Microbial Technology and Biotechnology

2

2.1 Introduction

Microorganisms since time immemorial have been exploited for the process of baking, brewing and food preservation. However, they also produce things which have immense value and are usually classified as primary metabolites that are essential for their own growth and secondary metabolites which are non-essential. Both primary and secondary metabolites have played a tremendous role in the development of chemical, pharmaceutical and food industries wherein they have been exploited for novel products and process development.

The advent of modern biotechnology began with the birth of recombinant DNA technology in 1972. Biotechnology currently refers to an array of enabling technologies which have applications in different industrial sectors. Genetic engineering, protein engineering and metabolic engineering are the three major disciplines which comprise biotechnology. The fourth discipline is biochemical process engineering which encompasses commercial production of biotechnological products. Modern microbial technology relies on the metabolic potential of microorganisms and the varieties of methods by which they have been harnessed. Genetically modified microorganisms (GMMOs) today find applications in the areas of human health, agriculture, environment, food, chemicals, paper and textile industries. By genetic engineering the molecular diversity and chemical selectivity of the microorganisms help in the production of desired products as well as cheaper and ecofriendly process development.

The different molecular methods which contribute to the development of GMMOs are (1) gene transfer methods to deliver specific genes in the desired host, (2) cloning vectors, (3) selectable marker gene for identification of recombinant microorganisms and (4) promoters to control the expression of desired genes. The most common recombinant microorganisms which express the genes of other organisms are *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha* and *Aspergillus niger*. A variety of products and processes have been developed through these recombinant microorganisms.

The strategies of creating a GMMO are based on (a) overexpression of the target gene in the native host or heterologous host, (b) alteration of gene sequence resulting in a change in the target protein sequence and (c) disruption or complete removal of the target gene or pathway.

In recombinant strains of *Corynebacterium glutamicum*, the increased number of *dapA* gene for dihydrodipicolinate synthase was introduced, and it enhanced the lysine titre when compared to the wild type (Eggeling et al. 1998). Site-directed mutagenesis and DNA shuffling have been

The chapter entails the application of microbial interventions in different industrial sectors. The role of GMMOs would also be discussed in light of their application for higher productivity or bioprocess development for green technologies.

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effectively used in improving the enzymes from bacteria and fungi for application in the detergent industry. Feedback regulation has also been manipulated at the molecular level and has been effectively applied in *Corynebacterium glutamicum* for isoleucine production. Several examples exist wherein recombinant microorganisms play an important role in the industrial sector for the production of specific/desired products as well as for the development of green and energy-efficient processes.

2.2 Healthcare Industry and GMMOs

Human insulin was the first recombinant protein which was produced through a genetically modified *E. coli* containing human insulin genes. This work was done at Genentech Inc, USA, and the USFDA approved the clinical use of this product in 1982. Recombinant interferon gamma-1b (Actimmune) has also been developed by Genentech with Boehringer Ingelheim for the treatment of chronic granulomatous disease in 1990. IFN-γ 1b was produced by expressing the genes encoding the interferon in *E. coli*. Similarly the first recombinant vaccine was Engerix-B® produced by the gene encoding for hepatitis B surface antigen expressed in *Saccharomyces cerevisiae*, the common baker's yeast. Till date 151 protein-based recombinant pharmaceuticals have been licensed up til 2009, by the FDA and EMEA, of which 29.8 % are being produced in *E. coli*, 18.5 % in *Saccharomyces cerevisiae* and 11.2 % in hybridoma cells while 39 % in mammalian cell lines. Some recombinant products produced by *Saccharomyces cerevisiae* and *E. coli* have been listed in Table 2.1.

In animals bovine somatotropin hormone regulates animal growth as well as milk production. The bovine somatotropin (bST) gene has been expressed in *E. coli* and was approved by the USFDA in 1994 under the commercial name Posilac™. Studies have indicated that bST-supplemented cows produced 10–15 % more

Table 2.1 Therapeutic interventions developed using recombinant GMMOs

Recombinant product	Application	Expression system	Approving authority, year
Dukoral	Oral cholera vaccine	*E. coli*	USFDA/EMEA, 2004
Actimmune	Interferon gamma-1b	*E. coli*	USFDA, 1990
Viraferon	Interferon alfa-2b	*E. coli*	EMEA, 2000
IPLEX	Mecasermin rinfabate recombinant (insulinlike growth factor)	*E. coli*	USFDA, 2005
Kepivance	Palifermin (keratinocyte-like growth factor)	*E. coli*	EMEA, 2004; USFDA, 2005
Humulin	Human insulin	*E. coli*	USFDA, 1982
Preotact	Human parathyroid hormone	*E. coli*	EMEA, 2006
Nesiritide	Recombinant natriuretic peptide B	*E. coli*	USFDA, 2001
Engerix-B	Hepatitis B vaccine	*S. cerevisiae*	USFDA, 1998
Gardasil	Human papillomavirus vaccine [types 6,11,16,18]	*S. cerevisiae*	USFDA/EMEA, 2006
Regranex	Human platelet-derived growth factor	*S. cerevisiae*	USFDA, 1997; EMEA, 1999
Valtropin	Recombinant somatropin	*S. cerevisiae*	EMEA, 2006
Regranex	Human platelet-derived growth factor for wound healing	*S. cerevisiae*	USFDA 1997; EMEA, 1999
Iprivask	Recombinant-specific inhibitor of human thrombin isolated from *Hirudo medicinalis*	*S. cerevisiae*	EMEA, 1997
Fasturect	Recombinant rasburicase (urate oxidase)	*S. cerevisiae*	EMEA, 2001

milk. The enzyme phytase helps in the release of phosphate from phytate which is a primary storage form in plants. It has been observed that ruminants possess the capacity to obtain phosphorus, an essential element from their feed; however, nonruminants like pigs and chicken are unable to obtain phosphorus. The gene of this enzyme has been isolated from *Aspergillus niger* and constitutively expressed in the industrial strain of *Aspergillus niger* for commercial production of phytase for treatment of commercial feed.

Apart from recombinant biopharmaceuticals for human and veterinary purposes, the genetically modified microorganisms also find applications in the development of diagnostic kits. The antigenic coat protein of HIV has been cloned and expressed in *E. coli* for the large-scale production of protein for the development of ELISA-based diagnostic kit. Similarly tau proteins have been implicated in Alzheimer's disease. To diagnose Alzheimer's disease non-invasively, antigens against tau proteins can be tested in human cerebrospinal fluid. The antigens against tau proteins have been produced in large scale using recombinant *E. coli* expressing Alzheimer's antigen for the development of enzyme-linked immunosorbent assay, INNOTEST h TAU.

2.3 GMMOs in Agriculture

Genetically modified organisms find limited applications in agriculture. *Bacillus thuringiensis* var. *kurstaki* is a soilborne bacterium which is exploited as a biopesticide since it produces unique crystalline proteins (δ-endotoxins or *cry* proteins) which possess larvicidal activities against different insect species. These toxins broadly are non-toxigenic to mammals, birds and fishes and thus can be effectively used as bioinsecticides.

Bt toxins have been introduced successfully into several plant-associated bacteria like *Pseudomonas* sp. and *Azospirillum* sp. for delivering Bt toxins inside the plants without altering their genomes. One important gram-positive bacterium is *Clavibacter xyli* ssp. *cyanodontis* which resides in the xylem of *Cynodon dactylon* (Bermuda grass). This bacterium also colonises *Zea mays* (corn) when introduced artificially, and thus, recombinant *Clavibacter xyli* expressing Bt toxins have been used as a bio-inoculant to provide corn resistance against insect attacks. It has experimentally shown moderate control of the European corn borer. Nitrogen assimilation is important for plant metabolism, and it has been reported that genetically modified rhizobacteria *Sinorhizobium meliloti* expressing the *nif A* gene of *Klebsiella pneumonia* exhibited better root nodulation in alfalfa (*Medicago sativa*) plants. Further it was also found that the recombinant *S. meliloti* significantly increased the plant biomass as compared to the wild type. Thus, possibilities exist wherein genetically modified microorganisms living endophytically or colonising in the rhizosphere could be suitably exploited for improving plant growth and yield without genetically modifying them.

2.4 Role of GMMOs in Chemical Industry

With the increasing societal concern about the environment, climate change and limited natural resources, there has recently been considerable effort exerted to produce chemicals and materials from renewable biomass. The common method of production of these polymers is directly through fermentation or polymerisation of the monomers produced by fermentation. Advances in metabolic engineering and molecular biology have helped in the systematic development of superior strains and processes for the production of polymers and monomers.

Polymers are macromolecules which are composed of a series of low molecular weight monomers. Microorganisms naturally produce polyhydroxyalkanoates (PHAs) in the form of granules that the organisms use as an energy storage material. They are genuine polyester molecules with properties similar to petroleum-derived polymers. Further they are biodegradable; the enzyme which depolymerises them is widely present in fungi and bacteria. *E. coli* has been transformed by transferring the PHA genes which

the microorganism is lacking for the commercial production of PHA since *E. coli* exhibits a robust growth and its metabolism is well characterised and apart from it lacks PHA depolymerase which is responsible for its degradation.

Similarly *E. coli* knockout mutant strain has also been developed for the commercial production of lactic acid exhibiting a production of 138 g/L of lactic acid with a yield of 0.99 g lactic acid/g glucose and an overall productivity of 3.54 g/L/h. Adipic acid is the building block of nylon-4, 6 and nylon-6, 6. Using a recombinant *E. coli* strain, cis, cis-muconic acid has been produced at a concentration of 36.8 g/L which is subsequently hydrogenated to adipic acid with 0.97 g/g conversion yield at room temperature. Similarly glucaric acid production has been carried out through *E. coli* by metabolically constructing a direct synthesis pathway by introducing myo-inositol-1-phosphate synthase from *Saccharomyces cerevisiae*, myo-inositol oxygenase from mouse and urinate dehydrogenase from *Pseudomonas syringae*.

Antibiotic production is significantly enhanced using GMMO's. Cephamycin path genes from *Streptomyces cattleya* was cloned in *Streptomyces lactamgems* led to 2.3 fold increase in Cephamycin C production. The precursors of semi-synthetic cephalosporins are 7-aminocephalosporanic acid (7-ACA) or 7-amino-deacetoxycepalosporanic acid (7-ADCA). *P. chrysogenum* was transformed with *Streptomyces lipmanii* cefD and *S. clavuligerus* cefE genes which allowed the production of intermediate deacetoxycephalosporin C (DAOC) at titers of 2.5 g/L, along with penicillin V. A recombinant *E. coli* was constructed containing a D-amino acid oxidase gene from *Trigonopsis variabilis* and the glutaryl-7-aminocephalosporanic acid acylase gene from *Pseudomonas* species which was capable of converting cephalosporin C to 7-ACA directly.

For the production of alcohol, a recombinant strain of E. coli was developed which expressed alcohol dehydrogenase II and pyruvate decarboxylase genes from *Zymomonas mobilis*. 43 g/L of ethanol production was achieved by this recombinant strain. *Lactobacillus plantarum* has been genetically engineered overexpressing the two sorbitol-6-phosphate dehydrogenase genes (srlD1 and srlD2) for high sorbitol production. It is used in food and pharmaceutical industries, and its annual requirement is approximately 500,000 tonnes. Thus, recombinant microorganisms play a critical role in commercial production of primary and secondary metabolites apart from the development of novel ecofriendly processes.

2.5 GMMOs in Textile Industry

The exploitation of microbial enzymes in the textile industry began in early 1900. To commercially use them in the industry on a large scale, these enzymes have to be produced at high levels. Heterologous hosts have been used for the production of enzymes to overcome the inherent limitations of the naturally producing microorganism. *Bacillus stearothermophilus* produces a heat-stable and broad-pH active α-amylase. The gene of this enzyme was cloned and expressed in *Bacillus licheniformis* for commercial production of the enzyme. A variety of recombinant enzymes such as cellulase, pectinases, proteases, etc., have been used in the textile industry which have been discussed in Chap. 9 of this book. Recombinant enzymes produced by GMMOs not only find applications in the textile industry but also other industrial sectors such as paper, environment, food, feed, biosensors, pharmaceuticals and fine chemicals.

2.6 Environmental Applications of GMMOs

Microbial bioremediation essentially refers to the use of microorganisms to detoxify the polluted environment which is due to heavy metals such as mercury and lead and organic compounds such as petroleum hydrocarbons, radionuclides such as uranium and plutonium and other compounds such as explosives, pesticides and plastics. There are two basic strategies of microbial bioremediation, bioaugmentation and biostimulation for the

remediation of polluted sites. The pioneering work in this field was carried out by Gunsalus and A. M Chakraborty by developing a recombinant *Pseudomonas*, *Pseudomonas fluorescens* HK44, for degradation of camphor, octane, salicylate and naphthalene. It is the first life form to be the subject of an intellectual property case. As soon as the prospect of releasing GMMOs for bioremediation became a reality, much of the research effort in the field was aimed at biosafety and risk assessment. There are many issues surrounding the use of GMMOs for use in bioremediation such as (1) their effectiveness as compared to their counterparts in the nature, (2) their influence on indigenous microbial community, (3) their fitness in nature and (4) their containment. There are only a few cases where the use of GMMOs turned out to be much better in performance than its non-manipulated counterpart. Commercial bioremediation largely relies on the naturally occurring microbes identified from the contaminated sites.

2.7 Food Industry and the Role of GMMOs

The enzymes produced by genetically modified microorganisms have been effectively used in the food industry for over a decade. The gene encoding for calf stomach chymosin was cloned and expressed in an industrial strain of *Kluyveromyces lactis*, a yeast that had been used for many years in the safe production of food ingredients. Chymosin produced through the recombinant yeast strains possesses the same chemical and biological properties as that of calf rennet. The preparation has been registered under the brand name Maxiren® and has been commercially available since 1988. Currently there are approximately 30 different enzymes, many of them in food use, that are produced by GMMOs. The recombinant brewer's strain having an amylase gene from *Saccharomyces diastaticus* together with a gene for copper resistance was approved in 1993; however, it could not go commercial because of the unwillingness of the industries to face a negative consumer reaction. Similarly recombinant strains of *Saccharomyces* for wine production have been developed but have not become a commercial reality till date.

Despite much research in the genetic and molecular studies on probiotic bacteria, *Lactobacillus* species, and *Saccharomyces* species, and modifications carried out, the applications of genetically modified microorganisms have not been realised in food processes.

2.8 GMMOs for Bioethanol Production

The major technical roadblock for the development of bioethanol industry is the lack of suitable microorganisms which can convert the biomass into fuel ethanol. However, in the last two decades, several microorganisms have been genetically engineered exploiting them as bugs which enhanced capacity to transform the lignocellulose biomass. Lignocellulose biomass basically comprises complex carbohydrates that cannot be fermented by brewer's yeast *Saccharomyces cerevisiae*. The success has been met with gram-negative microorganisms like *Escherichia coli*, *Zymomonas mobilis* and *Klebsiella oxytoca* which have been genetically engineered to metabolise a wide spectrum of sugars and produce ethanol. *Zymomonas mobilis* is homoethanol fermentative because it converts pyruvate into ethanol utilising pyruvate decarboxylase (PDC), which only consumes one NADH, H+ for each ethanol produced. The recombinant *E. coli* expressing the pdc and adhII genes were co-expressed under the control of native lac promoter, and the resulting construct was named as PET (production of ethanol) operon. This recombinant *E. coli* strain became homoethanol fermentative thereby producing ethanol exclusively. *Zymomonas mobilis* has high ethanol yield and productivity since it metabolises glucose anaerobically using the Entner–Doudoroff (ED) pathway as compared to the EMP pathway or glycolysis. However, the limitation of *Zymomonas mobilis* is that it ferments only glucose, sucrose and fructose. The first recombinant strain was engineered to ferment xylose.

The transformed strain CP4 (pZB5) grew on xylose, and the ethanol yield was 86 %. The recombinant microorganisms thus have played a significant role in the development of the bioethanol industry.

2.9 Summary

Thus, one can conclude that genetically modified microorganisms have a tremendous impact in the human healthcare sector and chemical industry as well as for bioethanol production. Advances in functional genomics and bioinformatics tools, combined with existing recombinant DNA technologies, will help us better understand the physiology and metabolic potential of the organisms we study and, in turn, will lead to the development of GMMs best suited to our needs.

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