutants. These are being used to measure the presence of some hazardous chemicals in the environment. Other genetic sensors that can be used to detect various chemical contaminants are also undergoing trials and include sensors that can be used to track how pollutants are naturally degrading in ground water. For example when gene such as the mercury resistance gene (*mer*) or the toluene degradation (*tol*) gene is linked to genes that code for bioluminescence within living bacterial cells, the biosensor cells can signal extremely low levels of inorganic mercury or toluene that are present in contaminated waters and soils by emitting visible light, which can be measured with fiber-optic fluro meters.

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