MICROBIAL ENZYMES AND BIOTECHNOLOGY

2ND EDITION

Edited by

WILLIAM M. FOGARTY & CATHERINE T. KELLY

Department of Industrial Microbiology,
University College, Dublin 4, Republic of Ireland

ELSEVIER APPLIED SCIENCE
LONDON AND NEW YORK

ELSEVIER SCIENCE PUBLISHERS LTD
Crown House, Linton Road, Barking, Essex IG11 8JU, England

Sole distributor in the USA and Canada
ELSEVIER SCIENCE PUBLISHING CO., INC
655 Avenue of the Americas, New York, NY 10010 USA

First edition 1983

WITH 76 TABLES AND 80 ILLUSTRATIONS

British Library Cataloguing in Publication Data

Microbial enzymes and biotechnology.—2nd ed.
1. Industrial microbiology. Use of enzymes
I. Fogarty, William M. II. Kelly, Catherine T.
660.62

ISBN 1-85166-486-6

Library of Congress Cataloging-in-Publication Data

Microbial enzymes and biotechnology/edited by William M. Fogarty & Catherine T. Kelly.—2nd ed.
p. cm.
ISBN 1-85166-486-6
1. Microbial enzymes—Biotechnology. 2. Microbial enzymes—
—Industrial applications. I. Fogarty, William M. II. Kelly,
Catherine T.
TP248.65.E59M53 1990
660'.634—dc20 90-3015
CIP

Photoset and printed in Northern Ireland by The Universities Press (Belfast) Ltd.

PREFACE

Biotechnology is now one of the major growth areas in science and engineering and within this broad discipline enzyme technology is one of the areas earmarked for special and significant developments.

This publication is the second edition of *Microbial Enzymes and Biotechnology* which was originally published in 1983. In this edition the editors have attempted to bring together accounts (by the relevant experts) of the current status of the major areas of enzyme technology and specifically those areas of actual and/or potential commercial importance. Although the use of microbial enzymes may not have expanded at quite the rate expected a decade ago, there is nevertheless intense activity and considerable interest in the whole area of enzyme technology.

Microbial enzymes have been used in industry for many centuries although it is only comparatively recently that detailed knowledge relating to their nature, properties and function has become more evident. Developments in the 1960s gave a major thrust to the use of microbial enzymes in industry. The commercial success of alkaline proteases and amyloglucosidases formed a bed-rock for subsequent research and development in the area.

This book is a collection of chapters covering the most important areas in enzyme technology. It is intended primarily as an update of recent developments and should provide an insight into advances in specific areas. One notable feature of the current edition, which should greatly widen its usefulness, is that the number of contributing authors has been increased and broadened in comparison with the first edition. The range of topics covered includes a number of emerging or expanding areas, such as enzymes in organic synthesis (S. M. Roberts), enzymes in antibiotics, steroid and other conversions (J. O'Sullivan), microbial lipases (S. E. Godtfredsen), cellulases (fungal—M. P. Coughlan; bacterial—F. Stutzenberger), glucose transforming enzymes (A. and W. Crueger), alkalophilic enzymes (K. Horikoshi), and microbiosensors and immunosensors (I. Karube, A. Seki and K. Sode).

One omission from this edition is that of microbial hemicellulases/xylanases—an area of considerable interest. Unfortunately, the contributor in question was unable, in the end, to provide a manuscript.

The editors are greatly indebted to the contributors who have given so generously of their time and expertise.

W. M. FOGARTY
C. T. KELLY

LIST OF CONTRIBUTORS

C. O. L. BOYCE
Novo-Nordisk A/S, Novo Allé, DK-2880, Bagsvaerd, Denmark

M. P. COUGHLAN
Department of Biochemistry, University College, Galway, Republic of Ireland

A. CRUEGER
Verfahrensentwicklung Biochemie, Bayer AG, Postfach, 5600 Wuppertal 1, FRG

W. CRUEGER
WV Umweltschutz, Bayer AG, 5090 Leverkusen, Bayerwerk, FRG

A. L. DEMAIN
Fermentation Microbiology Laboratory, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

W. M. FOGARTY
Department of Industrial Microbiology, University College, Belfield, Dublin 4, Republic of Ireland

S. E. GODTFREDSEN
Novo-Nordisk A/S, Novo Allé, DK-2880, Bagsvaerd, Denmark

K. HORIKOSHI
Department of Bio-engineering, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152, Japan
Present address: Department of Applied Microbiology, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

I. KARUBE
Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153, Japan

C. T. KELLY
Department of Industrial Microbiology, University College, Belfield, Dublin 4, Republic of Ireland

J. O'SULLIVAN
The Squibb Institute for Medical Research, Princeton, New Jersey 08543-4000, USA

H. OUTTRUP
Novo-Nordisk A/S, Novo Allé, DK-2880, Bagsvaerd, Denmark

L. W. POWELL
Beecham Pharmaceuticals, Clarendon Road, Worthing, West Sussex BN14 8QH, UK

S. M. ROBERTS
Department of Chemistry, University of Exeter, Stocker Road, Exeter EX4 4QD, UK

A. SEKI
Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153, Japan

K. SODE
Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153, Japan

F. STUTZENBERGER
Department of Microbiology, Clemson University, Clemson, South Carolina 29634-1909, USA

J. R. WHITAKER
Department of Food Science and Technology, University of California, Davis, California 95616, USA

CONTENTS

Preface v

List of Contributors ix

1. Cellulose Degradation by Fungi 1
   MICHAEL P. COUGHLAN

2. Bacterial Cellulases 37
   FRED STUTZENBERGER

3. Recent Advances in Microbial Amylases 71
   WILLIAM M. FOGARTY & CATHERINE T. KELLY

4. Microbial Pectolytic Enzymes 133
   JOHN R. WHITAKER

5. Glucose Transforming Enzymes 177
   ANNELIESE CRUEGER & WULF CRUEGER

6. Microbial Proteinases and Biotechnology 227
   HELLE OUTTRUP & C. O. L. BOYCE

7. Microbial Lipases 255
   SVEN ERIK GODTFREDSEN

8. Enzymes of Alkalophiles 275
   KOKI HORIKOSHI

9. Enzymatic and Microbial Transformation of Antibiotics and Steroids 295
   JOSEPH O'SULLIVAN

10. Regulation and Exploitation of Enzyme Biosynthesis 331
    ARNOLD L. DEMAIN

11. Immobilized Biocatalyst Technology 369
    LAWSON W. POWELL

12. Enzymes in Organic Synthesis 395
S. M. ROBERTS

13. Microbiosensors and Immunosensors 425
ISAO KARUBE, ATSUSHI SEKI & KOJI SODE

Index 453

Chapter 1

CELLULOSE DEGRADATION BY FUNGI

MICHAEL P. COUGHLAN

Department of Biochemistry, University College, Galway, Republic of Ireland

CONTENTS

1. Introduction 1
2. Cellulose—The Substrate 2
3. Cellulose Degrading Fungi 5
4. Fungal Cellulase Systems 7
   4.1. Hydrolytic Components 8
      4.1.1. Substrate Specificities of the Hydrolytic Components—Generalizations 8
      4.1.2. Multiplicity of Hydrolytic Components 9
      4.1.3. Structure/Function Studies 13
      4.1.4. Synergistic Interactions Between Endo- and Exo-acting Enzymes 19
   4.2. Oxidative Components 23
5. Cellulose Degradation—The Overall Mechanism 25
6. Conclusion 28
Acknowledgements 29
References 29

1 INTRODUCTION

Cellulose is the most abundant organic macromolecule on earth. It has been estimated that total biomass (fossil fuels excepted) amounts to about $1{\cdot}8 \times 10^{12}$ tonnes, 1×10^{11} tonnes being replenished each year by photosynthesis (Bassham, 1975; Stephens & Heichel, 1975). Since 40% of this biomass consists of cellulose (Brown, 1983), one may calculate that 7×10^{11} tonnes of this material exist, mainly in higher plants, and that annual productivity is about 4×10^{10} tonnes. The magnitude of these figures can be appreciated by noting that the rate of cellulose synthesis is equivalent to 70 kg per person per day (Lützen *et al.*, 1983) or to 50 000 barrels of oil per second in energy terms (Sienko & Plane, 1976). Indeed, current terrestrial biomass, at 640 billion

tonnes of oil equivalent, is equal to total proven fossil fuel reserves (De Montelambert, 1983). But, unlike the latter it is constantly being renewed. Lignocellulosic wastes or residues of forest, agriculture, industrial or domestic origin, are generated in great quantities especially in the more developed countries. Many reports have suggested that much of the demand for fuels and chemical feedstocks, currently met by oil, could be met by appropriate exploitation of such wastes (Avgerinos & Wang, 1980; Chartier, 1981; Eveleigh, 1982; Brown, 1983; Grohman & Villet, 1983; Hall, 1983; Sheppard & Lipinsky, 1983; Sinskey, 1983; Soltes, 1983; Eveleigh, 1984; Lloyd, 1984).

As yet there is little industrial-scale realization of this potential of cellulose as a source of food, fuels and chemical feedstocks. One is reminded of King Henry's words, 'Such are the rich, That have abundance and enjoy it not' (Shakespeare, 1600). This lack of realization is attributable to the recalcitrance of cellulose itself, exacerbated by its association with other polysaccharides and lignin *in vivo*. Thus, worthwhile saccharification of cellulosic biomass requires pretreatment to render it amenable to conversion and subsequent use of large amounts of enzyme. This involves costs that render the overall process uneconomical (Ladisch *et al.*, 1983; Mandels, 1985; Ladisch & Tsao, 1986). Nevertheless, the study of cellulases continues. These enzymes have other applications, including extraction of juices and flavours from appropriate plants and fruits, improvement of the quality of animal fodder and facilitation of brewing processes (Coughlan & Folan, 1979; Mandels, 1985). Moreover, there is an inherent scientific challenge in the study of the cooperative attack on an insoluble substrate by multicomponent systems of enzymes with subtly-overlapping substrate specificities.

Several excellent books and symposia proceedings are available to readers interested in the development of research on cellulose and cellulolytic enzyme systems. These include those by Gascoigne & Gascoigne (1960), Hajny & Reese (1969), Wilke (1975), Gaden *et al.* (1976), Ghose (1978) and Aubert *et al.* (1988). All of the relevant papers published between 1972 and 1982 are accessible on database (Anon., 1982). Several reviews provide an alternative means of bringing oneself up-to-date (Brown, 1983; Enari, 1983; Gilbert & Tsao, 1983; Coughlan, 1985; Eriksson & Wood, 1985; Ljungdahl & Eriksson, 1985; Marsden & Gray, 1986; Enari & Niku-Paavola, 1987; Eveleigh, 1987; Fan *et al.*, 1987). The emphasis in this chapter will be on fungal endo- and exoglucanases and their rôle in the degradation of cellulose. Bacterial cellulases are reviewed in the following chapter in this volume.

2 CELLULOSE—THE SUBSTRATE

Cellulose is a linear homopolymer of β-1,4-linked glucose residues in the chair configuration. The number of such residues, i.e. the degree of polymerization, is approximately 14 000 in native plant cellulose (Marx-Figini & Schultz, 1966), about 3500 in that produced by bacteria, such as *Acetobacter xylinum*

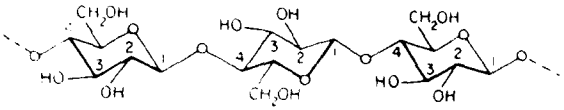

Fig. 1. Part of a cellulose chain showing the β-1,4-linked glucosyl residues rotated 180° with respect to neighbouring residues. Because of this rotation, adjacent linkages will be sterically different to an enzyme approaching the crystalline substrate.

(Marx-Figini, 1982), while that of commercial celluloses ranges from 50 to 5000 (Ljungdahl & Eriksson, 1985). Each glucose residue is rotated 180° with respect to its neighbours along the main axis of the chain (Fig. 1). X-ray diffraction and infrared spectral data indicate that anhydrocellobiose, $C_{12}H_{20}O_{10}$, is the basic recurring unit (Tonnesen & Ellefsen, 1971). The crystal unit cell is 10·5 Å × 8·35 Å × 7·9 Å (Blackwell, 1982; Eveleigh, 1987). The rotation of adjacent residues and the β-1,4 linkages give cellulose chains a flat ribbon-like structure that is stabilized by intrachain hydrogen bonding (see below). Sixty to seventy adjacent chains, having the same polarity, associate with one another through interchain hydrogen bonding and van der Waals interactions to form highly-ordered crystalline microfibrils (Blackwell, 1982; Rees *et al.*, 1982). Bundles of such microfibrils aggregate to form the strong, insoluble fibres characteristic of the primary and secondary cell walls of higher plants (Rees *et al.*, 1982).

Four crystalline forms (polymorphs) of cellulose, designated as I to IV, differing from one another in their X-ray and/or electron diffraction patterns, have been described in the literature (Rees *et al.*, 1982; Atalla, 1983, 1984). The two most common polymorphs are celluloses I and II. The former is akin to that found in nature while cellulose II is obtained by treatment of type I with concentrated (>15%) solutions of sodium hydroxide. On the basis of solid-state ^{13}C-NMR and Raman spectroscopic studies, Atalla (1983, 1984) and Atalla & VanderHart (1984) concluded that all native celluloses, i.e. celluloses of type I, are composites of two distinct crystalline forms, I_α and I_β. Cellulose types I and II differ from one another with respect to the equivalence, or otherwise, of the glycosidic linkages and of the primary hydroxyl group at C-6. In cellulose I, the C-6 hydroxyl on alternate residues participates, with the ring oxygen of the same residue and the hydroxyl at C-3 of the adjacent residue, in bifurcated intramolecular bonding similar to that observed in methyl-β-cellobioside (Fig. 2). This conformation, designated k_1, is the major factor stabilizing the glycosidic linkages in cellulose I. In this form, the C-6 hydroxyls on adjacent anhydroglucose units are non-equivalent as are the glycosidic linkages between the successive anhydroglucoses. I_α and I_β are two different crystalline lattices containing cellulose molecules in the same k_1 conformation. One or other form predominates depending on the source of the cellulose. Bacterial (*Acetobacter xylinum*) and algal (*Valonia macrophysa*) celluloses are comprised of 60–70% I_α whereas cotton, and presumably other plant

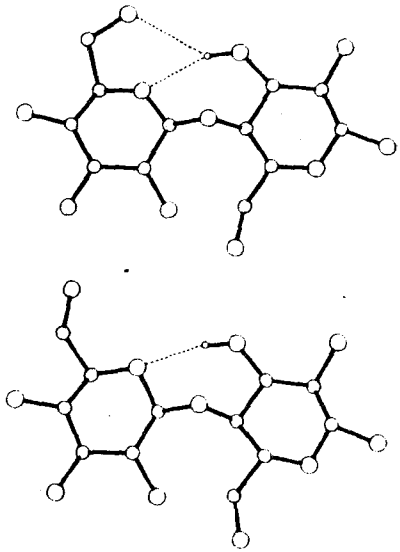

Fig. 2. Schematic representation of the anhydrocellobiose repeat units in the two ordered states, k_I (upper figure) and k_{II} (lower figure), based on the stable conformations of methyl β-cellobioside, with a bifurcated hydrogen bond (Ham & Williams, 1970), and cellobiose with one isolated hydrogen bond (Chu & Jeffrey, 1968), respectively. The computer-drawn figures were generated using molecular modelling (CHEMX, Chemical Design Ltd, Oxford, UK). Adapted from Atalla (1983).

celluloses, are 60–70% I_β. In cellulose II, on the other hand, non-equivalence is centred at the glycosidic linkages whereas the C-6 hydroxyls on adjacent anhydroglucose units are equivalent and exist in the conformation, designated k_{II}, characteristic of cellobiose (Fig. 2).

The resistance of native cellulose to enzymic hydrolysis is due in part to its intrinsic properties and to the fact that its association with lignin and the polysaccharides, hemicellulose and pectin, hinder access by cellulolytic enzymes (Crawford, 1981; Eriksson, 1981; Beldman *et al.*, 1984; Coughlan *et al.*, 1985; Marsden & Gray, 1986; Saddler, 1986; Schwald *et al.*, 1988). The degradability of cellulose by mixed rumen flora was shown to be inversely proportional to the crystallinity of the substrate (Dunlap *et al.*, 1976). Treatments that decreased substrate crystallinity enhanced degradability by *Trichoderma viride* and *Aspergillus niger* cellulases (Sasaki *et al.*, 1979). However, crystallinity indices are not of overriding importance in determining susceptibility of cellulose to enzymic degradation. The degree of swelling, fibrillar structure and pore volume may be more important factors (Marchessault & St-Pierre, 1980; Marchessault *et al.*, 1983; Grethlein, 1985; Puls *et al.*, 1985; Weimer & Weston, 1985; see also Section 4.1.4.).

Total pore volume of water-swollen cellulose is the sum of the volumes of the large number of small cell capillaries (up to 200 Å in diameter) between

adjacent microfibrils and by the fewer but larger gross capillaries (pit apertures ranging from 200 Å to 1000 Å in diameter) along the external surfaces of microfibrillar bundles (Cowling, 1975). Treatments that effect an increase in pore volume, hence providing access by cellulases to the inner microfibrils, and/or treatments that effect a decrease in particle size (i.e. increase in surface area) or degree of polymerization are considered to be the most appropriate ways of increasing the extent of saccharification (Marchessault & St-Pierre, 1980; Marchessault *et al.*, 1983; Puri, 1984; Grethlein, 1985; Puls *et al.*, 1985; Saddler, 1986). These disparate conclusions may not be contradictory, however, since it has been repeatedly stressed that it is difficult, if not impossible, to alter one property of native cellulose without modifying others. The physicochemical properties of the substrate that limit its degradation and the pretreatments that may be used to overcome these constraints have been extensively reviewed by Marsden & Gray (1986). The intrinsic properties of the enzymes may also limit conversion. Cellulolysis, in general, is inhibited by cellobiose, an endproduct of the process (Saddler *et al.*, 1986), and the enzymes may be unproductively adsorbed on lignin (Deshpande & Eriksson, 1984) or on the recalcitrant cellulose (Saddler *et al.*, 1986) remaining even after extensive saccharification. Reutilization of the adsorbed or the desorbed enzymes may be practical and economically worthwhile (Pourquié *et al.*, 1988) although the ratio of the individual components after desorption may differ from that of the unreacted enzyme mixture (Stutzenberger, 1988).

3 CELLULOSE-DEGRADING FUNGI

Much of the carbon dioxide fixed by photosynthesis is found in the cellulose of plants. As biomass undergoes turnover it is degraded and oxidized to carbon dioxide which is returned to the atmosphere. Ljungdahl & Eriksson (1985) state that while most (*c.* 90%) of the cellulose is degraded to carbon dioxide by aerobic microorganisms (eqn (1)), as much as 10% is converted by anaerobic microorganisms to methane and carbon dioxide (eqn (2)).

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (1)$$

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad (2)$$

Microorganisms that degrade cellulose are both abundant and ubiquitous in nature. They include fungi, bacteria and actinomycetes, aerobes and anaerobes, mesophiles and thermophiles. In what they described as a partial list of cellulolytic fungi, Ljungdahl & Eriksson (1985) named 60 different species. However, it should be emphasized that while many fungi can grow on cellulose, or produce enzymes that degrade amorphous cellulose, relatively few produce the 'complete' extracellular cellulase systems that degrade crystalline cellulose extensively *in vitro* (Mandels, 1975). *Trichoderma* and *Phanerochaete* species are the best known (Ljungdahl & Eriksson, 1985) but bacteria, such as *Clostridium thermocellum* (Béguin *et al.*, 1987; Tailliez *et al.*, 1989*a*, *b*), and the anaerobic rumen fungus, *Neocallimastix frontalis* (Wood *et al.*, 1986), may

be more promising for commercial exploitation. It was noted above that production of the large amounts of cellulase required for biomass conversion may account for as much as 60% of the total costs (Mandels, 1985). For this reason much effort and ingenuity has been expended on isolating hyper-cellulolytic mutants and in optimizing fermentation conditions. Productivities of 200–500 filter paper units (FPU) per litre per hour by mutants of *T. reesei* have been achieved using fed-batch techniques and soluble substrates (Watson *et al.*, 1984; Pourquié *et al.*, 1988). However, Eveleigh (1987) considers that productivities of as much as 1000 FPU per litre per hour may be achievable.

Wood decay is caused mainly by aerobic fungi. These microorganisms may be classified as white-rot, brown-rot or soft-rot species depending on the pattern of attack on the major components, viz. cellulose, hemicellulose and lignin, of wood (Eriksson & Wood, 1985; Ljungdahl & Eriksson, 1985). The white-rot fungi, a heterogeneous group, effect substantial degradation of all components, including lignin and leave erosion troughs in the vicinity of the fungal hyphae (Ljungdahl & Eriksson, 1985). Of the white-rot fungi, *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) has been the most extensively studied (see Sections 4.1 and 4.2). A sub-group of the white-rot fungi degrade the various components at approximately the same rate and so are called the simultaneous-rot fungi (Liese, 1970). This would indicate that metabolism of all components is integrated and interdependent. Indeed, it is now clear that metabolism of the polysaccharide components provides the energy and the hydrogen peroxide needed to degrade lignin (Kersten & Kirk, 1987; Eriksson, 1988). A second sub-group, called the white-pocket-rot fungi, preferentially degrade lignin leaving substantial amounts of cellulose and relatively sound wood surrounding the pocket (Blanchette, 1980*a*, *b*, 1982; Otjen & Blanchette, 1982). Such is the pattern of attack by *Phellinus pini* on conifers and by *Ionotus dryophilus* on oak (Blanchette, 1980*a*, 1982; Otjen & Blanchette, 1982).

Brown-rot fungi, generally Basidiomycetes, degrade cellulose and hemi-cellulose extensively but their action against lignin may be limited to removal of methoxyl groups (Cowling, 1961; Kirk & Highley, 1973; Ander & Eriksson, 1978). By contrast with white-rots, the brown-rot fungi degrade cellulose faster than the products are utilized and the point of attack on wood may be at some distance from the fungal cell wall (Blanchette *et al.*, 1978; Eriksson *et al.*, 1980). This would imply the operation of a diffusible factor(s) in the attack on polysaccharide components (see Section 4.2). Typically, the brown-rot fungal hyphae are localized in the wood cell lumen. They penetrate adjacent cells through pre-existing openings or by forming bore holes in the wood. Removal of cell wall substances begins in the S_2 layer of the secondary cell wall—the lignin-rich primary cell wall and middle lamella being resistant to degradation. In the latter stages of decay, i.e. when the bulk of the polysaccharides have been consumed, the cell walls collapse (Eriksson & Wood, 1985; Ljungdahl & Eriksson, 1985).

The soft-rot fungi, so called because they effect the softening of wood, are

found among the Ascomycetes and Fungi imperfecti (Ljungdahl & Eriksson, 1985). They catalyse the degradation of polysaccharides but are virtually inactive against lignin. Growth of such fungi begins in the wood cell lumen. This is followed by invasion of the secondary wall where they grow parallel to the fibre axes. Attack by these organisms is characterized by cylindrical boreholes with conical ends in the S_2 layer of wood or by an erosion of the cell walls beginning at the lumen.

Only in the relatively recent past have the rumen flagellates, *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis* been isolated in pure culture and recognized as being species of anaerobic phycomycetous fungi (Orpin 1975; 1977*a*, *b*; Heath *et al.*, 1983). They degrade cellulose and other polysaccharides (Orpin, 1977*c*; Orpin & Letcher, 1979; Bauchop & Mountfort, 1981). The nature of the cellulose fermentation products *in vitro* depend on whether such organisms are grown in monoculture or in coculture with methanogens, which utilize the endproducts of the fungal fermentation of cellulose (Bauchop & Mountfort, 1981). Electron microscopic studies have shown that in the rumen these anaerobic fungi develop preferentially on lignocellulosic materials to which they adhere (Bauchop, 1981; Orpin, 1981; Akin & Barton, 1983; Akin *et al.*, 1983; Akin, 1987). It is not yet known whether these rumen anaerobic fungi, reviewed recently by Orpin & Joblin (1988), are also engaged in cellulose conversion in anaerobic soils, muds and aquatic habitats.

The microorganisms engaged in cellulose conversion in nature do not operate alone. Whether aerobic or anaerobic, they act in consortium with other microorganisms including non-cellulolytic species. Under these conditions, both the rate and the extent of cellulose degradation is substantially greater than would be the case with a monoculture.

4 FUNGAL CELLULASE SYSTEMS

The oxidative and hydrolytic enzymes known to be associated with cellulose degradation by fungi are cellobiose:quinone dehydrogenase (cellobiose:quinone 1-oxidoreductase; EC 1.1.5.1), cellobiose oxidase/hydrogenase (cellobiose:acceptor 1-oxidoreductase; EC 1.1.99.18), lactonase (D-glucono-1,5-lactonohydrolase, EC 3.1.1.17), endoglucanase (1,4(1,3;1,4)-β-D-glucan 4 glucanohydrolase; EC 3.2.1.4), β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21), exoglucohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74) and cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91). To my knowledge cellobiose phosphorylase (EC 2.4.1.20), cellodextrin phosphorylase (EC 2.4.1.49) and cellobiose epimerase (EC 5.1.3.11) have been found only in bacterial systems (Coughlan, 1985; Coughlan & Ljungdahl, 1988). Procedures for the preparation of substrates and for assay, purification and activity staining of all of the relevant fungal and bacterial enzymes have recently been covered in detail by Wood & Kellogg (1988) and will not be repeated here.

4.1 Hydrolytic Components

The 'complete' cellulase systems of white- and soft-rot fungi that actively degrade crystalline cellulose are comprised, in the main, of endoglucanases (EG), exocellobiohydrolases (CBH) and β-glucosidases. The cellulase systems of most individual fungi are not 'complete' in that they lack the exo-cellobiohydrolase component (Wood & Bhat, 1988). However, the distinction between endo- and exo-acting components is not as simple as was considered heretofore and the necessity for development of a more informative nomenclature is urgent (Enari, 1983; Enari & Niku-Paavola, 1987; Coughlan & Ljungdahl, 1988; Knowles *et al.*, 1988*a*; see also discussion in Sections 4.1.3.4, and 4.1.4). Among the better characterized of the 'complete' systems are those of the white-rot fungi *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*; Eriksson & Pettersson, 1975*a*, *b*; Eriksson, 1978; Eriksson & Wood, 1985; Ljungdahl & Eriksson, 1985) and *Schizophyllum commune* (Paice *et al.*, 1984; Clarke & Yaguchi, 1985; Willick & Seligy, 1985; Clarke, 1987), of the soft-rots, *Fusarium lini* (Mishra *et al.*, 1983), *Fusarium solani* (Wood, 1971; Wood & McCrae, 1977), *Penicillium funiculosum* (Wood *et al.*, 1980; Wood & McCrae, 1982), *Penicillium pinophilum* (Wood & McCrae, 1986; Wood *et al.*, 1989), *Talaromyces emersonii* (McHale & Coughlan, 1980, 1981*a*, 1982; Moloney *et al.*, 1985), *Thermoascus aurantiacus* (Feldman *et al.*, 1988), *Trichoderma koningii* (Halliwell & Griffin, 1973; Wood & McCrae, 1975, 1978; Halliwell & Vincent, 1981) and *Trichoderma reesei* (formerly *T. viride*; Berghem & Pettersson, 1973; Berghem *et al.*, 1975; Shikata & Nisizawa, 1975; Gum & Brown, 1976, 1977; Anon., 1981; Nummi *et al.*, 1983), and of the phytopathogenic fungus, *Sclerotium rolfsii* (Lachke & Deshpande, 1988).

The brown-rot fungi produce endoglucanases but not cellobiohydrolases (Highley, 1975*a*, *b*). Oxidative degradation of cellulose appears to be more important to the brown-rots than to the white-rot and soft-rot species, although they also engage in such activity (see Section 4.2). The anaerobic fungus, *Neocallimastix frontalis*, isolated from the rumen of cattle (Pearce & Bachop, 1985) and sheep (Wood *et al.*, 1986) produces an extracellular cellulase with high activity against crystalline cellulose. This is the only member of this group whose cellulase system has yet been studied, so generalization is premature. It produces endoglucanase and β-glucosidase and probably cellobiohydrolase(s) as do the white-rot and soft-rot fungi (Wood *et al.*, 1986). Unlike the latter systems, however, the system produced by *N. frontalis* exists as a multienzyme complex (Wood *et al.*, 1988).

4.1.1 Substrate Specificities of the Hydrolytic Components—Generalizations

As a rule, endoglucanases are inactive against crystalline celluloses, such as cotton or Avicel, but hydrolyse amorphous celluloses and soluble substrates such as carboxymethylcellulose. Cellooligosaccharides are also substrates, the rate of hydrolysis increasing with increasing chain length (Wood & Bhat,

1988). Attack by these enzymes is characterized by random cleavage of β-glycosidic linkages resulting in a rapid drop in the degree of polymerization relative to the rate of release of reducing sugars. The products of action on amorphous cellulose include glucose, cellobiose and cellodextrins of various lengths. By contrast, true cellobiohydrolases degrade amorphous cellulose by consecutive removal of cellobiose from the non-reducing ends of the substrate. Such enzymes, when acting alone, do not attack cotton extensively (but see Section 4.1.4 for contrary opinions) whereas the extent of degradation of the microcrystalline substrate, Avicel, may be as much as 40% or more (McHale & Coughlan, 1980; Henrissat *et al.*, 1985; Wood & Bhat, 1988). With exo-acting enzymes, the rate of release of reducing sugars is greater than that expected from the decrease in the degree of polymerization or, when appropriate, the decrease in solution viscosity. Endoglucanases and exo-cellobiohydrolases act synergistically in the hydrolysis of crystalline cellulose (see Section 4.1.4).

Exoglucohydrolases have been found in only a few cellulase systems as yet. The enzymes from *Penicillium funiculosum* and *Talaromyces emersonii* catalyse the removal of glucose from the non-reducing ends of cellodextrins, activity decreasing with decreasing chain length (McHale & Coughlan, 1981*a*; Wood & McCrae, 1982). The exoglucohydrolases do not act synergistically with endoglucanases in the hydrolysis of crystalline cellulose (Wood & McCrae, 1982). The β-glucosidases complete the process of cellulose hydrolysis by cleaving cellobiose and by removing glucose from the non-reducing ends of smaller cellodextrins. However, unlike the exoglucohydrolases, their activity increases with decreasing degree of polymerization (McHale & Coughlan, 1981*a*). In a recent report on β-glucosidase, Bock & Sigurskjold (1989) say that high substrate specificity can only be obtained at the expense of the overall rate of reaction. In general, they conclude that the rate of enzymic reaction is optimized by evolving the lowest possible energy in the catalytic step while specificity is optimized by evolving the most stable enzyme–substrate complex. Thus, the need for compromise.

4.1.2 Multiplicity of Hydrolytic Components

Each of the major cellulolytic components synthesized by an individual organism exists in a number of forms. Thus, for example, culture filtrates of *S. pulverulentum* contain five separate endoglucanases (Eriksson & Pettersson, 1975*a*) and two distinct β-glucosidases (Deshpande *et al.*, 1978). The extracellular system of *T. emersonii* contains three β-glucosidases, four endoglucanases and as many as five exo-acting enzymes (McHale & Coughlan, 1981*a*, *b*; Moloney *et al.*, 1985). Eveleigh (1987) lists several possible causes of multiplicity, including: (a) microheterogeneity due to the complexing of cellulolytic components with other proteins, glycoproteins or polysaccharides; (b) macroheterogeneity based on the formation of multienzyme aggregates; (c) synthesis of variants of a single gene product as a result of infidelity in translation, differential proteolysis or glycosylation, and interaction with components of the culture broth; (d) the occurrence of multiple genes—in