

Biotechnology: Discoveries and Their Applications in Societal Welfare



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Abstract The present chapter describes the field of biotechnology from earliest known mention till modern-day developments. The introduction throws light on understanding the practice of using microorganisms for making food since prehistoric times and covers aspects on key discoveries in recent past like vaccines and drugs. The section journeys through important seminal contributions that laid the foundation of many branches of biotechnology which would eventually be applied for the benefit of the population. The gradual advancement in the field of knowledge about life, its complexities and processes governing these led to the generation of medicines, diagnostic tests, industrially important materials and environmentally sustainable products in future years. Biotechnology can be classified based on the broad spectrum of deliverables it caters to the society. Starting with the healthcare, which constitutes red biotechnology, the text details significant discoveries and inventions that have greatly enhanced biomedical research. Green biotechnology, mentions important methods which have been applied in the field of crop and livestock improvement to ensure food security. Blue biotechnology, an emerging area enlists and highlights important marine genetic resources which are being adapted for various demands of global economy. Finally, the last section details the application of biotechnology in the field of industry and environment for the generation of better raw materials and its clean-up, respectively.

Keywords Biotechnology · Health care · Gene · Agriculture · GM · Marine · Drugs · Environment · Industry

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1 Introduction

Biotechnology is the integrated use of biochemistry, microbiology, and engineering sciences in order to achieve technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof. (European Federation of Biotechnology 1981)

Biotechnology means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use. (Convention on Biological Diversity, United Nations 1992)

The term biotechnology has been defined in different ways by people and organizations. Scientific community by and large agrees that the science at its core involves the usage of other life forms for human betterment. Since time immemorial, people across various cultures have been using living organisms as food or medicine either in their natural or processed forms. These included whole microbes or parts of plants, animals or their derived products. Processing of raw food items required more elaborate methods which employed whole organisms, e.g. yeast. The technique of fermentation in preparation of edible items (e.g. bread, cheese, curd, wine, etc.) was a traditional knowledge across various civilizations. Since 6000 B.C., herdsmen of Central Asia knew the art of curd/yogurt making by carrying the milk in animal stomachs. Around the same time, the people of Babylon and Sumer (present-day Iraq) had the know-how of wine making by utilizing yeast. During 4000 B.C., people of ancient Egypt employed yeast in bread making (Bud 1993). Chinese in 500 B.C. used poultices made up of curds having growth of moulds in it as a source of antibiotics for curing boils, and farmers in Ukraine applied cheese moulds on wounds to cure infection. At around 470 B.C., Greek philosopher Socrates expounded the observation of shared features between the parents and their offspring. Another Greek philosopher Aristotle in around 300 B.C. stated that children inherit traits from their father (Daston and Lunbeck 2011).

Life in primitive times was principally dependent on agriculture and livestock. Man began collecting wild plants for consumption since 7000 B.C. First organized method of agriculture dates back to 3000 B.C. in China where crop rotation was practiced. Since then, man started observing plants closely. Theophrastus (322 B.C.) mentioned about the diseases affecting crop plants and pointed out bad air and nutrition as their cause (Singh 2018). Domestication of crop plants was a significant achievement as it ensured food security by preserving the seeds for next seasonal round of sowing. Animals that were domesticated for the first time were mainly cattle, sheep and goats as they helped in the cultivation of crops and also provided milk and meat. To carry forward the generation of animal, people began paying attention to animal breeding methods whereby they learned simple mating process that helped in propagation of livestock. Although, people at that time were unaware of the causative agent(s) behind these processes, yet they possessed experiential knowledge. As the thought process evolved, people began to provide a more rational explanation for biological phenomena they observed. Centuries later, from 1700 A.D. onwards numerous milestone discoveries were made that led to the improvement in our understanding

of heredity, causes and cures of diseases, better agricultural practices, etc. Important contributions in the field of basic biology expanded the horizon of knowledge leading to identification of their translational potential (Fig. 1). Many of the older practiced methods were better explained later when people began scientific quest to Understand natural processes. For example, fermentation was explained by Louis Pasteur who in multiple experiments demonstrated bacteria caused souring of milk due to lactic acid formation (Pasteur 1879). Around the same time, Edward Jenner’s discovery of smallpox vaccine was based on the observation of acquired immunity and heralded a new era of immunization-based preventive medicine (Riedel 2005). The curing of the wound infection was achieved through curd or cheese moulds in prehistoric times without knowing that poultices made up of them contained antibiotics. Alexander Fleming (1928) purified penicillin antibiotic from the moulds and demonstrated its curing abilities of bacterial infections. The discovery is considered to be one of the greatest scientific achievements in post World War II era as it was able to save countless lives (Aldridge 1999).

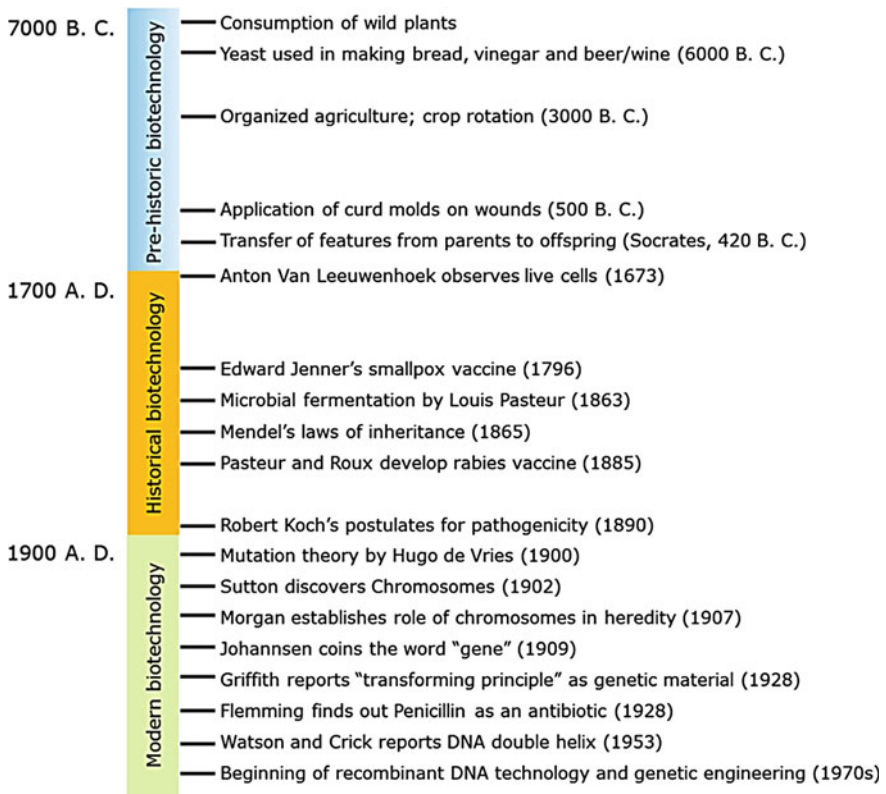


Fig. 1 Brief timeline of events in the development of biotechnology

2 Historical Biotechnology—1700s Onwards

This section describes selected milestone inventions and discoveries that influenced the society in the much needed way in those times. Hunger and disease have been the major challenges before the society since ever. The population was experiencing fatalities caused by several infectious diseases during the eighteenth and nineteenth centuries, e.g. smallpox, tuberculosis, rabies, etc. For most of the diseases, their causative agents were unknown at that time. Identification of disease pathogens and preventive immunization were major scientific breakthroughs of those times which greatly improved public health and increased life expectancy.

2.1 *Smallpox Vaccine*

Smallpox was the deadliest of all diseases of classical times that had the potential to decimate vast majority of population as can be read in history. Edward Jenner, an English physician, got the idea of developing a crude vaccine after observing that milkmaids working on a cow farm were immune to smallpox virus. This made him reason that may be the exposure of milkmaids to cowpox made them resistant to the smallpox virus and therefore such a challenge to a healthy individual may lead to a similar outcome. Jenner liked to experiment this idea for which he inoculated pus taken from a milkmaid suffering from cowpox into the arm of a boy named James Phipps who was found immune many days later. The observations were submitted to the Royal Society in 1797 (Plotkin 2014). Jenner established the field of vaccinology which has now become one of the exciting areas of biotechnology research with great commercial potential.



Edward Jenner. Photo Courtesy of the History of Medicine Division at the U.S. National Library of Medicine

2.2 *Rabies Vaccine and Germ Theory of Disease*

In 1885, another vaccine, this time against rabies was developed by Louis Pasteur and Emile Roux. Rabies developed as a result of bites from the dogs that were carrying rabies virus in their saliva. The virus is known to attack central nervous system that triggers encephalitis and kills the infected person. The vaccine was a great boon for the society because until then all rabies infections led to death (Hicks et al. 2012). The modern understanding of diseases came in the late nineteenth century from the studies done by Louis Pasteur and Robert Koch. Pasteur contributed to the development of “germ theory of diseases” which Koch later extended through his findings. Germ includes any microscopic pathogen like bacteria, virus and protists. The theory states that infectious diseases are caused by the growth and multiplication of germs after they invade human or animal body. Pasteur discovered the theory while examining a patient with puerperal fever whose blood was infected with pyogenic vibrio and suggested the use of boric acid as a potent and safe antiseptic to prevent the growth of germs (Ernst 2014). Germ theory of disease is still acceptable in medical sciences and has greatly improved the understanding of disease aetiology which has led to many preventive strategies and therapeutic interventions.

2.3 *Koch's Postulates*

Dr. Robert Koch, a German physician, in 1882, put forth his famous theory of establishing a link between the disease and the pathogen. He synthesized four important parameters from his studies on tuberculosis (TB) that became gold standard for judging a pathogen for its role in any disease. It must be noted that contemporary research on tuberculosis was unable to identify pathogen causing the disease, and Koch's postulates came out from the studies in which Robert Koch systematically showed the presence of the stained bacillus in the tuberculous tissue. Following are the Koch's postulates:

- (1) The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
- (2) The microorganism must be isolated from a diseased organism and grown in pure culture.
- (3) The cultured microorganism should cause disease when introduced into a healthy organism.
- (4) The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Until now, TB was wreaking havoc leading to mass deaths due to its spread; therefore, in the light of Koch's findings, public was provided better treatment and care. TB sanatoria were built where the patients were kept in isolation to prevent further

transmission (Walker et al. 2006). Koch was awarded Nobel Prize for Medicine and Physiology in 1905 in recognition of his contribution.

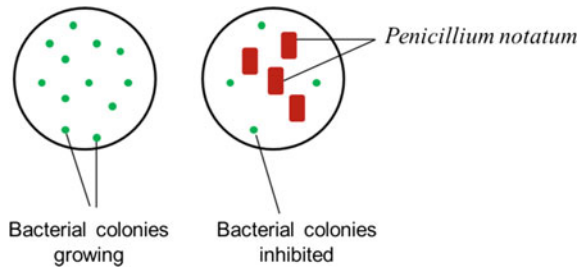
2.4 Fermentation Theory

Besides working in the disease biology, Pasteur also demonstrated for the first time the scientific basis of microbial fermentation process by propounding “fermentation theory” (1857). The theory rejected the idea of *spontaneous creation* and was later itself replaced by the germ theory of disease. It was perhaps the only scientific discoveries of its times that had the potential of industrial scalability. Through systematic experiments, Pasteur showed the role of yeast in fermentation process of ethanol production while also giving terms like “aerobic and anaerobic” (Barnett 2003). Fermentation till today continues to be one of the major areas of application of biotechnology on which many food, chemical and drug industries thrive. There are numerous health benefits of consuming fermented food products. Besides improving digestion, fermented edible items have also been shown to boost immunity and relieve from stress. Fermentation is also used to manufacture important industrial chemicals like ethanol, acetic acid; enzymes like cellulase for use in paper and pulp industry; antibiotic like penicillin by culturing fungus *Penicillium notatum* on a large scale (Parvez et al. 2006).

2.5 Penicillin Discovery

Professor Alexander Flemming (1928), a Scottish physician is credited with the discovery of first true antibiotic penicillin having a broad spectrum bactericidal activity. The antibiotic was discovered as an observation of inhibited bacterial growth on a plate having mould (*P. notatum*) growing. The “mould juice” was the antibiotic secreted out by the fungus which was effective in killing wide range of bacteria like *Staphylococcus* (causes abscess), *Meningococcus* (causes meningitis and sepsis), *Streptococcus* (causes pneumonia) and *diphtheria bacilli* (causes diphtheria) (Fig. 2). The antibiotic proved to be a massive life saver in post World War II era to treat wounded soldiers and general public. An otherwise wound infection that led to the development of abscess and subsequent gangrene could now be effectively treated and cured (Gaynes 2017). Fleming eventually received Nobel Prize for Physiology or Medicine in 1945.

Fig. 2 Schematic showing inhibitory effect of *penicillium* mould on growing bacteria



Prof. Alexander Fleming. Photo Courtesy of the History of Medicine Division at the U.S. National Library of Medicine

2.6 Cell Theory and Laws of Heredity

Observing cells for the first time under a microscope was a major breakthrough that totally revolutionized biological research in a way that was going to impact human life in years to come. Zacharias Jansen and his father Hans are credited with inventing the first compound microscope in 1590 (Helden 2010). It was Antonie Philips van Leeuwenhoek who for the first time saw living cells: bacteria, protists, blood cells rotifers, etc. (Lane 2015). Thereafter, some of the most path-breaking reports were published at successive intervals that included the discovery of nucleus by Robert Brown (1831), formulation of “cell theory” by Matthias Schleiden and Theodore Schwann (1839) and addition of new dimension to cell theory by stating “*Omnis cellula e cellula*: all cells arise from pre-existing cells”; by Rudolf Ludwig Carl Virchow (published in *Cellular Pathology*, 1858) that greatly enhanced our understanding of basic functioning of cellular physiology (Kuiper 2010).

Contemporary discoveries of laws of heredity/inheritance by Gregor Johann Mendel (1866; considered to be father of modern genetics) helped in understanding the transmission of traits from one generation to next. The work of Mendel was largely rediscovered by Hugo de Vries, Erich von Tschermak, Carl Correns and William Jasper Spillman. Through simple crossing experiments performed in garden

pea (*Pisum sativum*) plant, Mendel was able to conclude the presence of “dominant” and “recessive” traits. What he observed was, when two pure-bred varieties of pea plant (having tall and short traits) were crossed, then the second generation progeny had a mix of population: two were tall and short and remaining two were hybrids (Gros 1992). Initially, Mendel designated the traits as “factors”; however, it was Wilhelm Johannsen (1909) who introduced the term “gene” and William Bateson, the word “genetics” (1905) (Gerstein et al. 2007).

2.7 The Term “Biotechnology”

Karl Ereky, a Hungarian agriculture engineer coined the term *biotechnologie* in 1919 to explain the biological method of converting raw materials into useful products. He wrote a book titled “*Biotechnology of Meat, Fat and Milk Production in an Agricultural Large-Scale Farm*” in which he expressed his views on solving the problem of food crisis (Fiechter 2000).

2.8 Identification of DNA as Genetic Material

Present-day biotechnology revolves around DNA/RNA or protein-based science and its uses. However, to even make use of these molecules for societal application, a detailed and thorough knowledge about them was lacking in the first half of the twentieth century. Frederick Griffith, a British bacteriologist, in 1928 demonstrated the importance of *transforming principle* or the genetic material for the process of bacterial transformation (Lorenz and Wackernagel 1994). Later, in 1944, Avery, McLeod and McCarty showed DNA to be the real transforming principle. In 1953, James D. Watson and Francis H. C. Crick showed the double helix structure of deoxyribonucleic acid (DNA) for which they received the Nobel Prize in Medicine or Physiology (1962) (Crick and Watson 1954; Daston and Lunbeck 2011). With the basic knowledge of DNA, further deep studies in molecular biology became possible which led to the foundation of recombinant DNA technology and genetic engineering.

3 Modern Biotechnology—1970s Onwards

Nirenberg and Matthaei (1961) are credited with the discovery of the genetic code which helped in revealing the information contained in the genes. The triplet arrangement of bases in the mRNA (codons) is matched with the respective anticodons present on the tRNA which carries particular amino acid. During the process of mRNA translation, the codons direct the amino acids they encode to be incorporated

in the growing nascent polypeptide chain. The genetic code was further completed and presented as a table by the efforts of Har Gobind Khorana and Robert Holley. The code was found to be degenerated (64 codons encoding 20 amino acids) which meant simply that many codons encode the same amino acid. Nirenberg, Khorana and Holley shared the Nobel Prize in Physiology or Medicine in 1968 (Landmarks 2009).

Werner Arber and Matthew Messelson (1962) discovered “restriction endonucleases” or restriction enzymes that possessed the property of recognizing certain inverted repeat sequences (palindromic sequences) in a plasmid and digest it specifically. The plasmid was a covalently closed circular form of DNA that contained sites for many restriction enzymes called multiple cloning sites (MCS) which allowed incorporation of any gene. Nathans (1971) demonstrated the digestion of the phage DNA of simian virus 40 and resolving of the digested fragments by gel electrophoresis. These findings paved the new exciting area to work with DNA molecule and tune it to do molecular cloning that involved creating an exact replica of a gene (gene cloning). By this time, the central dogma of molecular biology was already in place that dictated the synthesis of protein to be guided by the sequence contained in the gene. People then began exploring the feasibility of cloning the genes that encoded for necessary proteins and began expressing them in suitable host systems (Arber and Linn 1969; Danna and Nathans 1971). The clone (plasmid carrying the gene of interest) created in this manner was called a *recombinant*. The work extended towards the production of recombinantly expressed proteins on an industrial scale, and thus several agricultural, pharmaceutical and other companies jumped into the fray of commercialization, patenting, licensing and mass production of them.

3.1 *Hybridoma Technology*

Cesar Milstein and Georges J. F. Kohler (1975) invented the hybridoma cells that were capable of synthesizing monoclonal antibodies (mAbs). The purification of single epitope recognizing antibody from the vast repertoire produced by the immune cells had been a challenge before the investigators. Milstein and Kohler fused a plasma B cell (secretes antibodies) with the myeloma cell (cancerous B cells) with the help of sendai virus and created what is called a hybridoma that secreted antibody recognizing single epitope. The antibody thus produced was monoclonal unlike the conventional polyclonal ones that recognized several epitopes (Fig. 3). The hybridoma was transplanted in mice peritoneum where it led to tumour growth and secretion of vast amount of mAb in ascites fluid (Milstein 1999). Milstein and Kohler got Nobel Prize for Medicine and Physiology in the year 1984 for the revolutionizing impact their study had on immunology. With the availability of mAbs, it became possible to selectively identify many tumour cells possessing unique antigens, their separation and subsequent purification. Today, mAbs are used extensively in biomedical and biotechnology research for immunodiagnostics of cancer and its immunotherapy;

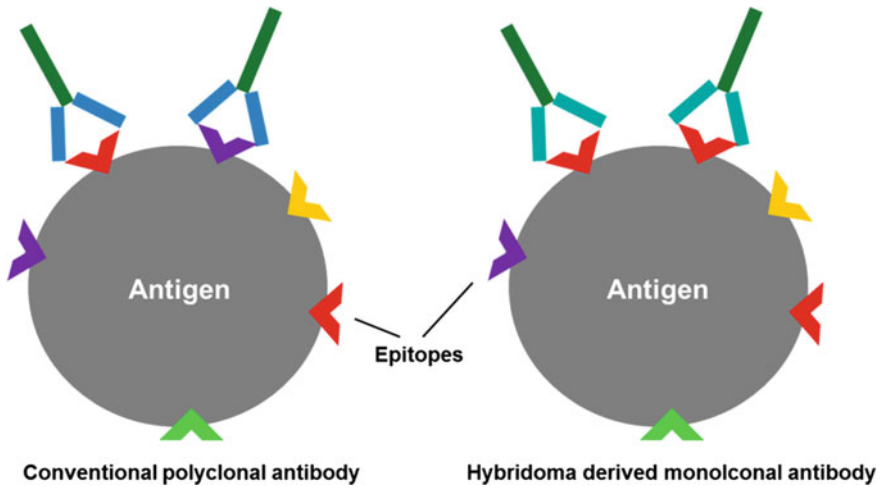


Fig. 3 Schematic illustrating the multiple versus single epitope recognition by a polyclonal antibody and hybridoma-derived monoclonal antibody, respectively

identification of infectious pathogens; affinity purification of single protein from a complex mixture, etc.

3.2 *Insulin: The First Commercial Recombinant Product*

Insulin is a hormone which is responsible for glucose metabolism in the body. Impaired levels of insulin are a hallmark of type 1 diabetes (T1D). The beta cells of islet of langerhans of pancreas produce insufficient or no insulin due to the autoimmune triggered death. Consequential to which the person affected manifests hyperglycaemia (increased blood sugar levels) symptoms. The patients of T1D are administered insulin by subcutaneous injection (Chiang et al. 2014; CDC 2017). The number of diabetics has increased from 108 million (in 1980) to 422 million (in 2014). It is one of the major causes of renal failure, blindness, cardiac attacks, strokes, etc. (WHO 2018). Herbert Boyer and Robert A. Swanson together founded Genentech, Inc. (headquartered in California, USA; now a subsidiary of Roche) in 1976 which became the first biotechnology-based company. Boyer and Swanson entered into the contract agreement to manufacture recombinant human insulin that was until now obtained from pigs. Boyer took the responsibility of scientific aspects of design, execution, purification, etc., while Swanson took care of the finances and legal aspects. The company came out with recombinant human insulin (expressed in and purified from *Escherichia coli*; Fig. 4) in 1978. It was licensed to Eli Lilly and Co. (to be sold as *Humulin*) and was approved by Food and Drug Administration, USA, in 1982 (US F&DA 1982; Hughes 2011).

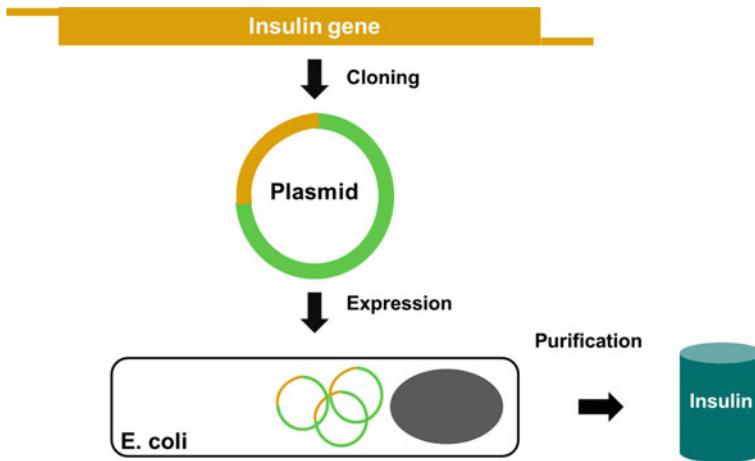


Fig. 4 Schematic depicting the workflow of recombinant human insulin production

Since the original production by Eli Lilly and Co. (Indiana, US); Sanofi (Paris, France) and NovoNordisk (Copenhagen, Denmark) have also come up with their recombinant insulin products (named Insuman and Novolin, respectively) (Landgraf and Sandow 2016). Today, insulin and its derivatives enjoy global sales of over \$4.5 billion. The technology of insulin production has moved ahead and is now produced in human cells besides yeast and other suitable expression systems for increased yield and better efficacy (Walsh 2005). *Arabidopsis thaliana*, lettuce and tobacco plants have been engineered for human insulin production and have been successful (Nykiforuk et al. 2006; Boyhan and Daniell 2011).

3.3 Human Growth Hormone (hGH) by Genentech

The human growth hormone (hGH or somatotropin) is released by the anterior pituitary gland. It is a protein composed of 191 amino acids and triggers cell reproduction, metabolism and overall body growth. Choh Hao Li, a US biochemist of Chinese origin synthesized and purified hGH for the first time (Cole 1996). Thereafter, Genentech pioneered its commercial production using the recombinant DNA approach and sold under the trade name Protropin (1985). The product got discontinued and is now sold as Nutropin since 1993 (Genentech 1993). Besides Genentech, there are other pharmaceutical companies selling recombinant hGH like Pfizer, Roche and NovoNordisk under various trade names. The hormone is administered subcutaneously to children and adults suffering with chronic kidney disease (CKD), Turner's syndrome and growth hormone deficiencies (GHD). The global market sales of hGH are estimated to be generating revenues worth \$5261 million by 2026 (TMR 2018).

3.4 Polymerase Chain Reaction (PCR)

Kary Mullis (1983) developed a method for exponential amplification of a piece of DNA using sequence-specific oligonucleotide primer and DNA polymerase (Fig. 5). The process was carried out in a specialized machine (thermal cycler) capable of rapid temperature switching (called thermal cycling). The method is now indispensably used in every basic and medical research laboratory for specific amplification or cloning of genes or any nucleotide sequence. The method proved to be a path-breaking invention in the field of biotechnology which made exact identification of any bacteria, virus, cell type or any gene possible. PCR had an immense impact on state-of-the-art methodologies of molecular biology practised worldwide. Mullis was awarded Nobel Prize in Chemistry in 1993. The PCR technique had direct application in the field of gene cloning, site-directed mutagenesis, parentage identification, prenatal sex determination, pathogen identification, DNA matching from crime scene sample (e.g. blood, semen, hair, skin, etc.), tumour cell testing, etc. For many infectious diseases, cancer stage identification or legal dispute of biological parentage, PCR has now become a standard method of diagnosis and investigation (Bartlett and Stirling 2003).

3.5 The Era of Genomics and Proteomics

Modern times is witnessing tremendous ramification of biotechnology in health care, agriculture and environment sectors. The wealth of data that has been generated by aforementioned discoveries is stored, annotated, analysed and interpreted in various ways. This enormous task has generated an entirely new arm of biotechnology called *bioninformatics*. The *in silico* approach towards solving biotechnology-based questions have been applied in the fields of genetics, molecular biology and protein science. The classical genetics approach aimed at studying one or few “genes to phenotype” on a case to case basis; whereas, *genomics* includes the entire genome characterization in one go. Whole genome sequencing, single nucleotide polymorphism identification, screening of chemical library for fishing out the genomic targets, creation of genomic libraries, etc., all together constitute genomics. The similar approach when applied to study the total RNA population dictated by the genomic

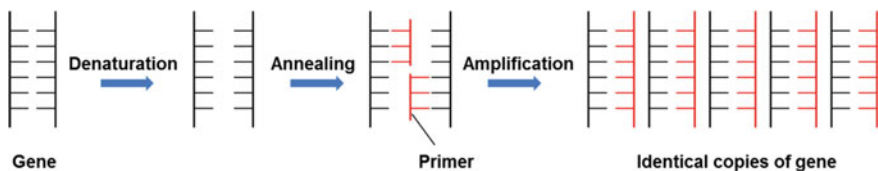


Fig. 5 Diagrammatic representation of the concept of polymerase chain reaction (PCR)

parent is known as *transcriptomics* which includes whole transcriptome profiling, RNA sequencing, microarrays-based gene expression analysis and their validation.

The idea of sequencing the entire human genome was conceived at National Institutes of Health, USA, (1984) from where the original funding for implementation of the idea came. The official launch commenced in 1990 and was termed complete in 2003. A parallel effort was undertaken by a company known as Celera Genomics which was spearheaded by J. Craig Venter. Currently, 92% of human genome stands sequenced which corresponds to euchromatic area; whereas, remaining heterochromatic region still awaits the process. The sequence-related information is stored in the publicly accessible databases like GenBank at NCBI (National Centre for Biotechnology Information, NIH, USA), DDBJ (DNA Database of Japan, National Institutes of Genetics, Shizuoka, Japan) and EMBL (European Molecular Biology Laboratory, Heidelberg, Germany). The three databases frequently exchange information with each other. The information generated by the human genome sequencing was hailed as a historic milestone in the field of biology and medicine. It was envisaged as a tool to identify many oncogenes, developmental disorders-related genes, identification of novel drug targets, throw light on understanding bacterial and viral pathogenesis, etc. (Collins et al. 2007). The information contained in the genome is eventually “executed” by the *proteome* (the sum total of all the proteins present in the cell). The science aimed at studying and a characterization of the proteome is called as proteomics. The term has virtually replaced the phrase protein science. Nowadays, any attempt either to study a single or complex of proteins is included in the term proteomics. Present-day state-of-the-art proteomic methodologies use protein purification strategies, 2-D gel electrophoresis, mass spectrometry analysis, peptide microarrays, Fourier resonance energy transfer (FRET), etc., to identify and characterize the function of proteins (Anderson and Anderson 1998). The protein-related information has been stored in free databases like UniProt Consortium comprising EBI (European Bioinformatics Institute, Hinxton, UK), SIB (Swiss Institute of Bioinformatics, Lausanne, Switzerland) and PIR (Protein Information Resources, Georgetown University, Washington, US) (Wu et al. 2002).

3.6 *Metabolomics*

The sum total of all the metabolites produced in the cell constitutes the metabolome of the cell. The science involving the study of the metabolome profile of cell or individual is known as metabolomics. The subject matter differs from genomics and proteomics in a way that it studies the actual processes that proteins (which are in turn dictated by the genome) are doing as end products. In this way, it rises above the molecules and takes the investigation to the “systems biology” level. Metabolome directly tells the fate of the cellular processes working in the cell. The study stems from background literature where clinicians or investigators used to analyse the presence of certain compounds in body fluids by the means available in those times. For example, physicians in ancient China employed ants to check the presence of

glucose in diabetes patients. Nowadays, modern techniques like gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) are used for identification of secondary metabolites in the biological samples (Sussulini 2017). METLIN (2005) was the first database of human metabolome that is maintained by the Scripps Research Institute (La Jolla, USA). It contains the mass spectrometry data (like molecular mass, structure and formulae) of the metabolites (Smith et al. 2005). A human metabolome repository is maintained at University of Alberta, Canada, named Human Metabolome Database (HMDB) that contains information of human body metabolites derived from NMR and GC/LC-MS analyses. At present, it is the most comprehensive and updated metabolomics database that is widely accessed (Wishart et al. 2007).

4 Areas/Branches of Biotechnology

Biotechnology has been classified in multiple ways. Recently, there was a colour-based rainbow-coding of various arms of biotechnology to provide a more holistic view. These colours comprised red, green, blue and white. Each colour specified the particular areas of application of biotechnology under its ambit (Fig. 6), e.g. red was

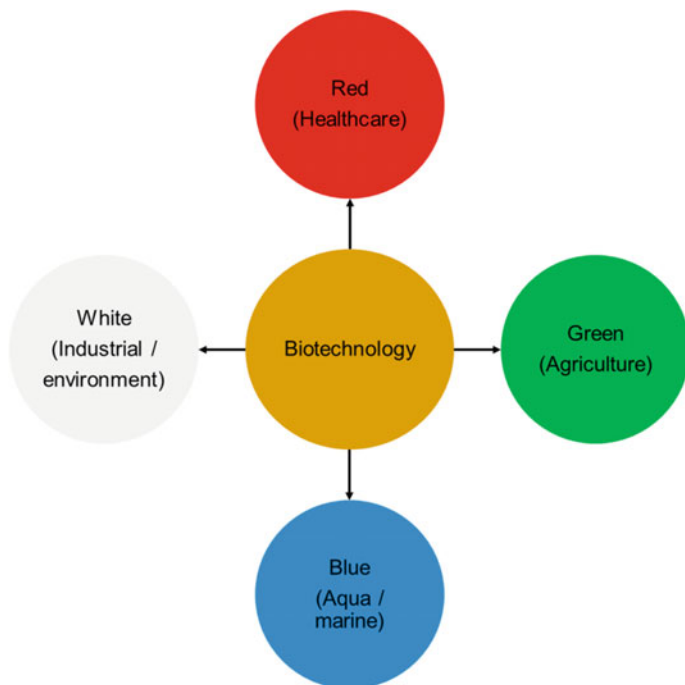


Fig. 6 Schematic description of the branches of biotechnology

constituted by biotech-based biomedical research; agriculture-oriented application was included in green; biotechnological application in marine or aquatic life forms and their usage comprised the blue and that pertaining to industry and environment was termed as white biotechnology (Frazzetto 2003; DaSilva 2004; Kafarski 2012). This section briefly discusses some of the recently emerging representative examples from such categories and highlights their significance in the improvement of lives on earth and its environment.

4.1 Red Biotechnology

The biotechnological inventions and discoveries pertaining to biomedical research or animal health have been collectively included in the umbrella term red biotechnology. It comprises significant developments in the field of stem cell biology, embryo manipulation, transgenic animals (with better nutritive or commercial value; like high milk-yielding cattle), biopharmaceuticals (recombinant vaccines, hormones and other therapeutic proteins production), forensics (DNA fingerprinting), genetic interventions like gene editing and gene therapy, disease diagnostics (pathogen identification, oncogene examination) and personalized medicine (SNPs-related disease predictions in cancers, infection and metabolic disorders).

4.1.1 Genetic Techniques

- **Recombinant DNA technology for production of vaccines**

“Prevention is better than cure”—Desiderius Erasmus

Perhaps, the aforementioned statement best describes the significance of vaccines in saving lives from diseases. Ever since Jenner laid the foundation of the field, vaccinology has come a long way from use of live-attenuated or heat-killed vaccines, to recombinantly expressed or the plasmid-based ones. In classical times, infectious virus was passaged for several generations in cell culture to get it mutated, and upon introducing back into the original host it was unable to infect yet elicit an immune response. In this way, it was said to have become “attenuated”. Examples of live-attenuated vaccines include polio, rotavirus, measles and H1N1 flu vaccines. Usage of chemicals like formaldehyde or heat to reduce their virulence resulted in inactivated or killed vaccines, e.g. cholera, typhoid or influenza vaccines (Benn et al. 2013).

The recombinant approach applied to manufacture vaccines is twofold: one is called the vectored approach whereby antigenic regions of the pathogen are delivered to patients via bacterial or viral vectors; second approach involves heterologous expression of antigen encoding genes in yeast systems like *Saccharomyces cerevisiae* or *Pichia pastoris* or mammalian and insect cell lines and large-scale purification of

the recombinant protein thereafter. The first approach is easy to handle and employs bacterial or viral vector for expressing genes encoding antigenic regions obtained from a wide variety of pathogens. Intramuscularly injected antigens use several viral or bacterial vectors like adenovirus (Ad5 serotype), adeno-associated viruses, recombinant *Mycobacterium bovis* BCG (rBCG strain), *Salmonella* (Shata et al. 2000; Rollier et al. 2011) (Fig. 7b). For antigens requiring post-translational modifications, the first approach proves to be inadequate and therefore use of yeast or mammalian and insect cell lines becomes indispensable. For this, the antigen encoding genes (e.g. Hepatitis B surface antigen) is cloned and expressed in yeast (e.g. *S. cerevisiae*) and cultured in fermenters on a large scale. The recombinant protein assembles in virus-like particles (called VLPs) which are secreted by yeast in the external medium from where it is purified (Fig. 7a). Glaxo Smithkline Plc., London, manufactures hepatitis B vaccine via this approach which is directed against its surface antigen under the brand name Engerix-B (Keating and Noble 2003).

A third category of recombinantly administered vaccines comprises DNA vaccines. The antigen encoding region is expressed in a vector that contains a bacterial origin of replication, a strong viral promoter (e.g. cytomegalovirus, CMV), an MCS region for cloning of the segment and an antibiotic selection marker. Modes of delivery of such vaccines have been intramuscular injection or via gene guns (Fig. 7c). The DNA is adsorbed on gold particles and bombarded on the localized site of action. The idea is to transfer the construct directly in the cells where it expresses and mimics the condition of natural infection. The method has been used for vaccination against TB, leishmaniasis, influenza, HIV, etc. (Yang et al. 1990; Oliveira et al. 1999). Improved methods now incorporate changes made in the construct so as to prevent its degradation inside the cells upon entry or to simultaneously co-express inflammatory cytokines to heighten the immune response (Belakova et al. 2007).

- **Gene editing: CRISPR-Cas** (clustered regularly interspaced short palindromic repeats—CRISPR associated)

Originally discovered as an immune system of bacteria conferring resistance against the invading viral DNA, the technology of CRISPR has been adapted into a toolkit for genome editing. The acronym describes a particular stretch of repetitive nucleotide sequences in the bacterial DNA that became part of the genome because of a previous viral infection. The sequences are capable of degrading viral DNA in case of a future infection by the same or similar DNA virus thus conferring immunity to the bacteria against viral attack. The repeat sequences were discovered in 1987 in *E. coli* by group of investigators in Osaka University, Japan. The group noticed the unusual feature of the repeats which was the presence of interrupting nucleotides between them; however, they could not assign any function to them (Ishino et al. 1987). The acronym CRISPR was given in 2002 by a group that published bacterial genomic loci which harboured interspaced repeat sequences (Jansen et al. 2002). Thereafter, a surge of reports describing the phenomenon was observed in the scientific community which not only established it as a novel form of natural bacterial defence mechanism but also opened the possibility of applying the method for artificial genome editing. The

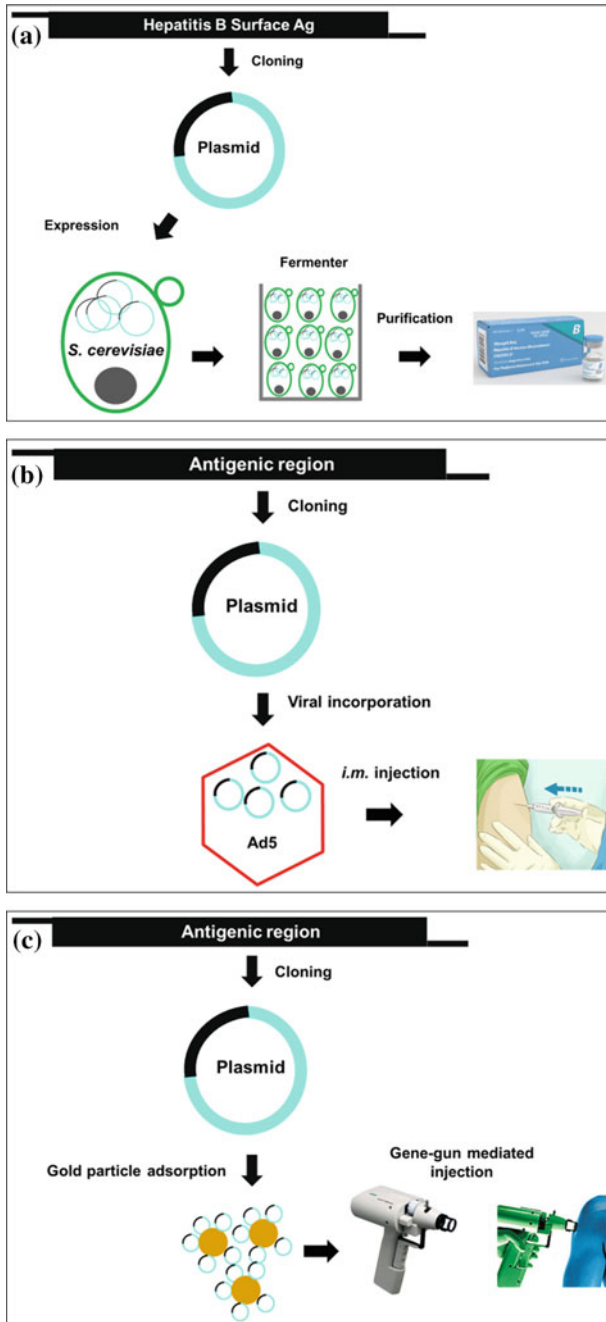


Fig. 7 Schematic describing types of recombinant vaccines. **a** An example of recombinant HbSAg vaccine. Recombinant vaccine administration. **b** Adenovirus (Ad5) mediated recombinant vaccine delivery. **c** Gene gun driven

technology holds immense possibility to correct genetic mutations causing diseases, genetically engineer crops or livestock of desired traits in a cheap and precise manner.

Simply speaking, whenever a DNA virus attacks a bacteria, then bacterial Cas enzyme captures spacer sequences from the invading DNA and integrates into the CRISPR loci in the bacterial genome in arrays. This array encodes an RNA transcript which matures through different types of CRISPR pathways (I, II and III). In type I pathway, the premature or precursor form of CRISPR-RNA or crRNA is cleaved by CRISPR-associated RNases leading to multiple crRNAs. The type III system employs an unknown RNase to generate final mature crRNA. The type II system which has been adapted for artificial genome editing (with some modifications) utilizes another trans-activating RNA or tracrRNA which hybridizes with the crRNA and together with Cas endonucleases binds and cuts the target DNA by introducing DSBs (Hsu et al. 2014). The three components, namely crRNA, tracrRNA and Cas9 endonuclease together constitute what is called as CRISPR system which is capable of performing its function even *in vitro* (Gasiunas et al. 2012; Jinek et al. 2012). For artificial purposes, the former two components have been combined, and a single guide RNA or sgRNA has been used successfully (Jinek et al. 2012). These findings have paved the way of translating the basic knowledge of this phenomenon into useful biomedical and agricultural applications.

In health care, the method has been applied to correct the genetic mutations causing Duchenne's muscular dystrophy (DMD), cystic fibrosis, haematological malignancies and HIV pathogenesis. Using the CRISPR technology in mouse model system, DMD gene expression was rescued by delivering its functional copy through adeno-associated virus vector. The muscular abilities of mice were found to be partially restored as compared to the control group exhibiting degenerating muscle conditions (Tabebordbar et al. 2016). The genome of HIV has been targeted to stop its replication in the infected cells without any toxicity. The achievement could be viewed as a significant advance over the currently practised method to contain or stop viral replication in preclinical systems (Hu et al. 2014). In a parallel study, the CXCR4 receptor of HIV was removed successfully from human T cells using the CRISPR system; a feat which could be mobilized vertically for edited bone marrow transplants from patients suffering from AIDS (Schumann et al. 2015).

There is growing concern about the potential misuse of CRISPR-Cas technology, particularly for the purpose of editing the genome of human embryos. Human embryo manipulation is legal in China and several states of USA. However, there are clear ethical concerns regarding the reckless use of the technology towards giving birth to only the ones with modified features. Such changes when introduced are heritable and are passed on for generations with unpredictable future outcomes. There has been a case of human embryo editing which attracted worldwide shock and condemnation. It called for an urgent action against the erring group simultaneous with rounds of brainstorm sessions held globally to chart out regulation(s) to prevent misuse of CRISPR technology (Cyranoski and Reardon 2015; Liang et al. 2015). Nevertheless, the method has invited global interest towards harnessing its potential in drug development by pharmaceutical companies, improvement of crop traits by

agriculture-based industries and general biomedical research towards understanding of biological processes governing several less characterized disease conditions.

● Gene therapy

The introduction of DNA into cells or organisms for therapeutic benefits is known as gene therapy. The first idea of gene therapy was proposed by Theodore Friedmann and Richard Roblin in 1972 by emphasizing on the beneficial aspects of supplying exogenous DNA to the organism for correcting the genetic defects (Friedmann and Roblin 1972). There are two types of gene therapy strategies, namely somatic cell gene therapy and germline gene therapy. Transfer of therapeutic DNA/gene into any cell other than germ cells or progenitor stem cells is called as somatic cell gene therapy. Such interventions do not affect the germ cells of the person and as such results are not heritable (Mavilio and Ferrari 2008). Two modes of delivery of the therapeutic DNA exists, namely *in vivo* and *ex vivo*. The delivery of the DNA directly into the patient's or model system's body is known as *in vivo* approach; whereas, transfer of gene into cells isolated from the body and then introducing them back after ascertaining its stable expression is called as *ex vivo* mode of gene therapy. The method of delivery of the DNA is divided into two categories: viral-dependent vectors and viral-independent vectors. The former employs the use of viruses devoid of their original genome and instead inserted with the functional copy of the gene for its delivery either into the body directly or in cultured cells. The latter group comprises liposome, gene gun, electroporation or dendrimers-based DNA transfer into the organism or cultured cells. Examples of viruses utilized for gene therapy include herpes simplex, human immunodeficiency, adeno-associated and vaccinia viruses (Mavilio and Ferrari 2008).

Adenosine deaminase (ADA) deficiency caused severe combined immunodeficiency (SCID); an autosomal recessive disorder (Hirschhorn et al. 1979) was the first target of somatic gene therapy in a clinical trial performed by R. Michael Blaese, W. French Anderson and Kenneth Culver in 1990. It was characterized by the lack of ADA enzyme which was essential for DNA synthesis. Using viral vector, the gene encoding the enzyme was delivered to the patient (Culver et al. 1990; Rosenberg et al. 1990). Haematological disorder like beta-thalassaemia is caused by reduced or no production of beta globin chain of haemoglobin. Using lentiviral vector-mediated beta globin gene delivery led to successful production of correct haemoglobin (Sadelain 2006). Gendicine was the first gene therapy drug approved for the treatment of head and neck squamous cell carcinoma in China. The drug enters the cells via receptor-mediated endocytosis and overexpresses p53 levels (Pearson et al. 2004).

Despite endowed with enormous potential of treating diseases and disorders, gene therapy has been marred by several challenges that has led to its decline in recent years. The lack of persistence of effect is the first of all issues that has been slowing down the pace of gene therapy. The functional copy of the administered gene is lost with successive cell divisions which lead to reducing effect of the gene therapy. As a result, the patient needs to get administered the therapy multiple times so as to maintain the therapeutic effect. Secondly, problem of immune challenge caused by viral vectors poses a serious impediment towards the usage of such vehicles for gene

delivery. The surface composition and pattern of many viruses elicit major immune response in the person administered with viral vector-based gene therapy. A second approach of using non-viral vectors has not met with much success. The low rates of gene delivery through such approaches are a major obstacle which has prevented their large-scale testing (Goncalves and Paiva 2017).

- **DNA fingerprinting or profiling**

The use of unique DNA sequence properties to identify an individual is known as DNA profiling or fingerprinting. The method was originally discovered by Sir Prof. Alec Jefferys in 1984 at the University of Leicester, UK. The genome between two unrelated individuals match up to 99% yet the unmatched portion offers enough to discriminate between them. Professor Jefferys also observed that the fingerprint in a child comprised half from both the parents. The classical method of fingerprinting relies on the selective detection of certain unique repetitive DNA sequences called as variable number tandem repeats (VNTRs) which are present in the genome in the form of minisatellites and microsatellites (Roewer 2013). The technique involved extraction of DNA from samples (e.g. blood, hair follicle, semen, nails or other body parts or secretions) and its restriction digestion and electrophoresis to generate band fragments on the basis of restriction sites present. The sample was then blotted to nitrocellulose membrane and repetitive sequences were detected using specific complementary radiolabelled probes. The minisatellites are six to hundred nucleotides long and repeat for hundreds of times in the genome (Tautz 1993). Microsatellites (also known as short tandem repeats or STRs) on the other hand are shorter in length (as the name suggests) ranging from one to five nucleotides repeating for some hundred times (Koreth et al. 1996).

The aforementioned method has now been replaced by a PCR-based technique which involves DNA extraction followed by amplification of microsatellite region. This has greatly improved the workflow of fingerprinting and has added to its rise in success. The method now is widely used for parentage identification, cell lines authentication, to check cancer progression but perhaps its greatest application has been in the field of forensics where it is used for identification of criminal. Owing to this, now many countries maintain a DNA database of their population with which the fingerprinting results are compared to find out the real culprit. In the USA, Coding for DNA Identification System (CODIS) is maintained by Federal Bureau of Investigation (FBI) for the identification of criminals by using DNA fingerprinting (Saad 2005).

The method also revealed through arbitrarily primed-PCR (AP-PCR), the mutations in the microsatellite regions of cancer tissues and established the process of a new mechanism of carcinogenesis. Since then fingerprinting has been successfully used for identification of cancer mutations in the microsatellites thereby allowing to identify the stages of progression (Perucho 1996). In an earlier study, 46 different cell lines (including cancer cells) were authenticated by using DNA fingerprinting of minisatellites (Gilbert et al. 1990). The case of disputed paternity is now routinely solved based on DNA profiling as mandated by the courts worldwide. As mentioned

earlier, based on the shared fingerprint of the VNTRs in the child and the suspected person, it is possible to nail down the biological father.

4.1.2 Embryological/Organismal Manipulation

- **Somatic cell nuclear transfer (SCNT)**

The technique involves transfer of a nucleus from donor cell to enucleated egg cell or ovum and allowing it to develop into an embryo. After many rounds of mitotic divisions, the embryo reaches blastula stage where it develops inner cell mass (ICM) containing embryonic stem cells (ESCs). From here, as the need demands the embryo can be implanted into a female animal if reproductive cloning is the objective or for therapeutic cloning, the ESCs can be extracted out and can be used for tissue regeneration by harnessing their pluripotent nature (Fig. 8) (McLaren 2000). Sir Hans Spemann (1928), a German embryologist, is credited with the discovery of the then *embryonic induction*, an idea considered to be the predecessor of modern-day SCNT. Through microsurgical needle, Spemann and his colleagues inserted a particular region of an embryo into another embryo which led to development of a new embryonic growth irrespective of the area where it was transplanted (Spemann 1938). Dolly (sheep) was the first organism born out of reproductive cloning through SCNT method in 1997 at Roslin Institute, The University of Edinburgh, UK. The investigators incorporated a nucleus from an adult cell into an enucleated ovum and allowed to develop the zygote till blastula stage of embryogenesis. Later, it was implanted into a female sheep that served as a surrogate mother for the developing foetus and gave birth to Dolly (Edwards 1999). Very recently, macaque monkeys

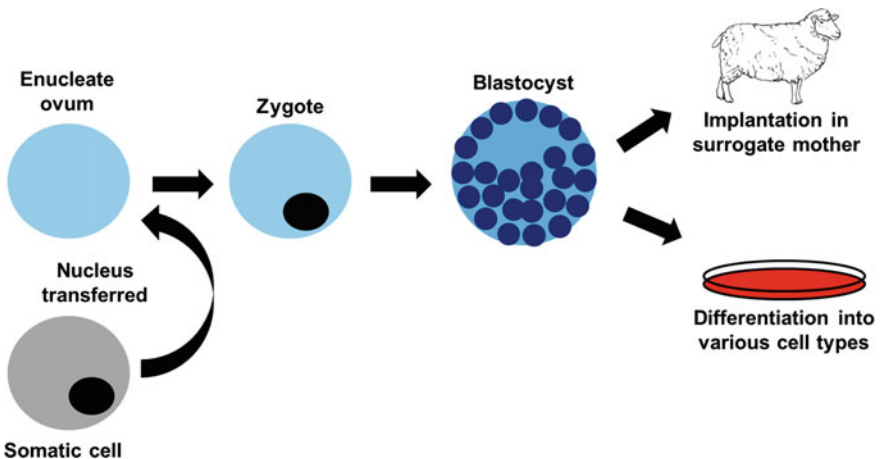


Fig. 8 Schematic depicting somatic cell nuclear transfer

were cloned in China which marked the first successful reproductive cloning using SCNT in primate species (Liu et al. 2018).

Therapeutic cloning is perhaps the best legacy that SCNT has left behind for the welfare of mankind in general. The power of generating blastocyst and extraction of ICM allowed the investigators to generate multiple tissues and organs of human body which has direct application in the treatment of several diseases. Researchers have been successful in generating pancreatic endocrine cells by the application of SCNT technique which were found to be capable of producing hormones like insulin, glucagon and ghrelin. An advancement like this holds immense possibility towards better treatment of type 1 diabetes in which insulin secretion is compromised (D'Amour et al. 2006). In another study done in mouse, investigators were able to regenerate spinal cord using motor neurons from ESCs, an achievement of medical importance in cases of paralysis (Liang et al. 2006).

● Stem cells and their applications

Stem cells are capable of differentiating into almost any cell type of the body and can also regenerate themselves. The property by which a stem cell can differentiate into myriad cell types is known as pluripotency, and such cells are known as pluripotent. As mentioned in the previous section, the inner cell mass (ICM) of a blastocyst houses the embryonic stem cells (ESCs) capable of generating all three germ layers and their cell types (Thomson et al. 1998). Besides the ESCs, there is another method of generating stem cells which involves reprogramming back a differentiated cell into a state of stemness. The stem cells thus created are known as induced pluripotent stem cell (Aoi et al. 2008; Chagastelles and Nardi 2011). Apart from stem cells derived from the embryo, the bone marrow also contains a population of haematopoietic stem cells (HSCs) which give rise to all the blood cell types. HSCs have been clinically used since 1960s (Good et al. 1969) and are now obtained from umbilical cord and placenta (Kogler et al. 2004).

The clinical uses of stem cells have opened a new avenue of research which has great potential for offering cure against several diseases. Cardiomyocytes generated from human ESCs (hESCs) have been successfully transplanted into mouse model system and were found to restore the beating function of heart (Laflamme et al. 2005). hESCs incorporated with gene essential for generation of cone photoreceptor cells have been successfully used for treating retinal pigment epithelial degeneration (Zhou et al. 2015). Another class of stem cells, known as mesenchymal stem cells (MSCs; capable of differentiating into cells of mesodermal origin) derived from bone marrow has been used to generate bladder tissue in baboons thus establishing a non-human primate model for potential human applications (Sharma et al. 2011). HSCs have been used for transplantation in patients suffering from various lymphomas, myelomas and immunodeficiency diseases (Eaves 2015). Prior to administering HSCs to the patient, the bone marrow is destroyed by high doses of chemotherapeutic drugs with or without radiotherapy (myeloablation). Thereafter, HSCs obtained from the patient are injected in the bloodstream from where they are able to reach and replenish the destroyed tissue. This mode of administering patient's own HSCs to him or her is known as autologous as compared to allogenic in which HSCs are obtained from

healthy donor with HLA match type (Russell et al. 2000). As pointed out earlier, mother's umbilical cord and placenta are two prime organs which are storehouses of HSCs; therefore, people are now getting their cord blood cells stored in blood banks. The stem cells derived from such banks can be very helpful in case of future haematological malignancies, inherited disorders or other genetic diseases.

4.1.3 Molecular Diagnostics

The use of DNA/RNA or protein(s) for medical diagnosis or prognosis of diseases or disorders is collectively referred as molecular diagnostics. The methods commonly utilize the detection of DNA or RNA converted to cDNA through PCR or hybridization-based techniques. Proteins are generally detected by using antibodies and antibody-dependent techniques like enzyme-linked immunosorbent assay (ELISA) or flow cytometry in a method called as immunophenotyping (Poste 2001). The methods are fast, precise and specific as compared to traditional diagnostic approaches.

• PCR-based detection

Since the inception of PCR by Kary Mullis, the method has evolved into a more advanced technique which has enabled the detection of DNA or RNA in real time through a technique called Real-Time or Quantitative PCR (qPCR). qPCR works on the principle of usual PCR except that the amplified product can be visualized by using fluorescent dye (e.g. SYBR green) or fluorescent-labelled complementary probes (TaqMan probes) whose signal rises as the amplified product accumulates (Kubista et al. 2006). qPCR is of great relevance in the field of molecular and diagnostic virology where one can identify viral serotypes in the patient's body sample. Since clinical samples are often limited, a PCR-based method of detection proves to be of immense advantage because the target gene can be amplified exponentially and can be monitored live. The method has been successfully applied to detect and quantify causative agents of several infectious diseases; viruses like HIV, hepatitis B virus (HBV), herpes simplex virus (HSV) 1 and 2, human papilloma virus (HPV) in patient's samples (Valones et al. 2009). For HIV, p24 gene is routinely used for amplification for detecting the presence of HIV-1 in patient's dried blood spots (Lakshmi et al. 2011). All four serotypes of dengue virus (DENV 1–4) are routinely detected in clinical laboratory using PCR methodology to amplify C and E genes using serotype-specific primers (Das et al. 2008). HBV has been successfully detected using TaqMan probe-based qPCR technique in clinical samples (Zhao et al. 2005). For bacterial strain identification, 16S rRNA gene (considered to be universal reference) of each strain is amplified through PCR. Bacteria like *Mycobacterium tuberculosis* and *Helicobacter pylori* are detected by their sequence-specific primers against 16S rRNA (Barghouthi 2011). Another target is the outer membrane protein (OMP) gene routinely used for detection of bacteria belonging to *Chlamydia* sp. which primarily causes respiratory illnesses. Many species of *Chlamydia* have been known to cause Alzheimer's, arthritis, atherosclerosis, etc. (Yamamoto 2002).

- **FISH (fluorescence in situ hybridization)**

The technique of FISH involves hybridization of fluorescently conjugated DNA/RNA probes with its target genomic region or mRNA. Its analysis requires an imaging system like a fluorescence microscope. The method can be performed on cells and fixed tissues. The samples (cells or fixed tissues) are permeabilized and incubated with the fluorescently conjugated DNA or RNA probes which then hybridize with their target. FISH-based diagnostic tests are routinely used for detection of HER2 expression levels in various cancers (Hicks and Tubbs 2005). FISH probes are widely used for detecting chromosomal translocations in the case of lymphomas (Tempest et al. 2008). Using the probes, BCR-ABL translocation gene has been successfully detected in peripheral blood granulocytes of patients suffering from chronic myelogenous leukaemia (CML) (Takahashi et al. 2005).

- **Immunophenotyping**

The method of detection of surface proteins through antibodies and flow cytometry is known as immunophenotyping. The method involves labelling cells with their respective cell surface markers, e.g. CD markers; CD8 in case of T cells or any other cell surface-expressed protein used for identification of cell type and developmental stage. Both high and low expression profiles of the markers are used as indicator of particular cell type and its developmental stage. These two parameters help in the diagnosis and prognosis of the disorder, respectively. The technique allows to discriminate between various stages of cells based on their development stage specific surface marker profile. This is helpful in deciding the prognostic strategy when a patient is undergoing therapy. The technique also enables to identify the lineage between cells of B or T lymphocyte origin. The method has been of great use in haematological disorders including malignancies like chronic lymphocytic leukaemia (CLL) (Ivancevic et al. 2014). Cancer cells tend to acquire drug resistance over the period of therapy which can be ascertained by immunophenotyping P-glycoprotein (MDR-1) or multidrug resistance-associated protein (MRP-1) (Jakab et al. 2005). In the case of HIV infection, CD8⁺ T lymphocytes decrease in population which can be accurately diagnosed by flow cytometry-based immunophenotyping. B cell lymphomas are detected by low expression profile of CD20 marker. Using the method, leucocyte adhesion deficiency disorders can be identified accurately by looking at the decreased expression profile of the leucocyte β 2 integrin receptor complex which comprises CD11/CD18 complex (Brown and Wittwer 2000).

4.1.4 Personalized Medicine

The utilization and practice of the knowledge of genetic information of a patient to evaluate disease risk assessment, drug response and deciding the therapy are known as personalized medicine (Mancinelli et al. 2000). Through genetic analysis like whole genome sequencing, it is possible to identify variations in the genes encoding receptors or enzymes that may predict the outcome of a particular drug treatment.

Advancement in the field of genomics after the completion of Human Genome Project has led to the generation of vast knowledge regarding underlying genetic differences in a particular population. This knowledge when applied to study disease-related predisposition markers or drug response of patients is known as pharmacogenomics (Vogenberg et al. 2010).

- **Genome-wide association studies (GWAS)**

The comparison of genetic differences associated with any particular trait or disease condition in a population between healthy and diseased subjects is known as genome-wide association studies. The common genetic differences taken into account while doing these analyses are single nucleotide polymorphisms (SNPs) which have been shown to be present in varying frequencies in a population of healthy and diseased individuals. Almost one million SNPs can be genotyped in one scan of a patient's DNA sample (Spencer et al. 2009). The first GWA study was reported in 2005 in patients suffering from age-related macular degeneration (leads to blindness in elder people). The study identified two SNPs present in complement factor H gene which were linked with the increased risk of the disease (Haines et al. 2005).

In a separate study performed by the Wellcome Trust Case Control Consortium (as reported in 2007), around 2000 patients for each of seven diseases, namely type 1 diabetes, type 2 diabetes, Crohn's disease, rheumatoid arthritis, bipolar disorder, coroner artery disease and hypertension were analysed for the variation in their SNPs using Affymetrix SNP array. This comprehensive effort revealed several new genetic loci associated with risk of diseases under study (Consortium 2007).

4.2 *Green Biotechnology*

Application of biotechnology in agriculture sector with the aim of increasing the productivity and ensuring food security is called as green biotechnology (Kafarski 2012). The methodology adopted for realization of aforesaid goal is somewhat similar to what is used in healthcare sector or red biotechnology due to the universality of molecular biology principles. Broadly, the branch includes gene manipulations done for crop or livestock improvement by increasing the crop or animal-based yield or conferring resistance against biotic or abiotic stress factors.

4.2.1 **Genetic Transformation in Plants**

There are a number of methods for incorporating foreign genes in plant systems, namely *Agrobacterium tumefaciens*-mediated gene transfer, electroporation, gene gun or biolistics and microinjection (Lorence and Verpoorte 2004). Among all these, *Agrobacterium*-dependent DNA transfer has been the most successful method, especially in dicotyledonous plants. The bacterium is a natural plant pathogen that upon infection transfers a portion of tumour-inducing plasmid (Ti plasmid) DNA called

T-DNA, which then triggers a tumour-like growth (called crown gall disease) in the plant (Nester Gordon et al. 1984). The T-DNA is flanked by border repeat sequences, which are retained while replacing the rest of T-DNA with the gene of interest for subsequent infection and incorporation of the gene by *A. tumefaciens* in the cultured plant cells (Quispe-Huamanquispe et al. 2017). Other technique like gene gun-dependent DNA transfer involves coating gold or tungsten particle with the desired plasmid DNA. The particles are then shot in the growing cultured cells for incorporation in the genome. *Bt* maize is an example of transgenic plant derived from gene gun-mediated DNA transfer (Slater et al. 2008).

• Transgenic plants

Also called genetically modified or engineered crops (GM/GE crops) are created by transferring genes to organisms from a different species which confers a trait not found in the recipient's species. The transgenic plants have been successfully created and field tried for their property to yield more, resist herbicides or insects, be resistant to drought or high soil salinity conditions, etc. (Banjara et al. 2012). The total produce of GM crops has increased from 1.7 million in 1996 to more than 175 million by 2013. The USA leads in the use of biotech crops (cotton, soybean, maize and canola) by accounting for over 40% of global produce. India and China are the biggest growers of *Bt* cotton (Clive 2015). *Gossypium* (cotton) species was genetically altered to express Cry protein (endotoxin) of *Bacillus thuringiensis* which is highly effective in killing lepidopteran insects. The crop was first commercially introduced by Monsanto, Inc. in the USA, in 1996 and later in India in 2003 (Rocha-Munive et al. 2018).

Crop plants of nutritional importance like rice have been genetically modified to produce golden rice variety. *Oryza sativa* (rice) was transformed into biosynthetic gene encoding β carotene which led to its higher yield as compared to wild-type strain. Two varieties were generated of different yield values of β carotene: golden rice-1 yielding 1.6 μg of vitamin A per gram of rice by transferring genes from daffodil and golden rice-2 yielding 35 μg of vitamin A per gram of rice by transferring genes from maize. The varieties are of considerable importance towards fulfilment of vitamin A deficiency which causes night blindness and xerophthalmia (Tang et al. 2009).

Transgenic plants of ornamental importance are now being generated with increased floral scent content or changed colours of flowers. The floral scents in the flowers are due to the presence of volatile organic compounds, namely terpenoids, benzenoid and aromatic amino acids (Piechulla and Effmert 2010). *BEAT* gene has successfully transferred from *Clarkia breweri* into *Eustoma grandiflorum* which induced fragrance in the petals of the recipient plant (Aranovich et al. 2007).

4.2.2 Genetic Modifications in Livestock

Microinjecting the embryos with foreign DNA or somatic cell nuclear transfer is the most feasible methods for developing transgenic animals of agricultural importance. The first method being the most widely practised in which embryos are microinjected with the gene of interest cloned suitably in an expression cassette and implanted back

in the female (Kues and Niemann 2011). The progeny is screened for the presence of the transgene by southern hybridization, pcr or DNA sequencing (Setlow and Hollaender 2002). The main objectives behind such modifications are increased milk and meat production, to render the livestock disease-free and utilize the animal for production of therapeutic proteins through the process called *biopharming*.

- **Recombinant human lactoferrin**

Human lactoferrin (hLF) is an 80 kD iron-binding glycoprotein present in milk and multiple bodily secretions. It is essential for iron uptake in body and kills several harmful iron requiring bacteria by sequestering iron away from them (Lonnerdal and Iyer 1995). Using native gene (comprising all introns and exons) instead of only cDNAs encoding the protein has been found to be yielding higher amounts of therapeutic proteins in the milk of mammals (Choi et al. 1991; Whitelaw et al. 1991). With the former approach, whole genes (having introns and exons) encoding human lactoferrin, transgenic cows have been produced, via both microinjection and nuclear transfer methods yielding ~3–3.4 mg/ml of human lactoferrin in their milk (van Berkel et al. 2002; Yang et al. 2008). The latter study utilized bacterial artificial chromosome for accommodating the hLF construct and bovine fibroblast cells for microinjecting the DNA. Somatic cell nuclear transfer led to the birth of two transgenic cows secreting levels of hLF as mentioned earlier. Biochemical assays revealed their iron-binding and release efficiency similar to the natural hLF.

- **Recombinant antithrombin**

In 2006, the European Medicines Agency and later in 2009, US FDA approved the first transgenically obtained drug—a recombinant human antithrombin III protein derived from the milk of goat, for surgical and childbirth applications. The transgenic goat was developed by the then GTC Biotherapeutics, Inc. at Massachusetts (USA) in collaboration with Louisiana State University Agriculture Center (USA) by injecting goat cells with human antithrombin gene (RD 2004). The protein is purified from goat's milk and is sold currently under the brand name *ATryn* by rEVO Biologics, Southborough, MA (USA) (Erickson 2009). The drug is a lifesaver for those who suffer from hereditary antithrombin deficiency which puts them at the risk of developing deep vein thrombosis or pulmonary embolism (Maksimenko et al. 2013).

- **Transgenic Salmon fish**

AquAdvantage or transgenic salmon fish was developed at Aqua Bounty in Canada in 2006. Wild-type Atlantic salmon (*Salmo salar*) eggs were injected with the growth hormone encoding gene from Chinook salmon (*Oncorhynchus tshawytscha*) under the regulatory elements of an antifreeze protein derived from an ocean pout (*Zoarces americanus*). Whereas a wild-type salmon feeds only in spring or summer and takes 3 years to attain its full length, the transgenically created salmon could reach the same length in less than two years. These fishes have triploid genomic content as compared to their diploid wild-type counterparts and are sterile, thus preventing the risk of interbreeding (Yaskowiak et al. 2006). The US FDA approved AquAdvantage

for human consumption only in 2015 while Canada did the same six months later (Waltz 2017).

4.2.3 Germplasm Conservation

For stable maintenance of the transgenic or wild-type traits of plants and animals, it is essential to preserve the organisms' genetic information so that the traits can pass on from generation to generation without getting exhausted. The body parts containing such genetic information which are viable despite being preserved at ultra-low temperature for long periods of time are collectively called as germplasm. For plants, seeds, calli, pollen, excised embryos or root/shoot bud tips comprise the germplasm. Animal germplasm includes semen, oocytes or embryos. With the advancement of techniques like artificial insemination, the need of storage and preservation of male and female gametes was realized. There are two principal modes of germplasm conservation of both plants and animals, namely *in situ* and *ex situ*. The preservation of the germplasm in the natural habitat of the organism is called as *in situ*; whereas, the artificial methods executed outside the boundary of natural habitat are known as *ex situ* modes of conservation, respectively. The method of cryopreservation is an example of the latter whereby the hereditary information containing components of plant or animal bodies is mixed with a cryoprotectant chemical and frozen in liquid nitrogen (Ruane et al. 2006).

Cryopreservation is achieved via two routes: slow freezing and vitrification (flash freezing). In the former method, the rapid formation of ice crystal is avoided by using chemicals like polyvinyl alcohol or synthetic biopolymers like alginates. The rate of cooling is brought down to 1 °C per minute using a freezing box and cryoprotectants like glycerol and dimethyl sulfoxide (Vutyavanich et al. 2010; Sambu 2015). Almost majority of animal cells, tissues and embryos are frozen by this method. Vitrification, also known as flash freezing, on the other hand bypasses the formation of ice crystals at all. The sudden plunge in ultra-low temperature (megakelvins per second) in cryoprotectant (e.g. ethylene glycol or sucrose) leads to amorphous ice formation which is different from ice crystal sheet formation in which water molecules arrange to create hexagonal lattice. The method was first used to freeze human oocytes which were used to deliver a healthy baby girl through *in vitro* fertilization (Kuleshova et al. 1999).

4.3 Blue Biotechnology

Officially, the term blue biotechnology has not been defined yet by any government/non-governmental organization. Although, the realm of blue biotechnology encompasses marine bioresources and their applications, a more widely acceptable definition of marine biotechnology is used alternatively for the purpose. The European Marine Board defines the area of marine biotechnology as following:

The application of science and technology for the production of knowledge, goods and services from (marine) biological resources. (Adapted from the Organization for Economic Co-operation and Development general definition of biotechnology 2005)

The field is relatively nascent as compared to other areas of biotechnology and not entirely independent from the rest. Blue biotechnology has clear overlaps with other areas of biotechnology because of the technical know-how derived from them. The marine realm of the biosphere comprises 70% of the total earth’s resources. Most of the phyla known till date belong to this realm. A majority of them have been used for societal applications. Broadly speaking, marine microorganisms, phytoplanktons, red, green and brown algae, cnidarians, sponges, echinoderms and mangroves are a few classes from which products of commercial value have been extracted (Blunt et al. 2013). Figure 9 describes the phyla wise contribution of marine life forms for biotechnological, industrial or domestic purposes (Arrieta et al. 2010).

The use of marine life forms on such an enormous scale has led to rise in marine organism’s gene sequence-related patents being filed/granted at the International Patents Office. These approvals have been granted in different sectors of biotechnology affecting various aspects of human life (Arrieta et al. 2010). Most of these range from the patents impacting human health to the field of biofuel generation as shown in Fig. 10.

The field starts with the discovery and bioprospecting of marine bioresources with potential biotechnological application. The commercial potential of these resources

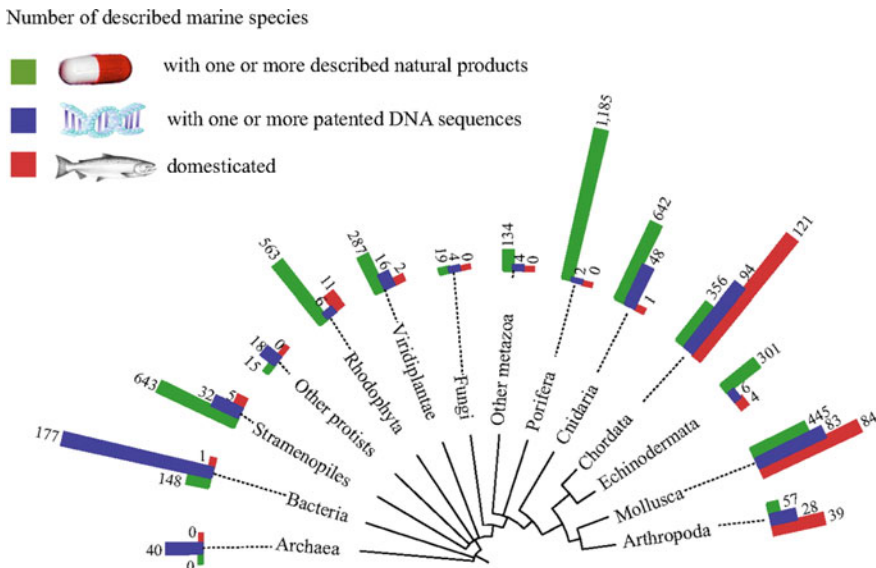


Fig. 9 Schematic depicting marine life forms usage for biotechnology or other human benefits. Each histogram indicates per cent organisms present in the respective taxon utilized for indicated purposes shown in the upper legend (reproduced with kind permissions from the National Academy of Sciences, USA and Dr. Jesus M Arrieta, Spain; (Arrieta et al. 2010)

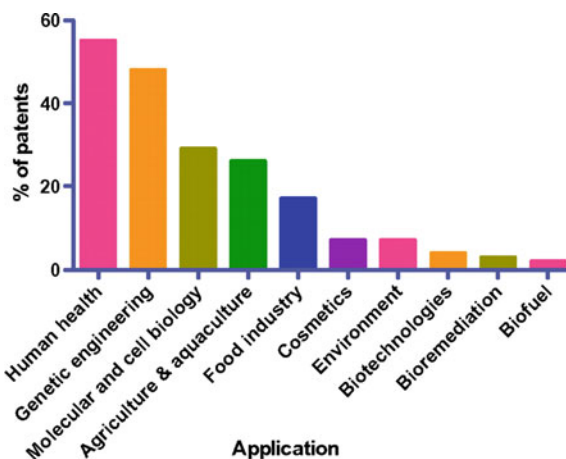


Fig. 10 Graphical representation of distribution of 460 patents deposited with International Patents Office in the area of marine biotechnology. Since one patent may fall in more than one category, sum total percentage exceeds 100% (reproduced with kind permissions from the National Academy of Sciences, USA and Dr. Jesus M Arrieta, Spain; (Arrieta et al. 2010)

ranges from health care in the form of novel pharmacologically active substances to the development of biofuels for providing clean energy. The European biotechnology industry generates annual revenue of € 754 million from the blue biotech sector (ECORYS 2014; ERA-NET 2017). Identification of key gene(s) and or other biologically active molecule and their characterization forms the next step. This involves the screening, selecting and identifying vast number of marine organisms for desired activity. Such organisms range from algal seaweeds to bacteria residing in deep sea hydrothermal vents. Usually, such organisms are difficult to grow in laboratory due to the requirement of pressure, temperature and light conditions in which they otherwise grow. Upon the establishment of successful growth conditions, the organism's DNA is isolated for the purpose of identification of the potential gene encoding the desired product. If the desired product is a biochemical, then its laboratory scale purification is undertaken. The gene or chemical compound identified in such manner is sequenced or assayed for activity in laboratories. Genes are matched to known databases to find out the uniqueness which indicates their evolutionary difference with other known organisms. An example is the famous GFP gene isolated from a bioluminescent marine cnidarian (*Aequorea victoria*), encoding a green fluorescent protein utilized in biomedical research.

After ascertaining the sequence, further research and development programmes are undertaken to characterize the genomic sequence for its expression, purification of the protein and standardization of biological/toxicological assays, and the efficacy in the biological process is formally reported. Examination of commercial feasibility is the deciding factor for the technology adaptation for upscaling to industries and sometimes also the major bottleneck. However, there are several products of marine origin which have seen the light of day, and these are being sold as food, medicine,

cosmetic ingredients, biofuels and bioremediation of the polluted environment. Both marine flora and fauna have contributed to the expanding market of such commercial products on which several industries falling in aforementioned sectors thrive. Alginates are extracted from brown seaweeds (class: Phaeophyceae) like *Laminaria*, *Macrocystis* and *Ascophyllum* and are used in food, textile and pharmaceutical industries. In food industries, it is used for increasing the viscosity like in making jellies. Another brown sea algae, *Fucus vesiculosus*, is used in its extract form as a dietary supplement. Algae-derived oils are good source of omega-3-fatty acids (monounsaturated and polyunsaturated fatty acids; EPA and DHA) that equates to oils obtained from salmon fish (Doughman et al. 2007). Microalgae, *Haematococcus pluivalis*, yields a red-coloured pigment called astaxanthin that is used in the manufacturing of fish feed and is sold by Fuji Chemical Ltd., Japan. Calcium alginates have been used in skin wound dressings for healing and micro-encapsulation of natural polyphenols from wine wastes (Lansdown 2002; Aizpurua-Olaizola et al. 2016). *Ecteinascidia turbinata*, a sea squirt, was identified by the National Cancer Institute (NIH, USA) for producing an anticancer compound. The compound ecteinascidin 743 was purified many years later and is now commercially sold as Trabectedin by PharmaMar S.A. (Spain) (Rinehart 2000). Ziconotide is a powerful analgesic drug which was first isolated from cone snail (*Conus magus*) in early 1980s by scientist Michael McIntosh at University of Utah (McIntosh et al. 1982). The compound which is a peptide got an FDA approval in 2004 and was sold by the name Prialt by Elan Pharmaceuticals, Ireland (Pope and Deer 2013).

Cosmetic industry has been deriving a considerable amount of raw material from marine bioresources. Brown algae, *Ascophyllum nodosum* and *Halopteris scoparia* (class: Phaeophyceae), contains compounds that protect skin from harmful UV rays of sun. The algal extract mix has been patented by Gelyma (France) and is sold by the name Actiseane by Biosil Technologies Inc., NJ (USA) (Andre et al. 2002). *Alcanivorax borkumensis* is a marine flagellar bacterium whose genome contains sequences encoding petroleum degrading gene products. The bacterium uses alkanes as its diet and therefore is employed for degradation of oil spills in oceans. It is therefore also known as “oil-eating” bacterium (Schneiker et al. 2006). Mutant form of *Alcanivorax* is used for production of polyhydroxy alkanooates (PHA) which are used for manufacturing biodegradable bioplastics (Sabirova et al. 2006). Several marine algae are currently explored and used as replacement of tradition fossil fuels for powering engines. *Botryococcus braunii* (family: Trebouxiophyceae) a colonial green microalga is known to secrete hydrocarbon rich oils in the exterior which has been recently purified using electric currents to disrupt the bacterial colony (Banerjee et al. 2002; Guionet et al. 2017).

4.4 White Biotechnology

The term white biotechnology is essentially European in origin. It is alternatively called as industrial biotechnology which simply means application of living cells,

microbes, yeast, fungi, plants and enzymes, etc., for industrial applications (Lee and Jang 2006a, b; Ribeiro et al. 2016). Microbes and enzymes are collectively referred to as *biocatalysts* which are used to manufacture paper, antibiotics, biopolymers, etc. (Heux et al. 2015). Since long time, even before the coinage of the term white biotechnology, living organisms have been used to manufacture products on an industrial scale by techniques like fermentation for making ethanol, acetic acid and various secondary metabolites. Fermentation simply means the decomposition of complex organic compounds to simpler products by enzymes secreted by bacteria and yeast. Modern-day reaction takes place in a steel vessel called the fermenter. The medium for fermentation is composed of carbohydrates, salts, nitrogen, trace elements, etc. The selection of growth medium and the starter culture that includes the inoculum of choice depends upon the product desired. Say, e.g. production of ethanol requires carbohydrate-rich molasses in the growth medium and invertase secreting yeast as inoculum. For production of cheese, lactic acid fermenting bacteria are added to the curdling milk in the fermenters. Examples of fermenting bacteria are *Lactobacillus*, *Enterococcus*, *Streptococcus*, etc., and are known for their beneficial effects on gut microbiota. These are typically used in inoculum as *starter culture* and fed into the fermenting food (e.g. milk for production of cheese). Characterization of the strain of inoculum (here in this case lactic acid-producing bacteria) is a norm as per the regulatory guidelines to ensure safety and maintain dietary consumption quality (Hill et al. 2017). The method of culturing microbes and obtaining commercially important products through the method of fermentation is briefly described by Fig. 11.

The major thrust areas at present are, namely the development of biodegradable polymers and clean green energy by creating biofuels using the tools available through white biotechnology. Since the use of plastics has greatly damaged the environment that does not require further elaboration, a shift towards the alternatives of synthetic non-biodegradable polymers has become imminent. Also, use of fossil fuels like petroleum is slowly being reduced due to its limited reserves. Various environmental protection agencies and government constituted panels have called for research looking into alternative fuel sources which are environment compatible.

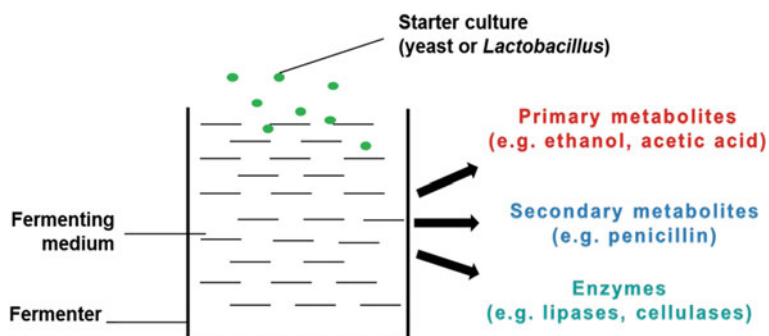


Fig. 11 Schematic describing the fermentation process and its products



Fig. 12 Switchgrass bunch grassland field (*Panicum virgatum*). Photo courtesy of Robert H. Mohlenbrock, hosted by the USDA-NRCS PLANTS Database/USDA SCS. 1991. Southern wetland flora: Field office guide to plant species. South National Technical Center, Fort Worth

Such fuels when derived from biomass meaning any plant or animal material are designated as *biofuels* as per US Environment Information Agency (EIA 2018). The most commonly used biofuels are bioethanol and biodiesel. Bioethanol is conventionally produced from carbohydrate-rich corn syrup or molasses. Recently, there has been a tilt observed towards the production of ethanol from cellulosic biomass. Switchgrass (*Panicum virgatum*; family: Poaceae) is a widely growing perennial bunch grass in most of the USA as shown in Fig. 12. The US Department of Agriculture (USDA) has selected the plant for production of bioethanol derived from the plant biomass because of its ease of growing and high yield. The ethanol thus produced is currently being mixed with gasoline at a final concentration of 10% (E10 fuel) and is used for automobiles (EIA 2018).

Biodiesel is a liquid fuel which is currently promoted as an alternative to diesel for its usage in public transport and commercial vehicles. It is manufactured by vegetable oils and animal fats by esterification of fatty acids with an alcohol (Omidvarborna et al. 2014). The National Biodiesel Board of America describes biodiesel as mono-alkyl esters of fatty acids and designate as B100 in its purest form. There are other parts mixed forms of biodiesel available, like B20 where it is 20% biodiesel of the total fuel. Plants utilized for the purpose of producing biodiesel include soybean, jatropha, rapeseed, etc. *Salicornia bigelovii* is a halophyte growing in coastal shorelines of America that has been identified as biomass feedstock for generating biofuel. It can survive well on salty water and is therefore suitable for large-scale cultivation by irrigating with salt-rich water, a common industrial effluent (Bullis 2010). A similar application of biomass lies in production of bioplastics which uses vegetable oils, straw, woodchips and food waste (McMillan 2010). Starch-based thermoplastics are used for making drug capsules by pharmaceutical companies. These plastics are actually a blend of several chemicals like polyhydroxy alkanooates and polylactic acid which are endowed with better water resistance and improved mechanical properties. Starch plastics are also used in the manufacturing of films used for packaging of food items (Halley and Avérous 2014). Another biodegradable polymer polylactic acid



Fig. 13 Chinese brake fern (*Pteris vittata*); a hyperaccumulator of arsenic. Photo courtesy of <https://suzandtell.wordpress.com/2015/02/18/the-chinese-ladder-fern/>

is being produced by fermenting metabolically engineered strain *E. coli* for supply to industries manufacturing it. Bacteria like *Citrobacter*, *Klebsiella* and *Enterobacter* have been known to produce 1,3-propanediol which is used for manufacturing adhesives, solvents and antifreeze agents (Lee and Jang 2006a, b).

Another area of growing importance is the field of phytoremediation that involves the usage of plant for environmental clean-up. The industrial pollutants are discharged into land, water and air which include heavy metals, organic compounds and high salt-containing water. These have become a growing menace for the society as they are severely damaging the environment and leading to rise in diseases and deaths. Heavy metals like cadmium, mercury and lead have been found to percolate down the soil and reach groundwater table thus polluting it and making it unfit for drinking or agricultural purpose. The plants that are able to extract these metals are used for the purpose of phytoremediation of the polluted soil. Plants capable of accumulating heavy metals more than 1000 mg/kg dry weight are called as hyperaccumulators (Reeves 2003). *Thlaspi caerulescens* (family: Brassicaceae) is being successfully used as hyperaccumulator for phytoremediation of cadmium and zinc. Arsenic remains one of the biggest threats to groundwater that is used for direct drinking purpose. A Chinese fern *Pteris vittata* of family—Pteridaceae (commonly known as Chinese brake fern) was demonstrated to be a hyperaccumulator of arsenic (Ma et al. 2001) (Fig. 13).

5 Summary

The field of biotechnology has evolved a lot since prehistoric times to what we see it today and continues to evolve further. Improvization of pre-existing knowledge and groundbreaking discoveries in the past centuries has led to significant overhauling of our understanding of biological processes. The causative agents of many

previously incurable diseases have now an identity, a method of diagnosis and a possible treatment regime due to which life expectancy has increased considerably from those times. Biotechnology has touched upon lives of people globally through its various defined and undefined branches. Seminal discoveries governing the basic process of life, their applications in the areas of health care, agriculture and environment have revolutionized the practice of science and adaptation of technology in an unprecedented manner.

It is reasonable to conjecture that given the magnitude of applications in societal welfare, the field of biotechnology will be going to be a major area of research and investment. However, the endeavours pertaining to the investment are to be taken up with certain precautions because many such areas need accumulation of enough body of work before final commercialization can be realized. Specifically, areas like blue biotechnology which holds immense possibility of discovery of several crucial drugs, food supplements, industrially required materials are first needed to be explored through deep research. Only thereafter, the true feasibility of the product on a commercial scale can be adjudged. Areas like red biotechnology have many promising inventions and discoveries in its basket. Gene editing tool like CRISPR method is hailed across the globe as a major breakthrough in the sciences that can cure several genetic diseases which are incurable till date. Application of the same in the field of green biotechnology can lead to the generation of crops and livestock with improved or superior traits for ensuring food and nutrition security of the public. Increasing pollution of the ecosystem has prompted the scientists to adapt these technologies for better environmental clean-up. The exploitation of phytoremediation for absorbing heavy metals and degradation of oil spills in the oceans is an exciting area of exploration. Increasing the industrial output by utilization of biologics or living organisms helps in generation of several biodegradable materials which avoids conventional hazardous chemical synthetic processes.

What remains to be done is an exhaustive list of tasks that needs to be taken up seriously to ensure better practice of biotechnology throughout its various branches. The formation of ethical guidelines for application of genetic techniques for embryo manipulation needs to be carefully drafted. This is essential to prevent the abuse of technology for personal fame and reputation at the cost of suffering of humanity. Release of GM crops with proper evaluation of pros and cons must form an integral part of crop improvement programs in poor and developing countries. The area of blue biotechnology is still in its infancy, and therefore, more impetus is needed to attract people towards exploring marine genetic resources. Marine resource exploitation should be carried out with the sense of responsibility without damaging the ecosystem. The concept of sustained development needs to be implemented in essence so as to propel further research while ensuring judicious use of the natural resources.

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