Practical Biotransformations

A Beginner's Guide

Gideon Grogan

York Structural Biology Laboratory, Department of Chemistry, University of York, UK

WILEY

A John Wiley and Sons, Ltd., Publication

Practical Biotransformations

Postgraduate Chemistry Series

A series designed to provide a broad understanding of selected growth areas of chemistry at postgraduate student and research level. Volumes concentrate on material in advance of a normal undergraduate text, although the relevant background to a subject is included. Key discoveries and trends in current research are highlighted, and volumes are extensively referenced and cross-referenced. Detailed and effective indexes are an important feature of the series. In some universities, the series will also serve as a valuable reference for final year honours students.

Titles in the Series

Protecting Groups in Organic Synthesis
James R. Hanson

Organic Synthesis with Carbohydrates
Geert-Jan Boons and Karl J. Hale

Organic Synthesis using Transition Metals
Roderick Bates

Stoichiometric Asymmetric Synthesis
Mark Rizzacasa and Michael Perkins

Catalysis in Asymmetric Synthesis (Second Edition)
Vittorio Caprio and Jonathan M. J. Williams

Reaction Mechanisms in Organic Synthesis
Rakesh K. Parashar

Photochemistry of Organic Compounds: From Concepts to Practice
Petr Klán and Jakob Wirz

Practical Biotransformations

A Beginner's Guide

Gideon Grogan

York Structural Biology Laboratory, Department of Chemistry, University of York, UK

WILEY

A John Wiley and Sons, Ltd., Publication

This edition first published 2009

Registered office
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, United Kingdom

Library of Congress Cataloging-in-Publication Data

Grogan, Gideon.
Practical biotransformations : a beginner's guide / Gideon Grogan.
p. cm. – (Postgraduate chemistry series)
Includes bibliographical references and index.
ISBN 978-1-4051-9367-2 (cloth) – ISBN 978-1-4051-7125-0 (pbk. : alk. paper)
1. Biosynthesis. 2. Enzymes–Biotechnology. 3. Microbial biotechnology.
4. Organic compounds–Synthesis. I. Title.
QD415.5.G76 2009
660.6–dc22

2009004187

A catalogue record for this book is available from the British Library.

ISBN Cloth: 9781405193672 (HBK)
ISBN Paper: 9781405171250 (PBK)

Typeset in 10/12 Minon by Laserwords Private Limited, Chennai, India
Printed and bound in Great Britain by TJ International Ltd, Padstow, Cornwall.

Contents

Foreword

'Biotransformations' is a fundamentally practical discipline in which the objective is to use enzymes and whole cells as catalysts for conversions of organic substrates. This subject is growing very rapidly, finding ever increasing applications ranging from pharmaceuticals and fine chemicals to food additives, cosmetic ingredients and more recently biofuels. Against this backdrop of an expanding demand for this technique, it is clearly important that we continue to train the next generation of 'biotransformers', hence the timely nature of this book by Gideon Grogan. Dr. Grogan has set out to provide a step-by-step guide to acquiring the basic techniques that are required to carry out practical biotransformations in the laboratory. He has put himself in the position of a scientist who possesses good basic laboratory skills but needs to be led by the hand in terms of how to set up a laboratory and how to acquire basic techniques (e.g. culturing microorganisms, using isolated enzymes, gene cloning) necessary for practical biotransformations. Crucially, in addition to the excellent text, the book is illustrated with many helpful pictures and diagrams. Books of this nature are not freely available and hence the availability of this manual will serve to provide an unmet need, particularly for organic chemists wishing to avail themselves of this emerging technology.

Professor Nicholas J. Turner,
Director
Centre of Excellence for Biocatalysis,
Biotransformations and Biocatalytic Manufacture
University of Manchester, UK

Preface

The development of new asymmetric catalytic methods is of fundamental importance to industrial synthetic chemistry. In addition to the importance of generating optically pure synthetic intermediates, the recent drive to adopt greener methods of synthesis has stimulated a growing interest in biologically catalysed reactions as a means of selective and environmentally benign synthesis. However, even when a biocatalytic method may be the best solution for a synthetic problem, there are obstacles that exist which bar it from being considered as a real option. Many of these are due to a lack of understanding of the basic techniques associated with biocatalysis, or a want of facilities or trained personnel.

This book is intended as a beginner's guide to microbes and enzymes and how to use them to do synthetic organic reactions in the laboratory. Rather than a list of reactions, or an overview of the literature, it is intended as a laboratory manual that can be readily referred to in an everyday experimental environment. It assumes very little knowledge of biochemical reactions or microbiology, but seeks, with appropriate advice on aspects of microbiological practice and associated safety, to help the uninitiated to begin to understand how biocatalysts work and how they can be used safely and efficiently for the generation of valuable intermediates and metabolites. It would therefore be suitable for undergraduate or postgraduate students of chemistry with little or no experience of biochemistry, microbiology or molecular biology. As a book intended primarily for those with a knowledge of synthetic organic chemistry, knowledge of synthetic reactions and techniques, analytical methods, such as TLC, NMR, UV spectroscopy, mass spectrometry and standard laboratory techniques, such as solvent extraction and column chromatography, has been assumed.

The book is not an exhaustive treatment of biochemistry, molecular biology or their techniques. Only those techniques, which are directly relevant to laboratory-scale biotransformations, or those that will help those unfamiliar with biocatalysis to engage with the relevant literature, which may include subjects such as microbiology or protein purification, are included. Different individuals will already have varying levels of exposure to the methods described herein, so it should be possible to dip in and out of the book depending on the level of previous experience.

Where more specialised techniques are required, e.g. more advanced molecular biology, or process improvement techniques such as immobilisation, the reader will be referred to more specialist texts in these areas. However, it is hoped

that this book will serve as a useful primer to new workers in laboratories that already undertake biotransformations but, more importantly, to encourage the uptake of biotransformations in synthetic organic laboratories that have until now not appreciated the benefits or inherent experimental simplicity of biocatalytic solutions.

Acknowledgements

I would like to thank Dr Mark Fogg and Dr David Nelson (University of York), Dr Robert Speight (Ingenza Ltd), Dr Andrew Wells (Astra Zeneca), Dr Richard Lloyd (Dr Reddy's) and Professor Nicholas Turner (Centre of Excellence for Biocatalysis, Biotransformations and Biocatalytic Manufacture (CoEBio3), University of Manchester) for their critical reading of the manuscript and their comments. I would also like to thank Phil Roberts for photography and assistance with graphics and Mark Thompson, Florian Fisch, Claudia Szolkowy, Sam Johnston and Laila Roper for additional photographs and protocols.

Chapter 1

Biotransformations, Microbes and Enzymes

1.1 Introduction

A biotransformation, as understood by the growing community of chemists and bioscientists who practise in the area, is the conversion of one chemical entity to another by the action of a biological system which, in our case will be primarily micro-organisms or enzymes derived from them. Micro-organisms and their extracts have been exploited for thousands of years for chemical reactions, largely for fermentation reactions leading to alcoholic beverages, vinegar or the production of foodstuffs such as yoghurt. The history of biotransformations shares much with the history of the study of the chemistry of the brewing process in the nineteenth century, on which an excellent essay is to be found in 'Introduction to Biocatalysis using Enzymes and Micro-organisms' by Professor Stanley M. Roberts and colleagues [1]. In the twentieth century, early interest in biotechnology was stimulated both by the use of organisms to produce bulk chemicals, such as sugars and amino acids, and also in the exploitation of microbes for pharmaceutical production for the chemical industry, after the discovery of penicillin. In terms of biotransformations science, such developments were crucial, as they encouraged research into the optimisation of large-scale fermentations of micro-organisms and the relevant enzyme biochemistry, and also encouraged the development of microbial culture collections that still serve as a valuable reservoir of biocatalysts today. However, it is in the last twenty or thirty years or so that microbes and enzymes have been recognised as very real solutions to many of the challenges facing synthetic chemistry in terms of their capabilities as catalysts of single-step reactions that are analogous to standard organic reactions. The first wave of applications in organic synthesis was neatly summarised in reviews by Professor J. Bryan Jones, one of the pioneers in preparative biocatalysis, in 1976 and 1986 [2], the latter having been cited over 740 times at the time of writing

this book. This review summarises over 350 reports of both applied studies of enzymes and related biochemistry that incorporated early developments including microbial hydroxylations, the use of lipase enzymes for kinetic resolutions and alcohol dehydrogenases for the asymmetric reduction of ketones. The early work of Jones, Professor George Whitesides [3] and others inspired a whole new generation of organic chemists to investigate the possibility of applying enzymes in organic synthesis and important developments were made in several laboratories around the world. The involvement and expertise of microbiologists, molecular biologists and enzymologists would prove crucial and the development of biotransformations science in the last two decades represents one of the best examples of synergistic relationships across disciplines in the biological and chemical sciences. Biocatalytic reactions are now reported in the literature and used in industry on a routine basis. Although it is not perhaps quite correct to say that there is an enzymatic equivalent for every type of reaction applied in abiotic chemical synthesis, the breadth of chemical catalytic diversity exhibited by enzymes is astonishing, particularly given that they are all made of the same, fairly small repertoire of twenty amino acids. Enzymes have been described that catalyse carbon and heteroatom oxidations, reductions of carbonyls and nitro groups, carbon-carbon bond formation, group transfer leading to the production of, for example, oligosaccharides and the hydrolysis of a number of functionalities including esters, amides, epoxides and even carbon-carbon bonds. All of these activities and more are now employed routinely in industrial synthetic chemistry by some of the world's largest chemical companies.

As both the level of interest and the amount of research in biotransformations science has grown, a number of textbooks exists that summarise the relevant literature and give examples of the application of enzymes in synthesis. In addition to the early volume by Professors Whitesides and Wong [3], notable amongst these are 'Biotransformations in Organic Chemistry' [4] by Professor Kurt Faber of the University of Graz, which is a convenient single volume that constitutes an excellent summary of the current state of the area, and 'Enzyme Catalysts in Organic Synthesis' [5], a larger volume edited by Professor Karlheinz Drauz of Degussa and Professor Herbert Waldmann of the University of Dortmund, with contributions from various authors on specific aspects of biocatalysis, with detailed sections on different types of enzymatic reaction and their application. In addition there are specific texts on certain enzyme classes, such as 'Hydrolases in Organic Synthesis' by Professors Bornscheuer and Kaslauskas [6], and in techniques such as high-throughput methodology in 'Enzyme Assays. High Throughput Screening, Genetic Selection and Fingerprinting', edited by Professor Jean-Louis Reymond of the University of Berne [7]. The interest in biotransformations has also fuelled the formation of several research Centres of Excellence throughout the world, notably in Iowa, USA, at the University of Graz in Austria, the University of Delft in the Netherlands and recently at the University of Manchester, UK. Many of

these run training courses in biocatalysis methodology for those in the synthetic organic chemistry industry. Several companies have also been established that offer biocatalytic solutions for industrial chemistry and these grow in number annually.

This recent and continuing appreciation of biocatalysis has probably arisen from three major considerations. First, the emerging recognition that the synthesis of single enantiomer forms of chiral drugs was going to be increasingly important in industrial chemistry in the late twentieth century and beyond, and that enzymes would be able to accomplish an important role as chiral catalysts. Second, that industry was to come under increasing pressure to develop environmentally benign methods of synthesis – 'green chemistry' criteria – that would be fulfilled by many of the natural characteristics of enzymes and microbes. Third, a revolution has been undergone in gene and protein engineering and techniques of molecular biology, ensuring that the manipulation of enzymes has become a standard technique in laboratories worldwide.

1.1.1 Biocatalysts catalyse selective reactions

The properties of enzymes as chiral catalysts has been appreciated for decades, and it is with the increase in demand for enantiopure drugs that there has been the growth of interest in enzymes in fine chemical synthesis. Enzymes are themselves chiral, being comprised of L-amino acids that go to make up their polypeptide chain. Within the active site of an enzyme, the three-dimensional, chiral environment ensures that the enantiomeric constituents of racemates are discriminated, and enzymes are therefore capable of catalysing kinetic resolutions, and also the desymmetrisation or chiral functionalisation of *meso*- or prochiral compounds. In addition, owing to their exquisite specificity, enzymes are also capable of catalysing *regioselective* reactions, functionalising only one of many chemically equivalent sites in a substance. An excellent example of this is the hydroxylation of progesterone by the fungus *Rhizopus arrhizus*, which occurs only at the 11 position of the steroid nucleus, and only in the α-configuration (Figure 1.1).

Biocatalysts are also capable of catalysing *chemo*- selective reactions, discriminating between chemically similar yet structurally different functions that may be

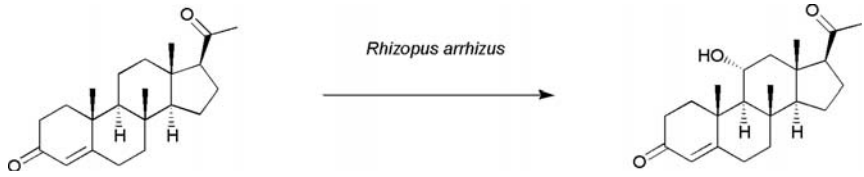

Figure 1.1 Regio- and stereoselective hydroxylation of progesterone by *Rhizopus arrhizus*

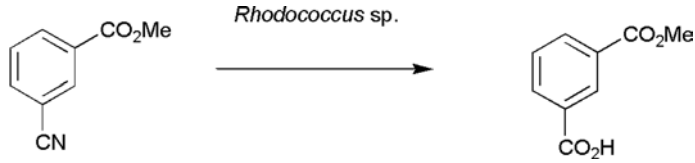

Figure 1.2 Chemoselective hydrolysis of an aromatic nitrile by a *Rhodococcus* species

labile under the same chemical conditions, such as in the preferential hydrolysis of a nitrile over a carboxy-ester, two acid-labile groups, by a strain of bacterium known as *Rhodococcus* (Figure 1.2).

1.1.2 Biocatalysts catalyse Green Chemistry

As both industrial and academic chemical laboratories come under increasing pressure to adopt environmentally benign methods of chemical synthesis, biocatalytic methods of synthesis offer several advantages. The twelve principles of Green Chemistry, proposed by Anastas and Warner in 1998 [8], are listed in Table 1.1.

Table 1.1 also highlights those principles to which enzymes and microbes conform either in part or totally. The most notable of these include the catalytic nature of biological catalysts, their derivation from renewable resources and inherent biodegradability and their ability to operate at neutral pH and ambient temperature and pressure. Enzymes are also often able to work in aqueous environments, thus removing the use of organic solvents. Their selectivity can also permit the direct functionalisation of molecules at one of many chemically identical sites, thus removing the need for protection/deprotection strategies. Whilst not providing a panacea for green chemical solutions, biocatalysts are certainly worthy of consideration where environmentally clean chemistry is an issue, and indeed, the Presidential award for Green Chemistry (2006) was awarded to a biocatalytic process, developed by Professor Roger Sheldon at the University of Delft in collaboration with Codexis Ltd in the USA, for the production of the side-chain of one of the world's most commercially significant pharmaceuticals, atorvastatin (Lipitor^{TM}) [9].

Another advantage of enzymes in the context of Green Chemistry is that they often provide a route to natural-equivalent materials where these may be required in, for example, the food or cosmetics industries. If a natural, i.e. non-genetically modified organism, is used to transform a natural substance, the product of that reaction may be labelled as 'natural', as having exploited both natural source materials and reagents in its production. Hence, despite the wealth of interest in recombinant biocatalysts, enzyme engineering and the associated advantages, there is still a great deal of interest in biotransformations catalysed

Table 1.1 The twelve principles of Green Chemistry, as proposed by Anastas and Warner [8]

	Green Chemistry principle	**Notes**
1	It is better to prevent waste than to treat or clean up waste after it is formed	
2	Synthetic methods should be designed to maximise the incorporation of all materials used in the process into the final product	
3	Wherever practicable, **synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment**	Biocatalysts are natural products with no inherent toxicity issues
4	Chemical products should be designed to preserve efficacy of function while reducing toxicity	
5	**The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary whenever possible and innocuous when used**	Biocatalysts are often employed in aqueous media.
6	Energy requirements should be recognised for their environmental and economic impacts and should be minimised. **Synthetic methods should be conducted at ambient temperature and pressure**	Most biocatalysts work at both ambient temperature and pressure
7	A **raw material feedstock should be renewable** rather than depleting whenever technically and economically practical	Biocatalysts are being intensively investigated for their application in renewables processing
8	**Unnecessary derivatisation (blocking group, protection/deprotection, temporary modification of physical/chemical processes) should be avoided whenever possible**	Biocatalysts are often both regio- and stereoselective, obviating the need for protection/deprotection steps
9	**Catalytic reagents** (as selective as possible) are superior to stoichiometric reagents	Biocatalysts are, of course, catalytic

(*continued overleaf*)

Table 1.1 (*continued*)

	Green Chemistry principle	Notes
10	Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products	Biocatalysts are, by their nature, biodegradable after use
11	Analytical methodologies need to be further developed to allow for real-time in-process monitoring and control prior to the formation of hazardous substances	
12	Substances and the form of a substance used in a chemical process should chosen so as to **minimise the potential for chemical accidents, including releases, explosions, and fires**	Again, the use of biocatalysts reduces the risk of these hazards

by naturally occurring organisms and exploiting the enzymatic systems within them.

Associated with the context of clean and sustainable chemical technology is the rapidly growing area of biorefining, in which renewable materials from sustainable resources will be transformed using either chemical or biocatalytic methods in biorefineries, to high-value products. The natural abilities of biocatalysts to degrade bulk natural materials, notably lignin and cellulose, will lend themselves well to the production of valuable platform chemicals such as sugars and phenolics that might themselves be transformed into high-value products using further enzymatic elaboration. Such industrial processes are often intensive and are hence dependent on engineered micro-organisms. One excellent example is in the microbial production of the polymer precursor propane-1,3-diol by DuPont in the USA (http://www2.dupont.com/Sorona/en_US/). In this process, corn starch is broken down to glucose, and then converted into the polymer precursor propane-1,3-diol by the action of an engineered biochemical pathway in the bacterium *Escherichia coli* (Figure 1.3). The propane-1,3-diol derived by biotransformation is then used to make the polymer Sorona™. Such biotransformations by engineered biochemical pathways may in future offer routes to many bulk chemicals from feedstocks that offer an alternative to non-renewable petrochemical sources.

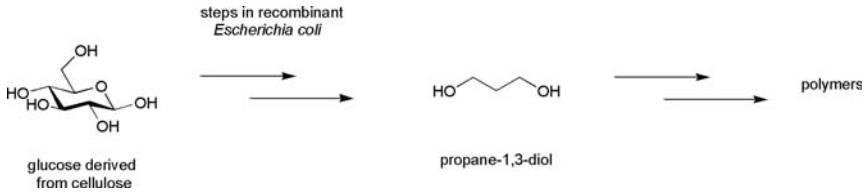

Figure 1.3 Production of propane-1,3-diol from corn-derived cellulose by DuPont

1.1.3 The impact of molecular biology on biotransformations chemistry

In the last twenty years, the ability to acquire, study and exploit the vast wealth of genetic diversity offered by the microbial world has extended to previously unimaginable proportions. The genomes of organisms are sequenced routinely and may be analysed and 'mined' for biocatalysts of interest, as well as the DNA of organisms that have never been isolated using the techniques of 'metagenomics' (see Chapter 2). The speed of genome sequencing is sure to increase over the coming years as the relevant technologies gather pace, offering rapid access to new enzymes and biochemical pathways. Allied to this is the development of bioinformatics, which, with the exponential increase in computer processor power, is offering user-friendly tools for the analysis of genomes and, in some cases, the prediction of the function of enzymes that are encoded within genomes. Laboratory tools for gene cloning and expression are becoming more accessible, affordable and user-friendly and are to be found in an increasing number of laboratories previously working in synthetic chemistry. Last, even the tools for protein engineering, including those that are dependent on structure ('rational engineering'), or random methods of mutagenesis dependent on so-called 'directed evolution' techniques, are becoming increasingly accessible to those not directly associated with the fields of molecular biology and structural biology. Whilst collaboration between groups in different disciplines is now common, there is a much greater appreciation of molecular biology and enzymology by those trained in synthetic chemistry, and the increasing significance of the now-established field of chemical biology should ensure that generations of new chemists are equipped with the knowledge and skills necessary to incorporate biological systems into their work.

Having reviewed the background to the current growth in biotransformations science, we will now examine biotransformations themselves, and the biocatalysts that are responsible for effecting the reactions.

1.2 Biotransformations

A working definition of a biotransformation for the purposes of this book is 'the transformation of a substrate to a recoverable end-product by either microbial or enzymatic means'. The term 'recoverable' makes an important distinction that separates the preparative biotransformations of interest from those 'biotransformations' that are described in the literature that refers largely to drug metabolism studies *in vivo*. The biotransformations that will be considered will be simple analogues of single-step synthetic organic reactions that convert substance A to product B through the action of a single primary catalyst, either enzyme or microbe, and will not therefore, consider multi-step, single-pot enzyme reactions (apart from where cofactor regeneration is an issue) or any aspects of 'pathway engineering'.

Before beginning a more detailed discussion on the nature of biotransformations and the types of reaction that can be catalysed, it would be useful to address briefly the characteristics of biocatalysts themselves, in order to familiarise the worker new to the area with some general considerations of micro-organisms and the enzymes that are derived from them. The range of natural biocatalysts is not restricted to the microbial world of course – there has been much work on catalytic antibodies and ribonucleic acids, for example – but our discussion will focus on micro-organisms and their enzymes, as these will be used most routinely in research and applications.

1.3 Microorganism

Whilst an extended discussion of microbial taxonomy or biochemistry is outside the scope of this book, we have attempted to provide below some information that will be useful in approaching the use of microbes for applications in biotransformations. For more detailed information, useful general introductions to microbiology and microbial biochemistry are provided by both Hans G. Schlegel's book 'General Microbiology' [10] and 'Brock Biology of Micro-organisms' [11].

Micro-organisms (eubacteria, archaea, fungi, algae, viruses) are ubiquitous, being able to exploit a huge range of substances for growth in order to survive or thrive in environments that are diverse in terms of growth substrates, temperature, pressure and salinity. It is this naturally evolved diversity that renders their biochemistry so fascinating and amenable to the catalysis of useful reactions and their application in chemistry. The micro-organisms typically used for biotransformations are *eubacteria*, unicellular micro-organisms such as *E. coli, Pseudomonas* or *Lactobacillus* that are encountered in everyday life, *yeasts* such as *Saccharomyces cerivisiae* (common baker's yeast) and microscopic

fungi such as *Aspergillus* and *Mucor*. There are fundamental differences in the biology and biochemistry of prokaryotic micro-organisms or prokaryotes (the eubacteria and archaea) and eukaryotic micro-organisms or eukaryotes (yeasts and fungi) that have a profound impact, particularly on the genetic manipulation of these organisms or the extraction and application of their genetic material.

1.3.1 Prokaryotes

The prokaryotes are single celled organisms typified by well known species such as *E. coli*. They are distinguished from the other major group, eukaryotes, in that they possess no membrane bound vesicles, known as *organelles*, within the cell but carry out their biochemical functions either at the cell membrane that surrounds the cell or in the soup of proteins, carbohydrates and other biochemicals that constitutes the *cytoplasm*. Their genetic material usually consists of a circular chromosome, sometimes also with a number of smaller circular pieces of DNA known as *plasmids*. For our purposes, prokaryotic organisms can be divided into two groups: the *archaea* and the *eubacteria*.

1.3.1.1 Archaea

The Archaebacteria or Archaea are a kingdom of life in their own right and consist of evolutionarily primitive bacteria that are thought to be the common ancestor of both prokaryotes and eukaryotes. They are distinguished from the other major kingdom of bacteria, the eubacteria, by a number of physiological features including an absence of peptidoglycan in their cell walls and their use of ether rather than ester linkages in their lipid chemistry. Archaea are often found to thrive in extreme environments, such as at high temperatures (thermophiles), cold temperatures (psychrophiles) or pressures (barophiles). Whilst their rather exotic growth requirements dictate that it is unlikely that whole cell preparations of Archaea would be used as biocatalysts, many of the enzymes that one finds in Archaea are, for example, highly thermostable, and are hence extremely interesting from the biotechnology perspective. It is therefore common to find applications of biocatalysts from thermophiles in the biocatalysis literature, from organisms such as *Aquifex, Thermotoga, Thermoanaerobium, Pyrococcus* and *Sulfolobus*, but these enzymes have almost always been prepared using recombinant techniques of the type described in Chapter 7.

1.3.1.2 Eubacteria

Prokaryotic organisms that have been exploited for biotransformations are drawn almost exclusively from this kingdom of life. The eubacteria can be divided into

two sub-groups based on their response to a type of staining used for microscopic visualisation called Gram staining. Strains of eubacteria are referred to as either Gram positive or negative, depending on whether the Gram stain can be removed by washing with ethanol. The difference in response is due to a difference in the structures of cell walls in these two groups – Gram-positive bacteria show a purple coloration and Gram-negative bacteria are red. Of the seventeen or so major classes of eubacteria or *phyla*, most biocatalysts have been drawn from only a few of these. Notable amongst these are the Gram-negative Pseudomonad group, which includes organisms such as *Pseudomonas, Burkholderia, Zymomonas* and *Agrobacterium* and the acetic acid bacteria, such as *Acetobacter* and *Gluconobacter*. The Pseudomonads (Figure 1.4) are distinguished by their ability to grow on a large range of carbon sources and possess many enzymes that are capable of processing both natural and xenobiotic compounds of different structural types. Such biodegradative capacity exploiting a wide range of enzymes is of course extremely useful when it comes to preparative biotransformations.

Acetic acid bacteria are capable of oxidising primary alcohols to carboxylic acids and have hence been used for the preparative oxidation reactions in the mode of potassium permanganate or Jones reagent. Gram-positive bacteria are often separated into those which are 'high-GC' or 'low-GC' – a reference to the proportion of the nucleobase cytosine or guanosine that features in their DNA sequence. Of the Gram-positive bacteria, the enteric bacterium *E. coli* is one of the most used bacteria in laboratories worldwide, providing both an early model for bacterial

Figure 1.4 Bacteria of the genus *Pseudomonas*, growing on an agar plate

genetics and routinely acting as a host strain for simple cloning experiments. It was also the first to have its entire genome sequenced [12]. *E. coli* is most often used as the host for enzymes from other organisms, expressing their genes in heterologous fashion using techniques described in Chapter 7. The low-GC spore forming Gram-positives such as *Bacillus*, and high-GC organisms such as *Corynebacterium* and *Rhodococcus* have biodegradative abilities that rival the Pseudomonads in enzymatic scope and substrate range. The high-GC Gram-positive bacteria actinomycete group that includes *Streptomyces* and *Rhodococcus* have also provided a host of valuable biocatalysts. The genus *Streptomyces* (Figure 1.5), which is most well-known for its ability to form a large amount of structurally diverse antibiotics, presents an impressive reservoir of enzymes that are used biosynthetically to make these valuable secondary metabolites. Such enzymes are often used to elaborate core structures such as macrocylic polyketides that have been assembled by large, processive enzyme complexes. Some of these interesting enzyme activities have been recruited for single-step biotransformations such as for preparative hydroxylation reactions.

In Chapter 3 we provide more detail about the growth requirements and characteristics of the bacteria used in biotransformations and give examples of preparative biotransformations catalysed by them.

1.3.2 Eukaryotes

The other major kingdom of life, the eukaryotes, provides other major examples of biocatalysts – these predominantly being drawn from the filamentous fungi

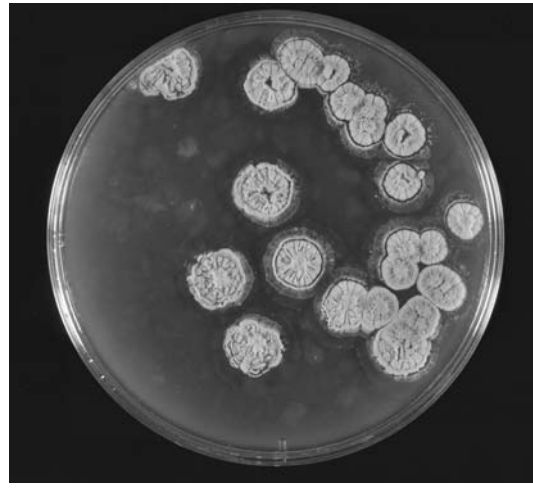

Figure 1.5 Bacteria of the genus *Streptomyces*, growing on an agar plate

and yeasts. The kingdom of eukaryotes covers many organisms from yeast to plants and mammals, but the 'higher' eukaryotes have of course seen a more limited application in biocatalysis. There are examples of the use of algae, plant cell cultures and indeed, cultured mammalian cells, and these have been shown to possess useful enzymatic activities. Our discussion will be mostly limited to the yeasts and fungi.

Eukaryotes, in contrast to prokaryotes, possess subcellular organelles, some of which are responsible for such biochemical processes as respiration (mitochondria) or, where relevant, photosynthesis (chloroplasts) and their genetic material is contained within the nucleus of the cell, bounded by a nuclear membrane. They can be single celled (yeasts) or multicellular (fungi).

1.3.2.1 Yeasts

Yeasts are single-celled eukaryotic organisms and, as one of the simplest forms of eukaryote, have been used as the model for many eukaryotic biochemical processes and genetics, including the first genome sequence of a eukaryotic organism, namely, that of *Saccharomyces cerivisiae* (Figure 1.6) [13].

They have also been hugely significant in biotechnology, initially as the active ingredient in both breadmaking and brewing (*S. cerivisiae; Schizosaccharomyces pombe*). Yeasts are easy to grow in the laboratory and, indeed are available not only from chemical suppliers but are also available in freeze-dried form from high-street

Figure 1.6 Freeze-dried *Saccharomyces cerivisiae*, a yeast often used for biotransformation reactions

supermarkets, so the applications of, for example *S. cerivisiae* or baker's yeast have been widespread, notably for their capacity to catalyse enantioselective reductions of carbonyl groups using alcohol dehydrogenase activities. Other yeasts, notably non-pathogenic strains of *Candida*, have also been used for reduction reactions, and have also provided enzymes such as formate dehydrogenase, one of the most important enzymes in cofactor recycling techniques.

1.3.2.2 Filamentous fungi

Filamentous fungi, sometimes called moulds, are multicellular organisms that grow often on dead or decaying matter, notably wood, which they are well-equipped to degrade using a powerful arsenal of ligninolytic and cellulolytic enzymes. On agar plates (Figure 1.7), the filamentous fungi usually appear as mats of *mycelia*, which are in turn made up of intertwining filaments called *hyphae*, which are extended filaments made up of many cells.

Some hyphae project upwards from the mat, and bear sporulation structures – the spores that are produced by such fungi are often visible as dark spots dusting the top of the fungal mat. Their capacity for biodegradation makes them, like many species of bacteria, extremely useful in the biotransformation of xenobiotic substrates, and they have been particularly highly prized as catalysts of *hydroxylation* reactions. Fungi such as species of *Penicillium* were shown in early studies in the 1950s at Eli Lilly and Upjohn to hydroxylate the steroid nucleus both

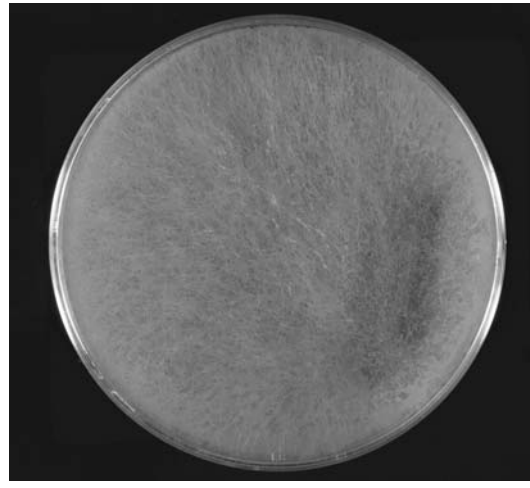

Figure 1.7 A filamentous fungus (*Mucor fragilis*) growing on an agar plate, showing characteristic mat made up of the fungal mycelium

regio- and *stereo*-specifically, and a wide range of substrates for hydroxylation by fungi have been described since. Other filamentous fungi commonly encountered in the biotransformations literature are *Aspergilllus, Rhizopus, Fusarium, Mucor* and *Beauveria*. Many of these have an established history in biotechnology as antibiotic producers, foodstuffs and as producers of commodity chemicals such as citric acid. Fungi, as eukaryotes, have more complex genetics than simple prokaryotes such as bacteria, but progress is being made on the genomics of fungi, and the genomes of *Neurospora crassa*, and a number of species of *Aspergillus* have been sequenced [14]. Macroscopic fungi such as ordinary mushrooms can also be a source of enzymes, such as tyrosinase, an oxidase which converts catechol to benzoquinone, but such organisms are of course less convenient to culture using standard laboratory techniques.

1.3.3 Plants and animals as sources of enzymes for biotransformations

There have been a number of reports of the use of plant cell cultures as biocatalysts [15], notably, tobacco and geranium, and also of useful enzymes from plants such as spinach and potato. It is certainly true that plants possess a fantastic biosynthetic capability that can be recruited for applied biocatalytic reactions, and indeed the sequenced genomes of organisms such as *Arabidopsis thaliana* have revealed many useful enzymes, such as glycosyltransferases, that can be exploited using biocatalysis [16]. However, plant cells in culture are comparatively difficult to grow reliably, and the exploitation of plant enzymes such as the glycosyltransferases of *Arabidopsis* have been accessed using recombinant biotechnology in easy-to-handle laboratory hosts such as bacteria and fungi.

Whilst animals were once the source of useful biocatalysts, notably the esterase from porcine liver (PLE) and alcohol dehydrogenase from equine liver (HLADH), the advent of recombinant biotechnology and the emergence of a huge and ever-increasing diversity of microbial biocatalysts of comparable or superior activity has reduced the use of these enzymes; indeed, the use of enzymes from animal sources is often precluded, particularly in the preparation of substances for human consumption and for which the use of animal products is now prohibited.

1.4 Organism Nomenclature

The nomenclature of micro-organisms is important as it enables us to distinguish between species of related bacteria/fungi and strains that may be available from different sources. As molecular biological techniques evolve, so do the rules of taxonomy and nomenclature and it is not unusual to find that an organism has been renamed. The name of a micro-organism is usually constructed from a prefix

that defines its *genus* and a suffix that denotes the *species*. This name is italicised. There is also usually a 'strain descriptor' which should be specific to one strain, or one that is genetically and biochemically distinct. This is often in the form of a mixed number-letter code at the end of the organism's name. In the context of biotransformations, it is the strain descriptor that is also vitally important, as it may only be one certain strain of, for example, *Pseudomonas putida* that possesses the activity required.

Hence, using a bacterial example, the genus *Pseudomonas* includes species such as *Pseudomonas putida, Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. The strain of the organism is usually specified with a suffix that often denotes either the accession number of that strain to a culture collection (see below) such as *Pseudomonas putida* ATCC (American Type Culture Collection) 17453 or, the code given to a strain by the researchers who discovered it, such as *P. putida* M10. Microscopic fungi and yeasts bear equivalent names, *Aspergillus niger, Mucor fragilis, Beauveria bassiana, S. cerivisiae* and so on; again qualified in each case by a strain descriptor such as *B. bassiana* ATCC 7159.

In addition, genetically modified organisms, particularly strains of *E. coli* that are routinely used for recombinant gene expression and thence as biocatalysts, become new strains once the foreign gene, located on a circular piece of DNA called a plasmid, has been taken up by the bacterium. Hence, the commonly used and commercially available expression strain of *E. coli* BL21 (DE)3, which has been transformed with (or taken up) a plasmid pXYZ, may be referred to as *E. coli* BL21 (DE3) pXYZ. An introduction to simple recombinant DNA techniques for creating recombinant or 'designer' biocatalysts is provided in Chapter 7.

Ultimately, it is important to stress that the enzyme complements of two different strains of the same species for example, *P. putida*, could be very different. The biocatalytic properties of an organism may well be restricted to the specific *strain* of an organism described by the literature and that strain must be obtained in an effort to repeat published results.

As we shall see, both whole cells of the organisms described above, and the enzymes that exist within those cells, may be used as biocatalysts. We will now consider the nature of enzymes and the possible useful chemical reactions that may be exploited in preparative applications.

1.5 Enzymes

Enzymes are the catalysts of the majority of biochemical reactions in living cells. They are proteins and as such are polymers made up of combinations of twenty amino acids, usually up to hundreds of amino acids in length, and typically have molecular weights in the range of 10 000–100 000 Da, although the polymeric chains can associate to form larger functional complexes.

The twenty amino acids (Appendix 1) each possess a distinct side-chain, conferring chemical and electronic properties which, when combined within the active site of an enzyme, perhaps with a small coenzyme or metal ion can confer the ability to catalyse a wide range of chemical catalytic processes including oxidations, reductions, hydrolyses and carbon-carbon bond formation. It is common to find these amino acids referred to by either a three-letter code (e.g. Tyr for tyrosine) or a single letter code (e.g. Y for tyrosine) when reading about enzymes in biocatalysis publications. Whilst each amino acid is itself relatively simple in structural and chemical terms, their combination to form proteins gives rise to several levels of *hierarchical structure* which are summarised briefly below:

- *Primary structure:* This refers to the sequence of the amino acids in the protein chain, which are connected together by amide (peptide) bonds formed in a condensation reaction on the ribosome – the cellular organelle of protein synthesis. It is common to see the primary structure of enzymes displayed as sequences of their amino acids in a single letter code (Figure 1.8).
- *Secondary structure:* This refers to local folding of the polypeptide chain, into discrete, three-dimensional structures, such as *alpha-helices* and *beta-strands*, the latter of which associate to form *beta-pleated sheets*. These structures are themselves often connected by loops and turns, which may themselves be ordered or disordered.
- *Tertiary structure:* The collection of helices, sheets, and other turns and loops is folded into a globular structure in enzymes that is termed the tertiary structure (Figure 1.9). This structure is stabilised by a number of usually weak binding interactions between amino acids and their side-chains, or the peptide backbone, including van der Waals contacts, extensive hydrogen bonding and electrostatic interactions ('salt bridges') between positively and negatively charged residues (such as lysine and aspartate, respectively). The structure is also stabilised by a number of interactions with water molecules.
- *Quaternary structure:* Should more than one polypeptide chain associate in order for an enzyme to function, this structure of associated proteins is known as the quaternary structure (Figure 1.10). For example, the quaternary structure of the lyase enzyme shown in Figure 1.10 is made up of three identical monomeric subunits.

```
MKQLATPFQEYSQKYENIRLERDGGVLLVTVHTEGKSLVWTSTAHDELAY
CFHDIACDRENKVVILTGTGPSFCNEIDFTSFNLGTPHDWDEIIFEGQRL
LNNLSIEVPVIAAVNGPVTNHPEIPVMSDIVLAAESATFQDGPHFPSGIV
PGDGAHVVWPHVLGSNRGRYFLLTGQELDARTALDYGAVNEVLSEQELLP
RAWELARGIAEKPLLARRYARKVLTRQLRRVMEADLSLGLAHEALAAIDL
GMESEQ
```

Figure 1.8 The primary structure of an enzyme, or its sequence of amino acids