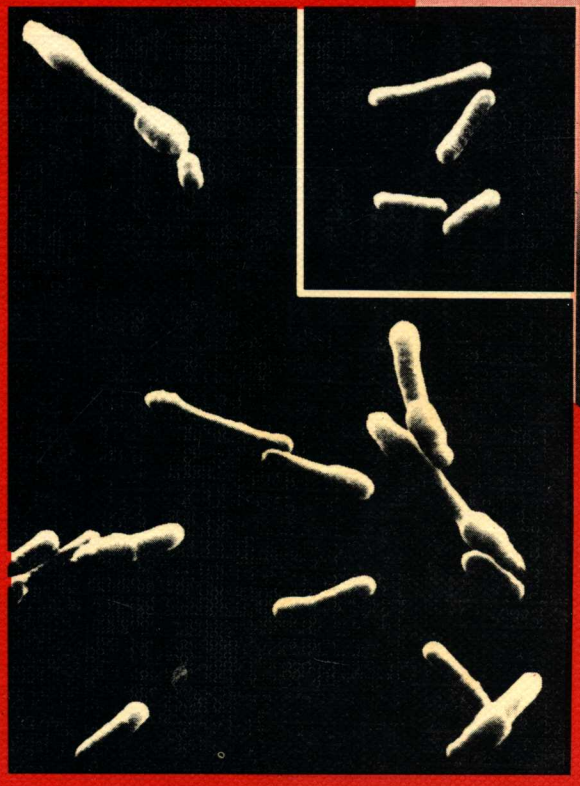
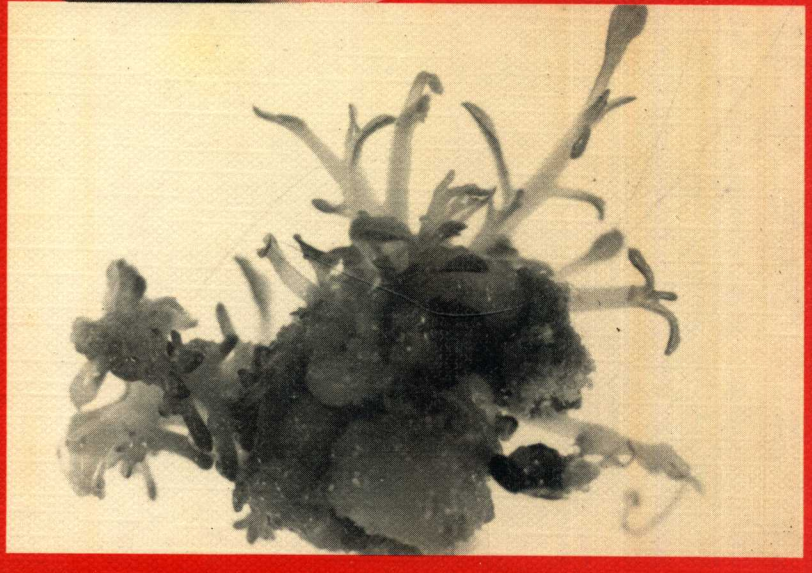
MODERN BIOTECHNOLOGY

S B Primrose





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S.B. PRIMROSE BSc, PhD
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Modern Biotechnology

Preface

Biotechnology is one of the buzz words of the decade. Rightly or wrongly it is often associated with easy money and, consequently, means all things to all people. So what is biotechnology? At its simplest it is the commercial exploitation of living organisms or their components, e.g. enzymes. Thus the science formerly known as industrial microbiology clearly falls within the definition of biotechnology and to many they are synonomous. But plants and animals are also exploited commercially in the practices known as horticulture and agriculture and these could come under the umbrella of biotechnology. By common consent the terms plant and animal biotechnology are restricted to that grey area encompassing the application of modern molecular biology and cell culture techniques to the manipulation of plants and animals, e.g. plant cell culture and transgenic animals, and which is not yet part of conventional plant and animal breeding.

This broad view of what constitutes biotechnology forms the basis of this book. The overriding aim has been to combine in a single text detailed information on recombinant DNA technology, protein engineering, industrial microbiology, monoclonal antibodies, plant and animal cell culture, new methods of plant and animal breeding and the legal, social and ethical issues which surround biotechnology. It provides a basic reference source for undergraduates taking courses in biochemistry, genetics and molecular biology and also for industrial specialists who seek further information on areas they are less familiar with.

The book is heavily biased towards the impact of in-vitro gene manipulation but then without gene manipulation biotechnology would not be a buzz word!

SANDY PRIMROSE

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Abbreviations

AAT	α_1-antitrypsin
ADA	adenosine deaminase
ADH	alcohol dehydrogenase
BPV	bovine papilloma virus
CaMV	cauliflower mosaic virus
cDNA	copy DNA
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
dpm	disintegrations per minute
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (USA)
EPSP	3-enolpyruvyl-shikimate 5-phosphate
FDA	Food and Drug Administration (USA)
FMDV	foot and mouth disease virus
GMP	good manufacturing practice
GVH disease	graft-versus-host disease
HAT medium	hypoxanthine, aminopterin and thymidine medium
HFCS	high-fructose corn syrup
HLA	human leucocyte antigens
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine phosphoribosyl transferase
MAB	monoclonal antibody
MEOR	microbial-enhanced oil recovery
MMT	mouse metallothionein
MS medium	Murashige and Skoog medium
NIH	National Institutes of Health (USA)
NMR	nuclear magnetic resonance
rbs	ribosome binding site
RF	replicative form
RFLP	restriction fragment length polymorphisms
RIA	radioimmunoassay
RuBPCase	ribulose bisphosphate carboxylase
SCP	single-cell protein
Ti plasmid	tumour-inducing plasmid
TK	thymidine kinase
VSV	vesicular stomatitis virus
VVM	volume (of air) per volume (of liquid) per minute

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Part I
Introduction

1/Biotechnology — Ancient and Modern

INTRODUCTION

Biotechnology is not a recent development. Microorganisms have been used to produce food such as beer, vinegar, yoghurt and cheese for over 8 millenia. Certainly the ancient Sumerians were familiar with beer and alehouses were an established part of Roman civilization. Wine also was popular with the Romans and they tried introducing grapevines into southern Britain for the express purpose of wine-making. References to wine and vinegar (Fig. 1.1) are scattered throughout the Bible, which is an indication that their production dates back to early times.

In many instances microbial contamination of food results in spoilage, although what is unpalatable to one race may be a delicacy to another! Occasionally microbial growth would result in beneficial changes such as improved flavour and texture and, more importantly, improved storage quality. Once these desirable changes had occurred, they would be self-perpetuating. In the absence of a knowledge of microbiology storage vessels would not be cleaned and the residual food would act as an inoculum. In many respects modern production of fermented foods is little different: open vessels are still used and a residue from one batch is used to inoculate the next one.

Ethanol was the first chemical to be produced with the aid of biotechnology. The origins of distillation are not clear but by the 14th century AD it was widely used to increase the alcoholic content of wines and beers. Indeed it was at this time when the 'auld alliance' between France and Scotland was at its prime that the French brandy manufacturers taught the Scots brewers to distil their beer to produce whisky! From the production of spirit beverages it was but a small step to the production of neat alcohol and approximately 25% of world ethanol production is still produced by this biological route.

Until just over a century ago it was not realized that microorganisms were involved in the production of alcohol and vinegar. The discovery came when a group of French merchants were searching for a method that would prevent wine and beer from souring when they were shipped over long distances. They asked Louis Pasteur for help. At the time many scientists believed that air acted on the sugars in these fluids to convert them into alcohol. Instead, Pasteur found that yeasts convert the sugars to alcohol in the absence of air. Such an anaerobic process is known as *fermentation*. Souring and spoilage occur later and are due to the activities of a group of bacteria, the acetic acid bacteria, which convert alcohol into vinegar (acetic acid). Pasteur's solution was to heat the alcohol just enough to kill most of the microorganisms present, a process that

20 And Noah began to be an husbandman, and he planted a vineyard:
21 And he drank of the wine, and was drunken; and he was uncovered within his tent.

Book of Genesis, Chapter 9.

46 And about the ninth hour Jesus cried with a loud voice, saying, Eli, Eli, lama sabachthani? that is to say, My God, my God, why hast thou forsaken me?
47 Some of them that stood there, when they heard that, said, This man calleth for Elias.
48 And straightway one of them ran, and took a spunge, and filled it with vinegar, and put in on a reed, and gave him to drink.

The crucifixion of Christ.
Book of Matthew, Chapter 27.

Fig. 1.1 Biblical references to alcohol and vinegar.

does not greatly affect the flavour of the wine or beer. This process is known as *pasteurization*, although we now know that a similar technique was used for the manufacture of sake in the Orient over 300 years earlier.

THE FIRST WORLD WAR AND THE RISE OF THE MODERN FERMENTATION INDUSTRY

Distillation apart, there was little change in biotechnology from the pre-Christian era until the early 20th century and, as is often the case with technological advance, the impetus was provided by war. At the start of the First World War the British naval blockade prevented the Germans from importing the vegetable oils necessary to produce glycerol for explosives manufacture. Consequently the Germans turned to the microbial production of glycerol by yeast (Fig. 1.2) and soon were able to manufacture over 1000 tons per month. For its part, Germany was able to hinder the British war effort since prior to hostilities it had been the source of acetone and butanol: the former was required for munitions and the latter for artificial rubber. The result was the British development of the acetone–butanol fermentation (Fig. 1.3) using *Clostridium acetobutylicum*.

The glycerol fermentation was short-lived. By contrast, the acetone–butanol fermentation survived until the early 1950s and during the Second World War some interesting innovations were made, such as the introduction of semi-continuous fermentation. Initially *Cl. acetobutylicum* was cultured in a large volume of medium until growth ceased. At this stage two-thirds of the medium was removed for extraction of acetone and butanol and the culture vessel filled up with fresh medium. With

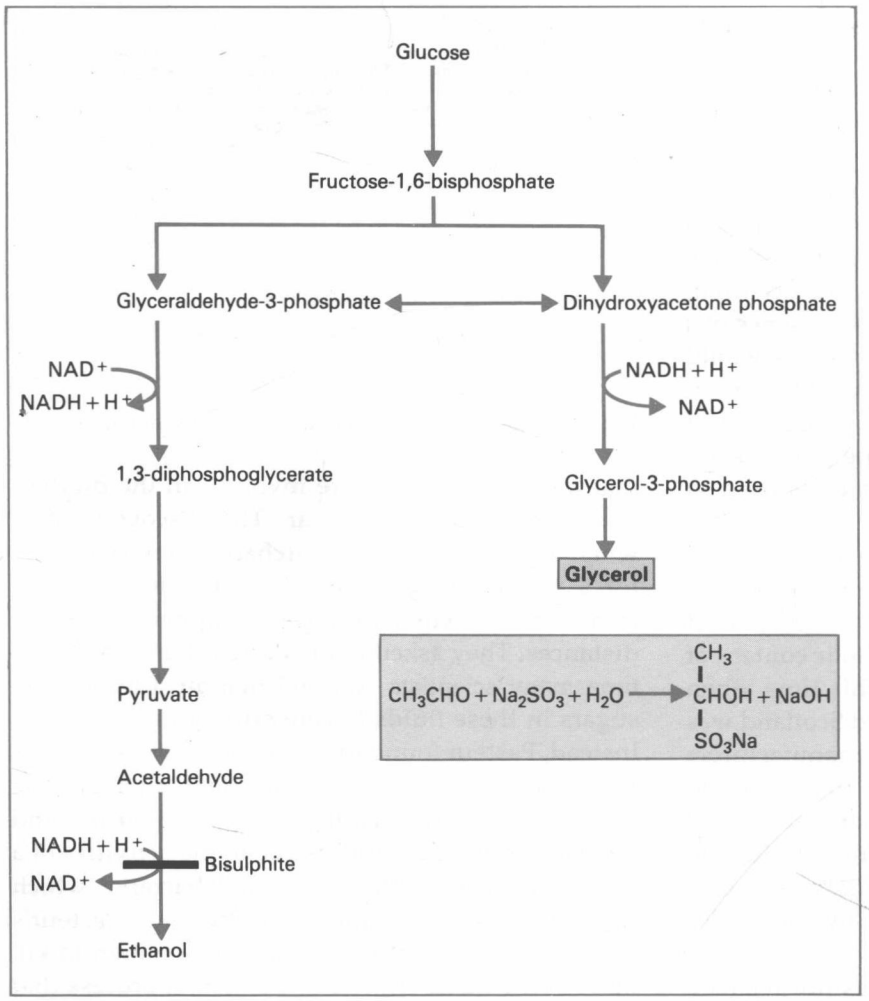


Fig. 1.2 The formation of glycerol instead of ethanol when bisulphite is added to a yeast culture. The bisulphite forms an addition product with acetaldehyde as shown in the inset.

such a large inoculum of cells complete conversion of sugar to solvents occurred in only 12 h. Later it was realized that it was possible to use non-sterile medium; although the yield of solvent was lower due to the growth of contaminating bacteria, the savings in fuel were substantial. In the immediate post-war period many organic chemicals, including acetone and butanol, became readily available from by-products of the petroleum industry and the fermentation process was discontinued. However, the acetone–butanol fermentation is an exceedingly simple process which might be of benefit to many Third World countries who cannot afford to spend vast sums of money on either petroleum itself or petrochemical-based products. Since these countries often have an abundance of the necessary cheap raw materials such as sugar or starch this fermentation process may make a comeback!

Present-day citric acid manufacture also has its origins in the First World War. Until then citric acid had been extracted from citrus fruits and the major producer was Italy. As men were called to arms the citrus groves were left untended. By the time hostilities ended the industry was in ruins and the price of citric acid had escalated. This paved the way for the introduction of a microbial process in 1923. Unlike *Cl. acetobutylicum*, the organism used to produce citric acid (*Aspergillus niger*) is an obligate aerobe and so must be cultured in the presence of oxygen. Initially large-scale culture was achieved by placing liquid medium in shallow metal pans and allowing the organism to grow on the surface. Later this method of *surface culture* was improved by absorbing the nutrient medium onto an inert granular support (Fig. 1.4(a)).

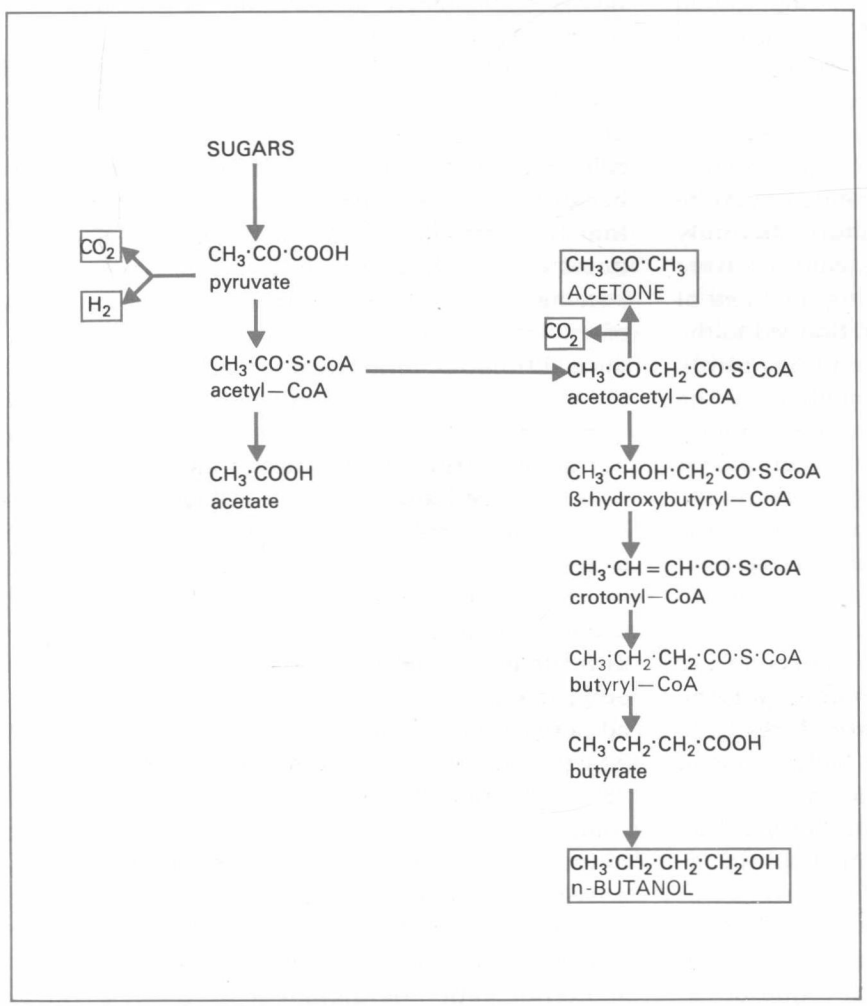
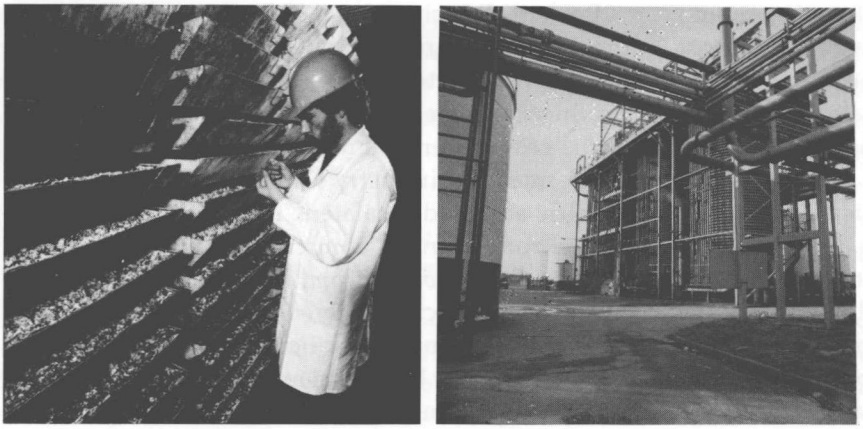


Fig. 1.3 The pathway of acetone and butanol formation by *Clostridium acetobutylicum*. Note that hydrogen gas is produced in addition to the two solvents. Not surprisingly, a number of manufacturing plants were destroyed by explosions!



(a) (b)

Fig. 1.4 Production of citric acid (a) by surface culture and (b) in a stirred tank reactor. (Photos courtesy of John and E. Sturge Ltd.)

PENICILLIN AND THE PRODUCTION OF FINE CHEMICALS

Penicillin was the name given by Fleming to the antibacterial substance produced by the mould *Penicillium notatum*. By 1940 when the first purified preparations became available the phenomenal curative properties of penicillin were obvious. The significance of this discovery was not lost on a country again at war. Like the *A. niger* used to produce citric acid, *P. notatum* is an obligate aerobe and had to be grown in surface culture. Not only was this labour-intensive but the cultures were prone to contamination, thus reducing the yield of penicillin. The need for aseptic operation led to the development of the stirred tank reactor (Fig. 1.4(b)), which to this day is the preferred method for the large-scale cultivation of microbes. Aseptic operation is achieved by sterilizing all the equipment with steam prior to inoculation and by keeping the pressure inside the vessel higher than atmospheric pressure. To meet the oxygen demand of the culture, sterile air is blown into the vessel and distributed throughout the medium by agitation.

A second contribution to modern biotechnology that was made by the penicillin programme was the development of strain selection procedures. The original *P. notatum* culture yielded only 2 mg of penicillin per litre of culture fluid but by screening many different *Penicillium* isolates a higher-yielding variant, *P. chrysogenum*, was identified. In an attempt to improve the yield still further the *P. chrysogenum* was exposed systematically to a variety of mutagens such as nitrogen mustard, ultraviolet radiation and X-radiation. After each round of exposure the survivors were screened and the highest-yielding variant carried forward for the next round of mutations. By combining fermentation improvements with the use of mutants the titre has been increased to over 20 g/l.

That microbes could produce antibiotics had been known for a long time but was considered of little significance. Once the clinical utility of penicillin was established, pharmaceutical companies began to consider antibiotics seriously. Following the discovery that *Streptomyces griseus* also produces a clinically useful antibiotic, streptomycin, it became standard practice to screen large numbers of environmental isolates for their ability to produce antibiotics. Since then it has been shown that the filamentous bacteria known as actinomycetes, a group to which *S. griseus* belongs, produce many hundreds of different antibiotics, including at least 90% of those known today. Gradually the screens became more and more sophisticated and the need to test more and more microorganisms led to the isolation of microorganisms from increasingly exotic sources. In recent times it has become increasingly difficult to find antibiotics which are both novel and useful; the number of new antibiotics discovered per year has remained constant but over the last 20 years the clinical success rate has dropped from 5% to less than 1%. Consequently pharmaceutical companies have redirected their screening efforts towards the identification of pharmacologically active fermentation products. As with the penicillin fermentation, once a microbe is identified which produces a useful metabolite, strain improvement and fermentation development are undertaken.

INDUSTRIAL USES OF PLANT AND ANIMAL CELL CULTURE

Animal cell culture has long been used in the production of viral vaccines but until the 1960s large-scale culture had not even been attempted. It was successful due largely to the vigorous application of the principles of aseptic operation formulated during the development of the penicillin fermentation. With some animal cell lines there is a problem not encountered with microbial cells, that is that the cells do not grow in suspension but require a surface for growth. To satisfy this requirement some novel solutions have been adopted. In some instances the surface area inside the vessel is increased by the addition of microcarrier beads which are maintained in suspension; in other cases, multiple plates are fitted inside the fermenter. Despite these successes the application of mass animal cell culture has been limited largely to the production of vaccines. New avenues have opened up with the realization that certain cell lines derived from human tumours can secrete sufficiently high levels of human proteins to warrant their use for commercial production. Thus the Bowes melanoma line overproduces tissue plasminogen activator which can be used to dissolve blood clots following coronary thrombosis. The Namalwa cell line overproduces interferons and currently is a commercial source of these antiviral and anticancer proteins. Nevertheless, the number of tumour lines identified that produce useful products is limited.

In the last ten years certain tumour cell lines have attracted a great deal of attention and these are the *hybridomas*. As their name implies, hybridomas are hybrid cells. They are created by fusing myeloma (a type of tumour) cells with antibody-producing spleen lymphocytes. Following fusion with the myeloma cell the lymphocyte acquires immortality and can be grown indefinitely in cell culture while continuing to secrete antibody. Since any given lymphocyte only synthesizes a single antibody species, all the antibody molecules made by culture of any particular hybridoma will be identical. Since all the antibodies in the preparation are identical they are said to be *monoclonal*, i.e. they are all derived from a single clone of lymphocytes. Monoclonal antibodies can be purified easily and find application in many different areas from diagnostic kits to cancer therapy and protein purification.

For a long time the ability to cultivate plant cells in the laboratory was considered no more than a curiosity. Although whole plants serve as valuable sources of agricultural chemicals, drugs, flavourings and colourings, very few of these compounds are produced in cell culture. When they are so produced, yields are low and classical mutation and selection techniques have not been particularly successful in improving them. Today plant cell culture is of considerable importance and this is a direct result of the development of methods for the regeneration of plants from individual cells. The benefits of this are twofold. First, regeneration enables hundreds of plants to be produced in a single experiment. During regeneration genetic variants are thrown off at a high frequency and these can be very useful to the plant breeder. Second, virus-free cells can be isolated and used to produce virus-free crops which will give increased yields per acre.

GENETIC ENGINEERING AND THE NEW BIOTECHNOLOGY

After the introduction of the penicillin fermentation there were virtually no significant new developments in industrial microbiology for 30 years. Most companies followed the practice of screening microbes for desirable metabolites or activities and then, however low the levels, implementing strain selection and fermentation development. In the late 1960s considerable excitement was generated by the prospect of using microbial cells (or *biomass*) as a source of protein, the so-called *single-cell protein* or SCP. The rationale for this was that globally there was a shortage of protein and with a rapidly expanding population this situation was going to become worse. However the introduction of SCP has not been a success. Development coincided with a rapid increase in oil prices and the introduction of improved, high-yielding varieties of crops (Fig. 1.5). The developed countries did not need SCP, they had a plentiful supply of protein from conventional sources; the underdeveloped countries, on the other hand, could not afford to buy SCP or even to build and run SCP plants. Thus developments in biotechnology, as in other areas, are subject to political and economic pressures. High quality science does not guarantee commercial success and there is little altruism in the business world.

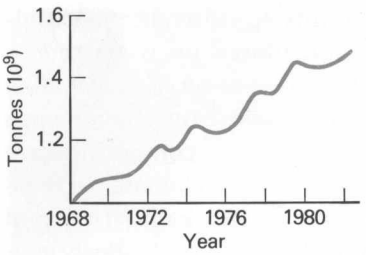


Fig. 1.5 Increase in grain production in the period 1968–1982. Similar increases in yield have been reported for other protein sources.

In the 1980s biotechnology has become a major growth area. This change has come about through a single development: the ability to splice together *in vitro* DNA molecules derived from different sources. This gene splicing ability is referred to as gene manipulation and because one is recombining pre-existing genetic sequences to create a novel combination the term *recombinant DNA technology* is also used. Whereas mutation and selection can be used to increase the level of pre-existing activity in a microbial cell, recombinant DNA technology can be used to confer on cells entirely *new* synthetic capabilities; for example, bacteria can for the first time synthesize human hormones. This technology is not confined to microorganisms: plant and animal cells and even intact plants and animals can also be modified.

Early work on the commercial applications of recombinant DNA technology centred on the production of proteins. There were two reasons for this. Not only did the technology enable many proteins, principally of therapeutic interest, to be produced for the first time but proteins are the immediate products of a gene. As genetic engineers have become skilled there has been a trend towards cloning all the genes associated with a biosynthetic pathway; for example, all the genes encoding the 29 steps in erythromycin biosynthesis have been cloned on a single DNA fragment. One outcome of this may be that ultimately the range of different organisms used in commercial biotechnology may diminish. Where low molecular weight compounds are required the pathways may be introduced to a limited selection of bacteria and fungi, e.g. *Escherichia coli*, *Streptomyces* sp., *Saccharomyces cerevisiae* and *Aspergillus* sp. Large molecular weight compounds may be produced in an even more restricted range of organisms and animal cells may grow in importance because they can effect post-translational modifications of proteins in an identical fashion to the intact animal (see p. 16).

Biotechnology, as practised today, is much more than recombinant DNA technology. It includes hybridoma technology, the use of cells and enzymes immobilized on inert supports and the ability to regenerate plants from isolated cells. However, if these are the performers, gene manipulation is the star of the show. The ability to splice genes has revolutionized the industry and is leading to the development of countless new products and improved methods for well-established processes. The aim of this book is to put into context all the new technologies and therefore no apology is made for the heavy emphasis on recombinant DNA technology. Having said that, it must be admitted that at the time of writing no company has made much money from the use of recombinant microorganisms but that monoclonal antibodies have lived up to their commercial promise.

Further reading

GENERAL

A general introduction to biotechnology can be found in the September 1981 issue of *Scientific American*, and the book by Steve Prentis *Biotechnology: a new industrial revolution* (1984, Orbis Publishing, London). The former concentrates on industrial microbiology, whereas the latter covers a similar range of topics to this book.

An up to date review of biotechnology but without the gene cloning emphasis is provided by I.J. Higgins, D.J. Best and J. Jones in their book *Biotechnology, Principles and Applications* (1985, Blackwell Scientific Publications, Oxford).

More advanced coverage is provided by the three special issues of *Science*, the official journal of the American Association for the Advancement of Science: volume 196 number 4286 concentrated on the methodology of gene manipulation, volume 209 number 4463 on the research applications of the techniques and volume 219 number 4585 on commercial biotechnology. There are a number of journals devoted solely to biotechnology. The best of these are *Bio/technology* (Macmillan, London) and *Trends in Biotechnology* (Elsevier, Amsterdam).

SPECIFIC

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Part II
Recombinant DNA Technology