ANALYSIS OF GENES AND ENZYMES INVOLVED IN THE DEGRADATION OF HEMICELLULOSE AND CELLULOSE BY *BUTYRIVIBRIO FIBRISOLVENS* H17c

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CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of "General regulations for the degree of Ph.D." I,as supervisor of the candidate L.-L. Lin, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication

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Contents

Abstract

B. fibrisolvens H17c is a Gram-positive obligate anaerobe which has been found in the rumen of most ruminants. Strains of *B. fibrisolvens* have been reported to exhibit activity toward cellulosic and hemicellulosic substrates.

The aim of this thesis was to screen a genebank of *B. fibrisolvens* H17c DNA and to isolate genes expressing cellulase and xylanase activity. Two genes encoding β-1-4-glucosidase (BglA) and endo-β-1-4-xylanase (XynB) were cloned in *E. coli* .

The β-glucosidase gene was cloned on a recombinant plasmid pLS215 which enabled *E. coli* cells to produce intracellular β-glucosidase activity. The gene was located on 3.74 kb fragment and was expressed from a sequence which has promoter activity in *E. coli* host. The nucleotide sequence was determined and the deduced amino acid sequence (830 residues) showed 41% identity with a β-glucosidase from *Ruminococcus albus*. Four extensively conserved regions were found in β-glucosidases of *B. fibrisolvens*, *R. albus* , *Kluyveromyces fragilis*, *Clostridium thermocellum*, and *Saccharomycopsis fibuligera*. The C-terminal region of *B. fibrisolvens* BglA contains the putative active site of β-glucosidase A3 from *Aspergillus wentii*. The cloned β-glucosidase had an apparent M_r of approximately 94,000. Optimum enzyme activity was obtained at pH 5.0 at 42°C. The enzyme hydrolyzed cellobiose to a limited extent and degraded cellodextrins to predominantly glucose.

The gene expressing xylanase activity was cloned on a recombinant plasmid pLS206. This gene was located on a 3 kb fragment and also expressed from a sequence which has promoter activity. The nucleotide sequence of *xyn*B was determined and the amino acids (635 residues) were deduced. The catalytic domain of XynB showed good similarity to that of the xylanases from alkalophilic *Bacillus* sp. strain C-125, *B. fibrisolvens* 49, and *Pseudomonas fluorescens* subspecies *cellulosa*, as well as to that of a cellobiohydrolase/endoglucanase of *Caldocellum saccharolyticum*. The enzyme which contains no typical signal sequence was located in the cytoplasm of the *E. coli* host. The purified xylanase had an apparent M_r of approximately 74,000. Optimun xylanase activity was obtained at pH 5.4 and pH 6.0 at 55°C. The enzyme activity was stimulated by Na^+, K^+, NO_3^-, and SCN^- but effectively inhibited by Ag^+, Cu^{2+}, Zn^{2+}, and Ca^{2+}.

Two major complexes, termed complex A (C_A) and complex B (C_B), of the extracellular xylanase and cellulase components of *B. fibrisolvens* H17c were eluted by column chromatography. C_A showed cellulase activity, while C_B had xylanase and cellulase activity. The xylanase and CMCase activity of C_B was eluted in a single peak with an apparent M_r > thyroglobulin (M_r 669,000). CMC zymograms under non-denaturing conditions showed 5 bands with CMCase activity from C_A and 8 from C_B. Xylan zymograms under the same conditions indicated 4 bands of activity in C_B. Under mild denaturing conditions the xylanase activity in C_B was found in 11 bands with M_r ranging from 45 kDa to 180 kDa. This

indicates that C_B exists as a multi-subunit protein aggregate of xylanases and cellulases.

Abbreviations

A	adenosine
A_{420}	absorbance
Ap	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C-terminal	carboxy terminal
CBH	cellobiohydrolase
Cm	chloramphenicol
CsCl	caesium chloride
CMC	carboxymethylcellulose
CMCase	carboxymethylcellulase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
DP	degree of polymerization
DTT	1,4-dithio-L-threitol
EDTA	ethylenediaminetetra-acetic acid
EG	endoglucanase
EtBr	ethidium bromide
h	hour(s)
HCA	hydrophobic cluster analysis
HEC	hydroxyethylcellulose
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
Km	kanamycin
l	liter
LB	Luria-Bertani broth
M10	*B. fibrisolvens* non-rumen fluid medium
MeUMb	methylumbelliferyl
min	minute(s)
mRNA	messenger RNA
MW	molecular weight
N-terminal	amino terminal
OD_{600}	optical density at 600nm
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame

Abbreviations(cont.)

p	plasmid
PAGE	polyacrylamide gel electrophoresis
PC	phosphate-citrate buffer
*pho*A	gene coding for alkaline phosphatase
pNPG	p-nitrophenyl-β-D-glucopyranoside
pNPC	p-nitrophenyl-β-D-cellobioside
pNPX	p-nitrophenyl-β-D-xylopyranoside
P_R	rightward promoter(phage lambda)
r	resistance(superscript)
RNA	ribonucleic acid
s	second(s)
SDS	sodium dodecyl sulfate
sp(p)	species
TAE	tri acetate EDTA buffer
TEMED	*N,N,N',N'*-tetraethylethylenediamine
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
U	units of enzyme activity
UV	ultraviolet (light)
v/v	volume/volume
w/v	weight/volume
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XP	5-bromo-4-chloro-3-indolyl phosphate
::	novel joint (fusion)
()	designates plasmid-carrier state
α	alpha
β	beta
Δ	delta
λ	lamda
μ	micro

Chapter 1

Literature Review

Chapter 1

Literature Review

1.0 General background

Lignocelluloses derived from higher land plants represent a major source of renewable organic matter. In form and availability they occur in whole trees, forestry processing wastes, agricultural wastes such as straw and bagasse, and in many forms of domestic, municipal, and industrial wastes. The quantity of available lignocellulosic biomass is around 1.8×10^{12} tonnes and approximately 1×10^{11} tonnes are synthesized annually by photosynthesis (Coughlan, 1985). The literature on the use of lignocellosic biomass, especially on the hydrolysis and biotransformation of the cellulose and hemicellulose to utilizable products and byproducts, is extensive (Coughlan, 1985; Saddler, 1986; Vallander and Eriksson, 1990; Parisi, 1989; Lynd, 1989; Smith *et al.*, 1987; Dale, 1987).

Lignocellulose, either in its native forms, or in any of the varieties of partly processed forms, is an extremely complex material. It contains three main components, lignin, hemicellulose, and cellulose. The respective quantities in different species are indicated in Table 1.1. These three components are closely associated and sometimes covalently linked to the others to form a variable and very complex material.

Table 1.1 Average composition of lignocellulosic materials (From Parisi, 1989)

Species	Cellulose	Hemicellulose	Ligin
Conifers	40-50%	20-30%	25-35%
Deciduous trees	40-50%	30-40%	15-20%
Cane bagasse	40%	30%	20%
Corn cobs	45%	35%	15%
Corn stalks	35%	25%	35%
Wheat straw	30%	50%	15%

Lignin, the major phenolic material present in secondary thickened walls, is formed when its major precursors, p-coumaryl, coniferyl and sinapyl alcohols are released into the cell wall and are polymerised *in situ* to form a three dimensional macromole(Kosaric *et al.*, 1985). The structural elements arising from the three phenylpropane precursors are p-hydroxyphenyl, quaiauyl and syringyl units respectively. Unlike the polysaccharides in which only the glycosidic linkage is involved in unit bonding, some twenty different types of linkages have been identified within the lignin macromolecule (Chesson and Forsberg, 1988). Most involve either aryl ether linkages or carbon to carbon bonds, the strength of these bonds accounting for the resistance of lignin to both chemical and microbial degradation. It remains uncertain whether lignins are totally random structures, or whether there is a degree of order and reproducibility of structure in lignins within the same plant. However, gross differences relating to the proportions of the three monomer types present can be

recognized in lignins of different phylogenetic orgins (Alder, 1977; Monties, 1985). Lignins are extensively bound to the matrix polysaccharides of the cell wall, the lignin-carbohydrate complex so formed varying in structure depending on the plant and plant part examined (Morrison, 1974; Chesson *et al.*, 1983; Ford, 1986).

The hemicelluloses form a distinct group of polysaccharides and have been classified according to the sugar residue present in the polymer (Aspinall, 1970; Wilkie, 1979). The polymers of galactose (1,3 and 1,4-β-D-galactans), mannose (1,4-β-D-mannans) or xylose (1,4-β-D-xylan) are three major types of hemicellulose. Unlike cellulose, they usually occur as heteroglycans having side chains containing different types of sugar residues (Wilkie, 1983). Xylan makes up the main component of hemicellulose and is second in abundance to cellulose (Biely, 1985). Isolated xylans are generally varied in degree of polymerization (DP) and highly branched polymers. The common substitutents found on the backbone of β-1,4-linked D-xylopyranosyl residues are acetyl, arabinosyl, and glucuronosyl residues (Wong *et al.*, 1988). Homoxylans, which consist of xylosyl residues exclusively, have also been isolated from esparto grass (Chanda *et al.*, 1950) and tobacco (Eda *et al.*, 1976).

Cellulose is a linear homopolymer of anhydroglucose units linked by β-1,4-glucosidic bond (Coughlan, 1985). The length of the macromolecule from different sources varies greatly and can be up to 14,000 glucose units (Coughlan, 1985). The average DP in naturally occurring cellulose is

around 1000 while cotton is found to have a DP of approximately 10,000 (Tsao, 1978). Each glucose unit is rotated 180 degrees with respect to its neighbouring residues forming the basic repeating unit of cellobiose. Individual linear polymeric cellulose molecules are linked together by intra and intermolecular bonds to form elementary fibrils (Alberts *et al.*, 1983). Both hydrogen bonds and van der Waals forces promote interchain binding (Rees *et al.*, 1982). Bundles of these fibrils are futher aggregated to form the insoluble cellulose fibres (microfibrils). The fibres contain areas of complete order (crystalline regions) and areas of lesser order (amorphous regions). Although the interchain forces are strong, the association between the elementary fibrils is seldom perfect and celluloses of greater than 90% crystallinity are relatively rare (Eveleigh, 1987). A few exceptions include cotton, cell walls of the alga *Valonia macrophysa* (Chanzy and Henrissat, 1985), and the cellulose ribbons produced by *Acetobacter xylinum* (White and Brown, 1981).

1.1 Degradation of lignocellulose

Despite the complexity of the lignocellulose substrate, it is broken down efficiently in nature by a multitude of microorganisms (Dekker and Richard, 1976; Bisaria and Ghose, 1981). Each organism appears to be specialized to play a particular role in this breakdown. It would also seem that each organism produces a wide range of degradative enzymes. The environmental conditions under which the degradation occurs largely dictates the types of

microorganisms that can be isolated. Generally fungi predominate in aerobic environments and bacteria in anaerobic environments, with certain exceptions.

1.1.1 Lignin degradation. The lignin polymer serves as a barrier that must be partly removed or at least morphologically changed before the wood polysaccharides can be attacked by the enzymes specific for these substrates (Eriksson, 1987). Fungi constitute the most active group of lignolytic microorganisms (Yang *et al.*, 1980). White-rot (eg. *Phanerochaetae chrysosporium*) and soft-rot fungi (eg. *Aspergillus fumigatus*) have been identified as major lignin degraders (Amer and Drew, 1980). Certain bacteria such as *Bacillus magaterium* (Crawford and Crawford, 1980) and certain *Streptomyces* species (Crawford, 1978) also show lignolytic activity. However, the role of bacteria in the degradation of lignin is probably limited by the lack of ability to penetrate through the woody tissue when compared to fungal hyphae (Amer and Drew, 1980). The normal way for white-rot fungi to degrade wood is by simultaneous attack on the polysaccharides and the lignin. The fungi seem to need polysaccharides or low molecular weight sugars to degrade lignin. The sugars thus derived are necessary to provide energy for growth and cell metabolism (Erikksson, 1987). They are also essential because it is the oxidation of these sugars that provides most of the hydrogen peroxide necessary for lignin degradation (Forney *et al.*, 1982; Faison and Kirk, 1983; Leisola *et al.*, 1985). The biochemical mechanisms of lignin degradation have been examined in only a few of the hundreds of known white-rot fungi, but

extensive studies of one fungus, *P. chrysosporium*, indicate that two types of extracellular heme-containing enzymes play important roles in the decay of lignin by white-rot fungi. These enzymes are peroxidases and include a group of ligninases (or lignin peroxidases) (Anderson *et al.*, 1985; Buswell *et al.*, 1984; Faison and Kirk, 1983; Gold *et al.*, 1984; Kuila *et al.*, 1985; Lobarzewski and Paszazynski, 1985; Tien and Kirk, 1984; Tien *et al.*, 1986) and a group of manganese peroxidases (Glenn *et al.*, 1986; Glenn and Gold, 1985; Paszazynski *et al.*, 1985; Paszazynski *et al.*, 1986). Ligninases appear to initiate lignin degradation by extracting one electron at a time from methoxylated aromatic rings, forming cation radical species which undergo further nonenzymatic reactions that lead to lignin decomposition (Kirk, 1987). Manganese peroxidases oxidise Mn^{2+} to Mn^{3+}, which may diffuse into wood cells and initiate additional oxidative reactions (Glenn *et al.*, 1986; Paszazynski *et al.*, 1986).

1.1.2 Hemicelluloses degradation. Hemicellulases (glycan hydrolases, EC 3.2.1) specifically degrade the glycans that make up the backbone chain of hemicelluloses, and therefore include β-D-galactanases, β-D-mannanases and β-D-xylanases (Dekker and Lindner, 1979; Dekker and Richards, 1976). The modes of action of galactanases and mannanases from bacterial, fungal and plant orgin have been described in detail (Dekker, 1985). Xylan is the major component of the hemicellulose group and is second in abundance to cellulose in nature. Therefore, the xylans and xylanases have been studied in much greater detail than the other hemicelluloses

and hemicellulases. Many fungi and bacteria are capable of producing xylan-degrading enzymes (Reilly, 1981; Biely, 1985; Dekker, 1985). Among the fungi, five genera including *Aspergillus, Botryodiploidia, Penicillium, Pestalotia* and *Trichoderma* are recommended as xylanase producers (Reese *et al.*, 1973). Certain yeasts from genera *Aureobasidium, Cryptococcus* and *Trichosporon* have also been reported to produce xylanases (Biely *et al.*, 1978).

Xylanases are produced by many bacterial genera, most of which are anaerobes. These include rumen bacteria of various genera such as *Fibrobacter* (formerly *Bacteroides*) (Dehority, 1967; Forsberg *et al.*, 1981), *Butyrivibrio* (Howard *et al.*, 1960; Hespell *et al.*, 1987) and *Ruminococcus* (Dehority, 1967). Mesophilic and thermophilic *Clostridium* species also produce xylan-degrading enzymes (Lee *et al.*, 1985; Berenger *et al.*, 1985). Aerobic xylanase producers include *Bacillus* and *Streptomyces* species (Dekker and Richards, 1976) as well as various plant pathogens such as *Agrobacterium, Corynebacterium, Erwinia, Flavobacterium, Pseudomonas* and *Xanthomonas* (Hayward, 1977).

1.1.2.1 Mode of action of xylanase. The enzymatic hydrolysis of xylan is accomplished by the action of endo-1,4-β-xylanase (EC 3.2.1.8) and β-D-xylosidase (EC 3.2.1.37). The first enzyme acts on xylan to generate small xylooligosaccharides, and the β-xylosidases hydrolyze dimers and trimers of xylose to the monomeric sugar (Biely, 1985). Most of the β-xylosidases show no activity towards xylan. Xylobiose is the best substrate for β-xylosidases and the

rate of hydrolysis decreases with increasing chain length of the xylooligosaccharides (van Doorslaer *et al.*, 1985; Matsuo and Yasui, 1984; Rodionova *et al.*, 1983).

Xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with side chains. The substituents seem to play an important role in limiting enzymatic hydrolysis. Some xylanases are able to cleave xylotriose but not arabino-xylotriose (Takenishi and Tsujisaka, 1975), indicating that the arabinosyl substituent protects xylosidic linkages from these xylanases. The shortest acidic xylooligosaccharides and arabino-xylooligosaccharides in various xylan hydrolysates may therefore be the limit dextrins of the respective xylanase preparations (Brillouet, 1987; Comtat and Joselean, 1981; Kubackova *et al.*, 1979). Debranching is carried out by separate enzymes which do not directly attack the xylan backbone but do aid in the degradation of xylan by removing side chains. They include α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1) and acetylesterase (EC 3.1.1.6) (Biely, 1985). Esterases act synergistically with endoxylanases to enhance the degradation of acetyl xylan (Biely, 1985; Biely *et al.*, 1986).

1.1.2.2 Multiplicity of xylanases. It appears that xylosidic linkages in lignocellulose are not all equivalent and equally accessible to xylanolytic enzymes. The production of an enzyme system, each enzyme with a specialized function, is one strategy that a microorganism may use to achieve superior xylan hydrolysis. Multiple

xylanases have been reported in numerous microorganisms (Dekker, 1985; Wong *et al.*, 1988). The occurrence of isomeric forms of xylanase can be due to substrate cross-specificity, multiple genes or post-translational modification.

The substrate specificity of xylanases is variable. Some enzymes hydrolyze only xylan (Sipat *et al.*, 1987), while others exhibit endoglucanase activity (Flint *et al.*, 1989; Lee *et al.*, 1987; Fournier *et al.*, 1985; Frederick *et al.*, 1985; Shei *et al.*, 1985; Uchino and Nakane, 1981). By using enzyme kinetic methods, Uziie *et al.* (1985) demonstrated that a bifunctional β-D-xylosidase-β-glucosidase from *Chaetonium tritalerate* contained a single active site with dual binding regions. A xylanase purified from *Cryptococcus albidus* does not hydrolyze xylobiose but does hydrolyze pNPX by using a complex transglucosylic pathway (Biely *et al.*, 1980a). A cell wall-bound β-glucosidase from the same organism also exhibits apparent xylosidase activity but in fact does not hydrolyze xylooligosaccharides (Peciarova and Biely, 1982). Substrate ambiguity among xylosidases also occurs and has recently been reported from cloned genes from *B. fibrisolvens* (Utt *et al.*, 1991), *Bacteroides ovatus* (Whitehead and Hespell, 1990) and *C. saccharolyticum* (Luthi *et al.*, 1990).

There is evidence suggesting that at least some of the multiple xylanases from a microorganism are distinct gene products. The amino acid compositions of the three xylanases isolated from *T. harzianum* E58 indicate that none

of these enzymes has a direct precussor product relationship with another (Wong *et al.*, 1986a). Other reports demonstrated immunological distinction among xylanases from *Aeromonas* sp. strain 212 (Kudo *et al.*, 1985), *Bacillus* sp. strain C-125 (Honda *et al.*, 1985b), *C. acetobutylicum* ATCC824 (Lee *et al.*, 1987), and *Streptomyces* sp. strain 3137 (Marui *et al.*, 1985). In addition, three distinct xylanase genes from *P. fluorescens* subsp. *cellulosa* have been isolated (Gilbert *et al.*, 1988), two of which show strong homology. The non-homologous gene is adjacent to an endoglucanase gene in the *Pseudomonas* chromosome, suggesting that the two genetic loci arise through gene duplication. Two MUC positive clones of *C. thermocellum* were also found to encode xylanases rather than cellobiohydrolase (Grepinet *et al.*, 1988).

Xylanase heterogeneity may rise from post-translational modifications as a consequence of different RNA processing, partial proteolysis or differences in the degree of amidation and glycosylation (Biely, 1985). Many xylanases are apparently glycosylated (Berenger *et al.*, 1985; Bernier *et al.*, 1983b; Fournier *et al.*, 1985; Frederick *et al.*, 1981; Grobacheva and Rodionova, 1977a; Morosoli *et al.*, 1986b; Yoshioka *et al.*, 1981), and some are apparently translated as precursors with peptide signal sequences (Fukusaki *et al.*, 1984; Hamamoto *et al.*, 1987; Morosoli, 1985; Paice *et al.*, 1986; Luthi *et al.*, 1990). Furthermore, expression of a cloned xylanase gene from *Aeromonas* sp. strain 212 in *E. coli* produced a 135 kDa xylanase which is smaller than the 145 kDa xylanase

characterized in this organism (Kudo *et al.*, 1985). These two xylanases appear to be differentially modified products from the same gene because they have similar hydrolytic, immunological, and physiochemical properties (Kudo *et al.*, 1985).

1.1.2.3 Structure of xylanases. When a number of different gene products are consistently produced by one organism for hydrolysis of xylan, it is reasonable to suggest that the individual xylanases will have distinct properties that contribute key functions to the overall xylanolytic systems of the different microorganisms. Perhaps these distinct xylanolytic functions are conserved among xylanolytic systems of microorganisms by evolutionary processes. The conservation of xylanases is suggested by the fact that xylanases purified from *Aspergillus, Bacillus, Clostridium, Streptomyces,* and *Trichoderma* species appear to have a conserved relationship with respect to their molecular weight and their pI values (Wong *et al.*, 1988). These species all produce two xylanases with low-MW/basic and high-MW/acidic characteristics (Wong *et al.*, 1988).

West *et al.* (1989) have noted the homology between the *C. fimi* exoglucanase domain and the xylanases from *Bacillus* sp. C-125 and *C. albidus* and have suggested that many of the glycosidases from *Cellulomonas, Trichoderma,* and *Bacillus* spp. may have evolved from the reshuffling of two catalytic domains. The similarities among *Caldocellum* xylanase/β-xylosidase (Luthi *et al.*, 1990), the exoglucanase of *C. fimi* (O'Neil *et al.*, 1986), the cellobiohydrolase domain of

Caldocellum celB (Saul *et al.*, 1989), and the C-terminal domain of *C. thermocellum xynZ* gene product also suggests the possibility of reshuffling of catalytic and binding domains.

There are several examples of conserved domains located at the termini of functionally related enzymes which interact with macromolecules (Teeri *et al.*, 1987). In the case of cellulases it has been suggested that they are non-catalytic but function in recognizing and mediating the binding of the enzymes to their substrates. In the case of xylanase XynZ of *C. thermocellum* (Grepinet *et al.*, 1988), a region of 60 amino acids is strongly similar to the conserved COOH-terminal region of EGA (Beguin *et al.*, 1985), EGB (Grepinet and Beguin, 1986), and EGD (Joliff *et al.*, 1986) of the same organism. The region contains two segments of 24 amino acids showing strong sequence similarity, linked by 10 residues. Two serine-rich regions in XylA of *P. fluorescens* subsp. *cellulosa* were also described by Hall *et al.* (1989). The conserved sequence in XylA can be removed without affecting enzyme activity. Likewise, the reiterated region can be deleted from *celD* without loss of activity of EGD (Grepinet *et al.*, 1988). Therefore, it would be difficult to argue in favour of these regions being important in enzyme function. A potential role of these homologous structures in *C. thermocellum* could be the anchorage of different enzymes to the multimolecular complex responsible for cellulose hydrolysis (Grepinet *et al.*, 1988).

A quantitative study provided evidence that the *C. albidus* xylanase catalyzes degradation of xylooligosaccharides by a multiple reaction pathway analogous to those catalyzed by lysozyme (Biely *et al.*, 1981b). This enzyme has a four subsite-binding site with catalytic groups in the middle (Biely *et al.*, 1981a). The *A. niger* xylanase also exhibits a mechanism of substrate degradation analogous to the *C. albidus* enzyme (Vrsanska *et al.*, 1982). Studies of egg white lysozyme reveal a general acid-base catalysis involving Glu-35 and Asp-52 as important catalytic residues (Quiocho, 1986). Subsequent studies have demonstrated that this catalytic region is conserved in xylanase enzymes (Morosoli *et al.*, 1986b; Utt *et al.*, 1991). However, investigations by chemical modification of an enzyme provide evidence for the involvement of tryptophan in the active site of xylanases from *Bacillus* and an actinomycete, *Chainia* (Deshpande *et al.*, 1990). A gene coding for xylanase from *B. subtilis* has been isolated and its complete nucleotide sequence determined (Paice *et al.*, 1986). The deduced amino acid sequence exhibited over 50% identity with a xylanase from *B. pumilus* (Fukusaki *et al.*, 1980) but it does not appear to be homologous with cellulases and lysozymes (Paice *et al.*, 1986). Knowles *et al.*(1987) have pointed out that the homologies demonstrated between lysozyme and cellulases are too weak to draw any conclusions about their significance. The same conclusion could apply to xylanases.

1.1.2.4 Applications of xylanases. In paper manufacturing, the use of purified xylanases to selectively hydrolyze xylan while retaining cellulose is necessary for yielding

desirable pulp characteristics. The feasibility of using xylanases for cellulose purification is highly dependant on the intrinsic selectivity of the enzymes, the absence of cellulolytic activities, and the availability of enzyme preparations in inexpensive bulk quantities. One strategy for obtaining cellulase-free xylanases is to transfer a xylanase coding gene into a non-cellulolytic microorganism. Convincing results include the production of extracellular xylanases by *E. coli* expressing cloned xylanase genes from *Aeromonas* sp. strain 212 (Kato *et al.*, 1986) and from *Bacillus* sp. strain C-125 (Honda *et al.*, 1985a). Xylanase is also produced by a cellulase-negative and xylanase-negative double mutant of *Streptomyces lividans* carrying a xylanase gene cloned from *S. lividans* 1326 (Mondou *et al.*, 1986).

Along with that of cellulases and pectinase, the use of xylanases has also been suggested in applications such as the clarification of juices (Biely, 1985), the preparation of dextrans for use as food thickeners (Thompson, 1983), and the production of fluids and juices from plant materials (Woodward, 1984). Furthermore, partial xylan hydrolysis in animal feed may improve cellulose accessibility to ruminal digestion and thus improve nutritional value of the feed (Wong *et al.*, 1988). Xylanases may also be used to prepare materials for scientific research. Selected xylanases may be suitable for the production of branched/unbranched, short/long, or labelled xylooigosaccharides, model compounds for studing mechanisms of xylanase action. Similarly, antibodies for detecting various xylanases can be useful for

characterization of xylanase production, plant cell walls, and plant pathogenesis (Wong *et al.*, 1988).

1.1.3 Cellulose degradation. There are a great many microorganisms which have been classified as cellulase producers (Table 1.2). Fungal cellulase producers include species of *Trichoderma*, of *Penicillium*, of *Aspergillus*, of *Fusarium*, of *Clostridium*, and of *Sporotrichum*. Among the mesophilic fungi, *T. reesei* has been extensively studied and over-producing mutants of this organism have been isolated (Coughlan, 1985). Bacterial cellulase producers appear to be divided into two groups. The bacteria of group one produce the complete cellulolytic complex required for crystalline cellulose hydrolysis. These included members of the genera *Ruminococcus, Bacteroides, Clostridium, Cellulomonas, Cellvibrio, Pseudomonas,* and *Acetovibrio*. The second group seems to produce only components of the cellulase system and are not able to hydrolyze crystalline cellulose (Gong and Tsao, 1979a; Bisaria and Ghose, 1981). *Bacillus subtilis* and *Clostridium acetobutylicum* as well as other *Bacillus* species fall into this group (Fogarty and Griffin, 1973; Horikoshi *et al.*, 1984; Lee *et al.*, 1985a).

The cellulases produced by cellulolytic organisms are a collective name, comprising a multiplicity of activities. These enzymes can be divided into three major classes : (1) endo-β-1,4-glucanase (β-1,4-glucano 4-glucanohydrolase, EC3.2.1.4), commonly known as CMCase, which attacks the glucan chain at random internal sites, (2) exo-β-1,4-glucanase (β-1,4-glucan cellobiohydrolase, EC 3.2.1.91),

commonly known as Avicelase, which removes cellobiose units from the non-reducing end of the polymer, and (3) β-1,4-glucosidase, some of which are cellobiase (EC 3.2.1.21), which hydrolyzes cellobiose to glucose (Coughlan, 1985).

Table 1.2 Cellulose-degrading microbes (From Kosaric *et al.*, 1985)

Family	Genera	Species
A) Fungi		
Mesophilic	*Trichoderma*	
		T. reesei
		T. koningii
	Penicillium	
		P. funiculosum
		P. citrinum
	Aspergillus	
		A. wentii
		A. niger
	Fusarium	
		F. solani
		F. oxysporum
Thermophilic	*Chaetonium*	
		C. cellulolyticum
		C. thermophile
	Sporotrichum	
		S. pulverulentum
		S. thermophile
B) Bacteria		
Mesophilic	*Acetovibrio*	
		A. cellulolyticus
	Pseudomonas	
		P. fluorescens
	Cellvibrio	
		C. fulvus
		C. gilvus
	Cellulomonas	
		C. fimi
	Ruminococcus	
		R. albus
		R. flavefaciens
	Bacteroides	
		B. succinogenes
Thermophilic	*Clostridium*	
		C. thermocellum
C) Actinomyces		
Mesophilic	*Streptomyces*	
		S. flavogrisens
		S. griseus
Thermophilic	*Thermomonospora*	
		T. curvata

The classification of an enzyme is based on its activity on substrates. (1) Endoglucanases hydrolyze soluble substituted cellulose such as carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) (Gilkes *et al.*, 1984; Wirick, 1968a; 1968b), but either fail to hydrolyze or exhibit weak activity on crystalline and other cellobiohydrolase specific substrates. A recommended test for endoglucanase activity is to measure the decrease in viscosity of CMC as a function of the release of reducing sugars from the substrate (Gilligan and Reese, 1954). (2) Cellobiohydrolases hydrolyze model substrates such as p-nitrophenyl-β-D-cellobioside (pNPC) and methylumbelliferyl-β-D-cellobioside (MUC) to a glycone and cellobiose (Deshpande *et al.*, 1984; Van Tilbeurgh *et al.*, 1982). They release cellobiose from crystalline cellulose and show weak activity against endoglucanase specific substrates. In contrast to endo-acting enzymes the exo-type enzymes cause only a slow increase in specific fluidity. (3) β-glucosidases appear in the literature as a rather undefined term for enzymes which catalyze the hydrolysis of β-glucosidic linkages between aryl, alkyl, or saccharide groups (Woodward and Wiseman, 1982). Many β-glucosidases are capable of producing glucose from cellooligosaccharides and show a great variation in their patterns of substrate specificity (Woodward and Wiseman, 1982). Some fungal β-glucosidases even show β-1,4-D-glucan glucanohydrolase activity when removing glucose residues from the nonreducing ends of β-glucan chains (Wood and McCrae, 1982a; Schmid and Wandrey, 1987). (4) Cellodextrinases are defined as the enzymes which release cellobiose units from short cellulooligomers

and pNPC. These enzymes show weak activity on crystalline cellulose and have been found in bacteria (Huang and Forsberg, 1987; Wang and Thomson, 1990; Berger *et al.*, 1990).

1.1.3.1 Mode of cellulase action. Degradation of crystalline cellulose to water soluble cellooligomers is considered to be due to the synergistic action of endoglucanases and cellobiohydrolases. The action of endoglucanases is thought to initiate the splitting of internal sites of the polyglucan chain and to create new sites for action of cellobiohydrolases. Both types of enzymes have been found to act synergistically and cooperatively on crystalline cellulose substrates (Eveleigh, 1987; Coughlan and Ljungdahl, 1988; Wood *et al.*, 1988). Two stereospecific classes of endoglucanase (A:A') and cellobiohydrolase (B:B') can be theoretically predicted, based on the fact that the cellobiose repeating unit occurs in two stereospecific forms (Eveleigh, 1989). The hydrolysis of cellulose into glucose is completed by the action of β-glucosidase on cellodextrins and cellobiose. Hydrolysis of cellobiose may occur by cleavage of the glycosidic bond to yield two molecules of glucose. This reaction is catalyzed by cellobiase, which is present in bacteria and yeasts (Kadam *et al.*, 1988; Rayal and Guerineau, 1984). A second mode of action consists of Pi-dependent phosphorolysis of cellobiose into one molecule of glucose and one molecule of glucose-1-phosphate. This action is catalyzed by cellobiose phosphorylase (EC 2.4.1.20), an enzyme which has been described in bacteria (Alexander, 1968; Barras *et al.*, 1984; Schmiz *et al.*,

1983; Helaszek and White, 1991) and fungi (Heale and Gupta, 1971). Finally, a different mechanism of phosphorolysis which yields glucose and glucose-6-phosphate has also been described (Palmer *et al.*, 1971).

Understanding the mechanism of enzymatic cellulose degradation has been limited by the lack of substructural observations demonstrating the mode of cellulase action proposed above. High-resolution electron microscopy has permitted the visualization of the macromolecular fine structure of cellulose as it is degraded (White and Brown, 1981; Chanzy and Henrissat, 1985; Sprey and Bochem, 1991). According to the study on *T. reesei* cellulase (cellobiohydrolase CBHII and endoglucanase EGII), the fibrils are eroded only at one end by CBHII. The subsequent addition of endoglucanase EGII results in selective attack at amorphous zones along the length of the fibrils, followed by surface stripping by CBHII (Chanzy and Henrissat, 1985). Previously White and Brown (1981) found no visible change in cellulose structure by CBHI of *T. reesei*, but did demonstrate synergistic activity between CBHI and EGIV on crystalline cellulose ribbons of *A. xylinum*. Recently, Sprey and Bochem (1991) reported that an endoglucanase of *T. reesei* alone could initiate *Sinapis alba* cellulose decomposition by an erosion process in the microfibril structure to form subfibrils, which were then hydrolyzed progressively from the microfibril periphery to its core. The evidence also shows that CBH enzymes may be the key components in fungal hydrolysis of crystalline cellulose. Two immunologically distinct cellobiohydrolases of *T. reesei*

have been showed to act synergistically in the hydrolysis of crystalline cellulose (Fagerstam and Pettersson, 1980). *T. reesei* CBHI binds all over the cellulose surface and can independently break down *Valonia* cellulose crystals (Chanzy *et al.*, 1983). Other researchers have similarly noted the independent action of cellobiohydrolases on crystalline cellulose and not on soluble substituted forms (Halliwell and Griffin, 1973; Nummi *et al.*, 1983; Sasaki *et al.*, 1979).

1.1.3.2 The structure and function of cellulases. Hydrophobic cluster analysis (HCA) has recently proven to be a useful method for comparing amino acid sequences (Gaboriaud *et al.*, 1987; Benchebrit *et al.*, 1988). The approach can detect similarities in the three-dimensional folding in regions of low sequence identity (eg. 10%). In addition, HCA is effective in finding homologous domains which are separated by variable segments of widely differing sizes(Henrissat *et al.*, 1988). Genes coding for cellulolytic and xylanolytic enzymes have been grouped into six families (A-F) by HCA (Henrissat *et al.*, 1989). Furthermore, the comparison reveals a high degree of protein sequence conservation within the catalytic regions of enzymes from taxonomically unrelated organisms (Beguin, 1990; Gilkes *et al.*, 1991).

Catalytic domains. The removal of certain parts of a cellulase gene without loss of cellulase activity has been shown to be the case for many cellulases. Deletions of 233 base pairs (bp) from the 3' end of the *C. thermocellum*

endoglucanase D gene (Chauvaux *et al.*, 1990) and 327 codons from the 5' end and up to 245 codons from the 3' end of the *C. thermocellum cel* H (Yague *et al.*, 1990) which did not affect enzyme activity, are two examples of catalytic "cores" which behave independently. Gilkes *et al.* (1988; 1989) showed that both *C. fimi* Cex and CenA enzymes cloned in *E. coli* are cleaved by an extracellular *C. fimi* protease in a specific manner without loss of activity against soluble substrates. The catalytic domains of the Cex and CenA enzymes can also function independently, and it was shown that a fusion protein between the two catalytic domains produced a bifunctional enzyme which is able to degrade endo- and exo-type substrates (Warren *et al.*, 1987b).

The known cellulase and xylanase sequences can be grouped into nine distinct families according to their catalytic domains (Table 1.3) (Gilkes *et al.*, 1991). Families, A, B, F and H contain fungal and bacterial enzymes. Family E contains bacterial enzymes and plant enzymes, thereby raising the possibility of gene transfer. At present, family C contains only fungal enzymes, whereas families D and G contain only bacterial enzymes. Cellulases and xylanases vary widely in the numbers of amino acids, but their catalytic domains tend to be more uniform in size.

Table 1.3 Families of cellulase and xylanase catalytic domains (After Gilkes *et al.*, 1991)

Family	Organism	Enzyme	Catalytic domain[a] Terminus	No. of amino acids
A				
	Bacillus sp. strain 1139	Egl	N	385
	Bacillus sp. strain KSM-635	Egl		<555
	Bacillus sp. strain N-4 gene pNK1	CelB	N	307
	Bacillus sp. strain N-4 gene pNK2	CelA	N	305
	Bacillus sp. strain N-4 gene pNK3	CelC	N	~350
	Bacillus lautus	CelB		
	Bacillus polymyxa	Egl		
	Bacillus subtilis N-24	Egl		
	Bacteroides ruminicola	Egl	N	
	Butyrivibrio fibrisolvens A46	CelA		
	Butyrivibrio fibrisolvens H17c	EndI	N	~385
	Caldocellum saccharolyticum	CelB[b]	C	388
	Clostridium acetobutylicum	Egl	N	~300
	Clostridium cellulolyticum	CelA	N	~380
	Clostridium thermocellum	CelB	N	>469
	Clostridium thermocellum	CelC		
	Clostridium thermocellum	CelE	N	340
	Clostridium thermocellum	CelH	C	~305
	Erwinia chrysanthemi	CelZ	N	305
	Fibrobacter succinogenes	Egl3	C	~416
	Robillarda sp. strain Y-20	Egl		
	Ruminococcus albus F-40	EglI		
	Ruminococcus albus SY3	CelA		
	Ruminococcus albus SY3	CelB		
	Trichoderma reesei	EglIII	C	327
	Xanthomonas campestris	EngXCA	N	~350
B				
	Cellulomonas fimi	CenA	C	284
	Microbispora bispora	CelA	N	~290
	Streptomyces sp. strain KSM-9	CasA		
	Trichoderma reesei	CbhII	C	385
C				
	Humicola gisea	CbhI		
	Phanerochaete chrysoporium	CbhI	N	~425
	Trichoderma reesei	CbhI	N	~425
	Trichoderma reesei	EglI	N	363
	Trichoderma viride	Cbh	N	~435
D				
	Bacillus circulans	Bgc		
	Cellulomonas uda	Egl		
	Clostridium thermocellum	CelA	N	>384
	Erwinia chrysanthemi	CelY		

Table 1.3-continued

Family	Organism	Enzyme	Catalytic domain[a] Terminus	No. of amino acids
E				
	Butyrivibrio fibrisolvens H17c	CedI		
	Cellulomonas fimi	CenB	N	607
	Cellulomonas fimi	CenC	Internal	589
	Clostridium thermocellum	CelD	N	>543
	Clostridium stercorarium	CelZ	N	474
	Dictyostelium discoideum	SGSP270	N	~450
	Persea americana	Egl		
	Persea americana	CelI		
	Persea americana	Cel2		
	Pseudomanas fluorescens subsp. *cellulosa*	Egl		
F				
	Bacillus sp. strain C-125	XynA		
	Butyrivibrio fibrisolvens	XynA	N	~350
	Caldocellum saccharolyticum	CelB[b]	N	347
	Caldocellum saccharolyticum	XynA		
	Caldocellum saccharolyticum	ORF4		
	Cellulomonas fimi	Cex	N	315
	Clostridium thermocellum	XynZ	C	~350
	Cryptococcus albidus	Xyn		
	Pseudomonas fluorescens subsp. *cellulosa*	XynA	C	345
	Pseudomonas fluorescens subsp. *cellulosa*	XynB	C	272
	Thermoascus aurantiacus	Xyn		
G				
	Bacillus circulans	Xyn		
	Bacillus pumilus	XynA		
	Bacillus subtilis	Xyn		
	Clostridium acetobutylicum	XynB		
H				
	Aspergillus aculeatus	Egl		
	Erwinia carotovora	CelS		
I				
	Ruminococcus flavefaciens	CelA		

[a] Deduced from positions of putative linkers, sequence and truncation experiment.
[b] Catalytic domain 1 (family F, no.3) and domain 2 (family A, no.12) of the bifunctional cellulase of *Caldocellum saccharolyticum*.

Enzymatic hydrolysis of cellulosic substrates presumably proceeds by general acid catalysis promoted by carboxylic acid residues (Asp, Glu) and involves a carbonium ion

intermediate, which is stabilized either by a carboxylate group or by a histidine residue (Evelebil and Sternberg, 1988). The active site modification studies of CBHI of *T. reesei* have shown that Asp (Glu) groups in the 115-130 region are implicated and could be functionally important (Tomme and Claeyssens, 1989). The active site of *T. reesei* CBHII is located at the carboxy-terminal end of a parallel b barrel, in an enclosed tunnel through which the cellulose threads (Rouvinen *et al.*, 1990). There are two aspartic acid residues (Asp^{175} and Asp^{221}) located in the center of the tunnel. The Asp^{175}-Ala^{175} mutant shows only ca. 20% of wild type activity and the Asp^{221}-Ala^{221} mutant has no measurable activity (Rouvinen *et al.*, 1990).

Since active and/or substrate binding sites are often highly conserved, Henrissat *et al.* (1989) have listed the conserved Asp, Glu and His residues in the homologous regions of ten sequences of family A. These data provide useful information in identification of potential calalytic residues. Several of the major families contain enzymes with substrate specificities indicating that such enzymes share similar overall structure and active residues (Beguin, 1990). Therefore the variety of enzyme specificities required for the synergistic hydrolysis of cellulose may be due to subtle changes within similar sequences and not necessarily due to totally different enzymes (Beguin, 1990).

Non-catalytic domains. Many cellulases contain conserved sequence elements in addition to their catalytic core domains and signal sequences for protein secretion. The

non-catalytic domains are commonly connected to the catalytic domains by protease-susceptible hinges, rich in proline and hydroxyamino acids (Ong *et al.*, 1989). The size of linking sequences varies considerably from 6 to 59 amino acids (Gilkes *et al.*, 1991). Some of them are relatively rich in arginine, glutamine, glutamate, and hydrophobic amino acids (Gilkes *et al.*, 1991). These kinds of linkers are similar to Q-linkers, a recently proposed class of interdomain linkers found in a number of bacterial regulatory and sensory transduction proteins (Wootton and Drummond, 1989).

Shuffling of conserved catalytic and non-catalytic domains has been observed within different cellulase families (Warren *et al.*, 1986; Knowles *et al.*, 1987; Yablonsky *et al.*, 1989; Schenfens and Wong, 1987). The conserved, non-catalytic domains have been shown to be involved in the binding of cellulolytic enzymes to crystalline cellulose (Ong *et al.*, 1989). Recently, Beguin (1990) identified four types of conserved, non-catalytic domains among the cloned cellulases and xylanases (Table 1.4).

The *C. fimi* type non-catalytic domain was identified as a substrate binding domain and has been extensively studied. CenA and Cex from *C. fimi* bind tightly to cellulose (Gilkes *et al.*, 1984; Langsford *et al.*, 1984). Comparison of the predicted amino acid sequences showed that both enzymes contain a conserved Pro-Thr(PT) box and a non-catalytic binding domain of approximately 100 amino acid residues (Miller *et al.*, 1988; O'Neill *et al.*, 1986; Wong *et al.*,

1986b). The CenA binding domain is located at the NH_2 terminus, while the Cex binding domain is positioned at the COOH terminus (Warren *et al.*, 1987a). The unglycosylated versions of CenA and Cex produced by recombinant *E. coli* clones were cleaved by a *C. fimi* extracellular serine protease at the COOH end of the PT-box, and this prevented binding of the cellulase to the cellulose substrate. The generated catalytic domains retained activity on soluble substrates, but showed reduced binding to cellulose (Gilkes *et al.*, 1988). Furthermore, distinct fragments devoid of cellulase activity but capable of binding independently to cellulose have been observed. These binding domains offer interesting prospects for the construction of fusion proteins. The fusion of the CenA binding domain to the alkaline phosphatase protein (Greenwood *et al.*, 1989) and the Cex binding domain to a β-glucosidase enzyme of an *Agrobacterium* sp. (Ong *et al.*, 1989) illustrated the use of the binding domains to immobilize an enzyme on an inexpensive inert support, such as cellulose, or to use them for affinity purification of a protein.

The predicted amino acid sequence of *T. reesei* CBHI, CBHII, EGI, and EGII displayed no overall homology, either with one another or with enzymes from other organisms (Knowles *et al.*, 1988; Paice *et al.*, 1984). However, a 36-amino acid region of high homology occurs in all four enzymes. This domain has no catalytic activity in CBHI and CBHII, but has some cellulose binding activity (Van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988; Johansson *et al.*, 1989). It is a compact, wedge-shaped molecule whose structure has been

determined by two-dimensional nuclear magnetic resonance (Kraulis *et al.*, 1989). These enzymes contain a similar non-catalytic domain linked to the enzymatically active core protein by a polypeptide rich in serine and threonine residues (Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986). Proteolytic cleavage produces a catalytic domain that has full activity on small synthetic substrates, but only partial activity to natural solid cellulose (Tomme *et al.*, 1988).

Table 1.4 Conserved, non-catalytic domains of cellulase and xylanase enzyme (After Beguin, 1990).

Domain	Function	Organism	Position in the protein
T. reesei	Substrate binding	*T. reesei* CBHI	COOH end
		T. reesei CBHII	NH_2 end
		T. reesei EGI	COOH end
		T. reesei EGIII	NH_2 end
		P. chrysosporium CBHI	COOH end
C. fimi	Substrate binding	*C. fimi* EXO	COOH end
		C. fimi EGAN	H_2 end
		M. bispora EGA	NH_2 end
		B. fibrisolvens EG	COOH end
		P. fluorescens EGA	COOH end
		P. fluorescens EGB	NH_2 end
		P. fluorescens XYNA	COOH end
C. thermocellum	Substrate binding?	*C. thermocellum* EGA	COOH end
		C. thermocellum EGB	COOH end
		C. thermocellum EGD	COOH end
		C. thermocellum EGE	Middle
		C. thermocellum EGH	COOH end
	Binding to cellulosome?	*C. thermocellum* XYNH	Middle
		C. thermocellum EGA	COOH end
B. subtilis	Secretion?	*B. subtilis* EG	COOH end
		B. circulans EGI	COOH end
		C. saccharolyticum EGB	Middle

The *C. thermocellum* type non-catalytic domains have been described in Chapter 1.1.2.3. This model reveals the fact

that all *C. thermocellum* cellulases which contain the conserved segments are associated with the cellulosome (Beguin, 1990). The first 12 residues of the conserved segments bear significant resemblance to the Ca^{2+} binding sites (Kretsinger, 1980) of various Ca^{2+} binding proteins and led to the question whether these segments are perphaps involved in Ca^{2+} binding. However, Chauvaux *et al.* (1990) showed that the removal of the conserved segments did not affect the binding of *C. thermocellum* EGD to Ca^{2+}.

The cellulose-binding fragment of Avicelase I of *C. stercorarium* corresponds to the C-terminal half of the protein and comprises two types of duplicated non-catalytic domain (Jauris *et al.*, 1990): the central region is composed of the closely conserved domains B and B', which are flanked by the more distantly related domains C' and C. In addition, single copies of domain C are present in endoglucanases from *B. subtilis* and *C. saccharolyticum* (Jauris *et al.*, 1990). The position of non-catalytic domains were found at the COOH terminus of the *B. subtilis* EG (Mackay *et al.*, 1986), the *B. circulans* EG1 (ref in Beguin, 1990), and between the two catalytic domains of the *C. saccharolyticum* EGB (Saul *et al.*, 1989). The function of these domains has not been elucidated. However, proteolytic removal of the non-catalytic domain of *B. circulans* EGI enzyme resulted in a marked reduction in exported EGI enzyme (ref. in Beguin, 1990). As suggested by Beguin, this domain may be involved in secretion of the cellulase enzyme (Table 1.4).

1.1.3.3 Induction of cellulase and xylanase. The mechanism by which cellulosic materials trigger cellulase formation is not well understood, although evidence is now available that an initial attack on the cellulose molecule is carried out by low constitutive levels of cell surface bound cellulases (Kubicek, 1987; Kubicek *et al.*, 1988; El-Gogary *et al.*, 1989). Cellobiose has been proposed as the natural inducer of cellulase activity (Mandels *et al.*, 1971). However, although at low concentrations, it is a good inducer, at high concentrations, cellobiose inhibits cellulase synthesis (Gong and Tsao, 1979b). The response of organisms to cellobiose concentration varies from strain to strain. In *Sporotrichum pulverulentum*, the induction of endocellulase is less sensitive to the effects of cellobiose concentration than other fungi (Eriksson and Hamp, 1978). In some cases, cellobiose even failed to induce the synthesis of cellulase in *Trichoderma* spp. (Royer and Nakas, 1990; Kawamori *et al.*, 1986; Nisizawa *et al.*, 1971; Sternberg and Mandels, 1979).

It has been proposed that the actual inducer of cellulase in *Trichoderma* spp. is not cellobiose but an enzymatically modified product of cellulose or cellobiose (El-Gogary *et al.*, 1989; Nisizawa, 1971). Sophorose has been considered the actual *in vivo* inducer for a long time (Kubicek, 1987; Mandels *et al.*, 1962; Sternberg and Mandels, 1979). However, sophorose induces only an incomplete array of cellulases (Sternberg and Mandels, 1979; Messner *et al.*, 1988) and hence further inducers might exist. Induction of cellulases in *T. reesei* by cellobiose-1, 5-lactone has been proposed (Iyayi *et al.*, 1989).

Kubicek-Pranz *et al.* (1990) reported a low degree of oxidation of cellulose molecules had a stimulatory effect on cellulase formation by *T. reesei*, whereas a higher degree became inhibitory. However, the *in vivo* role of the oxidized compound is unclear. A report by Vaheri (1988) showed that enzymatic cellulose hydrolysis led to the formation of acidic degradation products of cellulose. This provided evidence for the involvement of an oxidative cell-wall bound enzyme in cellulose breakdown (Vaheri, 1983). An extracellular enzyme which utilizes molecular oxygen to oxidize cellodextrins to the corresponding aldonic acids has been isolated from culture filtrates of the white-rot fungus *S. pulverulentum* (Ayers *et al.*, 1978). This enzyme, termed cellobiose oxidase, shows a high activity towards all of the cellodextrins tested (cellobiose through cellohexaose) (Ayers *et al.*, 1978). These facts indicate that oxidation processes are significant in cellulose degradation.

Lactose induces both xylanase and cellulase activity of *Trichoderma longibrachiatum* after a lag time of several hours (Royer and Nakas, 1990). A slow rate of induction of *T. reesei* cellulase activity by lactose has also been observed (Sternberg and Mandels, 1979). Lactose has been referred to as an inducer of cellulase (Gong *et al.*, 1979a) and as a nonrepressive carbon source (Biely *et al.*, 1980).

Xylobiose and xylan are shown to induce specific xylanases of *T. reesei* (Hrmova *et al.*, 1986), and mixtures of xylobiose with cellobiose or cellopentaose rapidly induced cellulases and xylanases activity (Royer and Nakas, 1990).

Induction of cellulase by combinations of xylobiose with cellulose oligomers is difficult to rule out since none of these substrates was an effective single inducer (Royer and Nakas, 1990). A further complication is that xylobiose-induced xylanases may be capable of modifying cellulose oligomers to form inducers of cellulase activity. Indeed a specific xylanase of *C. albidus* is able to transfer a cellobiose unit to a xylosyl acceptor (Biely and Vrsanska, 1983). These data suggest that the regulation of xylanase and cellulase activity may be closely linked.

Studies by Howard *et al.* (1960) with *Butyrivibrio* species indicated that both xylanase and xylobiase activities are expressed when cells are grown on xylan or wheat flour pentosan. The xylanase activity of *B. fibrisolvens* AcTF2 is induced by xylan, whereas the xylobiase activity appears to be constitutively produced (Hespell *et al.*, 1987). However, an enzyme pattern inverse to that of strain AcTF2 is observed with strains 49 and H17c (Hespell *et al.*, 1987). Carbon source-dependent induction has also been reported in *Ruminococcus* spp. (Fusee and Leatherwood, 1972; Smith *et al.*, 1973). In contrast, the cellulase of three rumen bacteria (Hiltener and Dehority, 1983), *R. flavefaciens* (Pettipher and Latham, 1979) and *Fibrobacter succinogenes* subsp. *succinogenes* S85 (Mcgavin *et al.*, 1990; Huang and Forsberg, 1990) appear to be constitutive in nature. In *Streptomyces*, the biosynthesis of activities for hydrolysis of cellulose and xylan is inducible and controlled separately (Kuepfel and Ishaque, 1983; MacKenzie *et al.*,

1987). As the above finding illustrates, induction process of cellulases and xylanases are extremely complex.

1.1.3.4 Substrate preference. The preferential utilization of one substrate to the exclusion of another was first reported for microorganisms (Epps and Gale, 1942). Monod (1947) later studied this phenomenon and referred to it as the glucose effect. Magasanik (1961) proposed the term catabolite repression to describe the mechanism when it was known that substrates other than glucose could produce the same effect. The discovery of cyclic AMP in microorganisms by Markman and Sutherland (1965) led to the elucidation of cyclic AMP-dependent catabolite repression (Perlman and Paston, 1968; Zubay *et al.*, 1970). However, observations by some workers showed that cyclic AMP was absent in some bacteria which carried out sequential substrate utilization (Bernlov *et al.*, 1974; Hasan and Durr, 1974). Two additional mechanisms were proposed to explain sequential utilization not explained by catabolite repression. The first of these is catabolite inhibition in which the sequential utilization is mediated by an interference of the preferred substrate with the enzyme system responsible for the utilization of the other substrates (McGinnis and Paigen, 1973; Standing *et al.*, 1972; Thompson *et al.*, 1978). The second proposed mechanism, inducer exclusion, functions by exclusion of the inducer substrate at the cell membrane , so that induction of the specific catabolic enzyme system is prevented. The detailed mechanism of inducer exclusion is not yet known, although the studies of Saiser and Roseman (1976) suggest it is phosphotransferase mediated.

In most cellulolytic microorganisms examined, cellobiose is cleaved into glucose by cellobiase (Haliwell, 1979). However in others such as *Cellvibrio gilvus* (Swisher *et al.*, 1964). *R. flavefaciens* (Ayers, 1959; Ayers, 1958; Helaszek and White, 1991), *C. thermocellum* (Alexander, 1961), and *Cellulomonas* (Schimz *et al.*, 1983), the cellobiose is converted into glucose-1-phosphate and glucose by cellobiose phosphorylase. This enzyme results in conservation of the β-1,4 bond energy during activation of glucose with inorganic phosphate (Huttermann and Volger, 1973). Cellulolytic species that contain cellobiose phosphorylase prefer cellobiose to glucose as an energy source for growth (Ng and Zeikus, 1982; Halaszek and White, 1991; Herlcher and King, 1958; Hungate, 1950; Ng *et al.*, 1977; Sijpesteijn, 1951; Sato and Takhashi, 1967; Huttermann, 1973). However, cellobiose is utilized in preference to glucose by *T. curvata* with no evidence of cellobiose phosphorylase involvement (Bernier and Stutzenberger, 1987). The ruminal bacterium *Streptococcus bovis* has higher affinities for sucrose and maltose than for glucose or cellobiose (Russell and Baldwin, 1979), but glucose and sucrose are used in preference to maltose and cellobiose (Russell and Baldwin, 1978). In *B. fibrisolvens*, the addition of maltose and sucrose causes a decrease in the rate of cellobiose utilization (Russell and Baldwin, 1978), suggesting preferenial use of maltose and sucrose as carbon source. *Selenomonas ruminantium* also shows high affinities for glucose, maltose, sucrose, and xylose (Russell and Baldwin, 1979), but glucose, sucrose and xylose are used in preference to maltose (Russell and Baldwin, 1978).

1.1.3.5 Substrate uptake. Uptake is often a key characteristic which determines the success of bacteria in natural environments (Matin and Veldkamp, 1978). Bacteria usually take the advantage of using different approaches to taking up sugars. *C. thermocellum* and *C. thermohydrosulfuricum* transport glucose by glucose permease and ATP rather than a phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) (Ng and Zeikus, 1982), and *B. succinogenes* contains a highly specific active transport system for glucose (Franklund and Glass, 1987). However some ruminal bacteria favour the PEP-PTS for glucose because the system mediates the simultaneous transport and phosphorylation of a sugar without expending additional ATP in a kinase reaction. *S. ruminantium* possesses a PEP-PTS for glucose (Martin and Russell, 1988), while xylose uptake is inducible and involves a high energy phosphate compound and possibly a binding protein (Williams and Martin, 1990). Another ruminal bacteria, *S. bovis*, also contains a constitutive PEP-PTS transport for glucose (Martin and Russell, 1987).

Cellobiose transport has been studied with *C. thermocellum* (Ng and Zeikus, 1982), *Erwinia carotovora* (Barras *et al.*, 1984). *R. flavefaciens* (Helaszek and White, 1991), *S. bovis* (Martin and Russell, 1987), *Streptomyces granaticolor* (Jiresova *et al.*, 1987) and *Trichoderma curvata* (Bernier and Stutzenberger, 1987). Evidence was found for the involvement of both PEP-PTS-dependent transport (Martin and Russell, 1987) and PEP-PTS-independent transport of cellobiose (Ng and Zeikus, 1982; Barras *et al.*, 1984).

Kinetic investigations of cellobiose transport have been done with *R. flavefaciens* (Helaszek and White, 1991) and *S. granaticolor* (Jiresova *et al.*, 1987), and these results indicated active transport of cellobiose.

1.1.3.6 Effects of sugars on cellulase activity. The expression of extracellular β-glucosidases by *P. chrysosporium* (Smith and Gold, 1979), *T. viride* (Berg and Pettersson, 1977; Sternberg, 1976), *Schizophyllum commune* (Wilson and Niederpruem, 1967) and a *Monilia* sp. (Dekker, 1981) have been shown to be repressed by glucose. In some yeasts, β-glucosidase is produced constitutively in a synthetic medium without glucose (Herman and Halvorson, 1963; MacQuillan and Halvorson, 1962; MacQuillan *et al.*, 1960), the rate of β-glucosidase synthesis is significantly increased at low concentrations of glucose, but repression occurred at higher concentrations. However, the expression of β-glucosidase from *Candida wickerhamii* is repressed by glucose and derepressed by anaerobiosis (Freer and Detroy, 1985).

Cellobiose has been reported to cause repression of cellulase production in *C. thermocellum (Johnson et al.*, 1985), *S. pulverulentum* (Despande *et al.*, 1978) and *B. cellulosolvens* (Murray, 1987). In addition, the cellulase system of *B. cellulosolvens* is subject to feedback inhibition by cellobiose, while glucose has no significant effect on either cellulase production or activity (Murray, 1987). The cellulase of *A. cellulolyticus* is not subject to end product inhibition by either glucose or cellobiose

(Mackenzie and Bilous, 1982), whereas the cellulase of *S. flavogriseus* was inhibited by cellobiose but not by glucose (Mackenzie *et al.*, 1984). In contrast to various responses described for other bacteria, both glucose and cellobiose have been reported to cause inhibition of cellulase of *R. albus* (Smith and Hungate, 1973).

The precise mechanism by which sugars inhibit cellulase synthesis is not known. They could inhibit by affecting the transcription of cellulase mRNA. In *C. fimi*, the transcription of *cen*A, *cen*C, and *cen*X is CMC-inducible, while constitutive transcription in the presence of glycerol and glucose is only observed for *cen*B (Greenberg *et al.*, 1987a; 1987b; Moser *et al.*, 1989). Cellulase synthesis in *Thermomonospora fusca* and *T. curvata* is subject to dual control by induction and growth rate-dependent repression by readily metabolizable carbon sources. The latter could be alleviated by addition of cyclic AMP (Lin and Wilson, 1987; Wood *et al.*, 1984). Four endoglucanase genes of *C. thermocellum* have also been shown to be regulated at the transcriptional level (Mishra *et al.*, 1991). Furthermore, Teeri *et al.* (1983) isolated the genes of two cellobiohydrolases and two endoglucanases by a differential cDNA hybridization screening approach with cDNA of induced and repressed *T. reesei* mycelia. Hence, these genes should be controlled mainly at the transcriptional level. A fact also reported for carbon source control of cellobiohydrolase I and II formation by *T. reesei* (Messner and Kubicek, 1991). In contrast, Nisizawa *et al.* (1972) reported that carbon

catabolite control of cellulase synthesis takes place mainly at the translational level.

1.1.3.7 The existence of gene clusters. Howard and White (1988) reported the detection of multiple enzymatic activities in a single clone from *R. albus*. Huang *et al.* (1989a) also found a similar result with a bacteriophage clone encoding CMCase, MUC^+, and β-glucosidase activities. Subcloning data showed the cellulase genes might be expressed as a unit under a type of operon control (Huang *et al.*, 1989). A study with *B. ovatus* has reported the cloning and expression of xylanosidase and arabinosidase activities (Whitehead and Hespell, 1990). These activities were both encoded by DNA within a cluster of genes involved in hemicellulose degradation. The *C. saccharolyticum* genes coding for enzymes involved in degradation of xylan also exist as a cluster (Luthi *et al.*, 1990), and close linkage between xylanase and cellulase genes has been reported for *P. fluorescens* subsp. *cellulosa* (Gilbert *et al.*, 1988). Recently Luthi *et al.* (1991) found that the mannanase gene is located on the same genomic fragment as *cel*B (Saul *et al.*, 1990) and both genes were located on the same phage clone. The *B. fibrisolvens xyl*B gene was located between two incomplete ORFs within 4.2-kb region and these three genes are proposed to be part of a single operon (Utt *et al.*, 1991).

1.1.3.8 Adhesion of cellulolytic bacteria. Studies with *C. thermocellum* have shown that the cellulose-binding activity of cells and their ability to hydrolyze cellulose

are associated with a compact multi-enzyme complex on the cell surface, termed a cellulosome (Bayer and Lamed, 1986; Lamed *et al.*, 1986; Lamed *et al.*, 1983). The investigation of ultrastructural localization of the cellulosome showed the entire cell surface is covered with a multitude of polycellulosomal protuberant organelles (Bayer *et al.*, 1985). After binding of the bacterial cell to cellulose, some of the polycellulosomal protuberances are transformed to yield an amorphous or fibrous network which appears to connect the cellulose-bound cellulosomes to the cell surface (Lamed and Bayer, 1988). The *C. thermocellum* cellulosome comprises both enzymes and specific proteins which form a very stable structure, 18μm in diameter with a *Mr* of approximately 2×10^6 kDa (Lamed *et al.*, 1983). Similar cell surface structures are found in other cellulolytic bacteria, including *B. cellulosolvens*, *A. cellulolyticus* (Lamed *et al.*, 1987), and more recently, *R. flavefaciens* and *F. succinogenes* (Miron *et al.*, 1989). The occurrence of protuberant structures on the surfaces of ruminal cellulolytic bacteria appears to be a carbohydrate-dependent response (Miron *et al.*, 1989) and a general consequence for bacterial interaction with and degradation of cellulose (Lamed *et al.*, 1987).

In the rumen, fibrolytic microorganisms rapidly colonize plant particles after their ingestion (Akin, 1979; Latham *et al.*, 1978). *In vitro* evidence also shows that *R. flavefaciens* (Roger *et al.*, 1990) and *R. albus* (Morris and Cole, 1987) adhere instantly to cellulose. Adhesion of cellulolytic bacteria brings the cell into close contact

with its specific substrate and concentrates hydrolytic enzymes on cellulose. Some physicochemical factors do affect the adhesion of bacteria to cellulose. Several workers have reported that CMC has inhibitory effect on adhesion (Minato and Suto, 1978; Kudo *et al.*, 1987; Rasmussen *et al.*, 1989; Morris, 1988). Adhesion is also inhibited by the presence of methylcellulose (Morris, 1988; Roger *et al.*, 1990; Rasmussen *et al.*, 1989). White *et al.* (1988) and Rasmussen *et al.* (1989) have shown that methylcellulose has surfactant properties at high concentrations and suggested that hydrophobic interactions are involved in the adherence of *R. flavefaciens*. A number of researchers have implicated phenolic compounds and lignin as inhibitors of growth and cellulolysis by rumen bacteria (Borneman *et al.*, 1986; Chesson *et al.*, 1982; Varel and Jung, 1986). Richards (1976) suggested that lignified regions of the plant cell wall might prevent attachment of rumen microorganisms, and Varel and Jung (1986) found that phenolic compounds inhibited attachment of *B. succinogenes* to filter paper. Adhesion of bacteria is sensitive to temperature (Roger *et al.*, 1990; Gong and Forsberg, 1989) and pH (Roger *et al.*, 1990; Morris, 1988) changes. The adherence of *R. flavefaciens* FD-1 to cellulose is inhibited by formaldehyde (Rasmussen *et al.*, 1989). Furthermore, glutaraldehyde, trypsin and pronase treatments markedly reduce the extent of adhesion (Gong and Forsberg, 1989). However, the adhesion of *F. succinogenes* to crystalline cellulose is enhanced by increasing the ionic strength (Gong and Forsberg, 1989) and the attachment of *R. flavefaciens* to cellulose is affected by the removal of divalent cations

(Roger *et al.*, 1990). The addition of 3-phenylpropanoic acid was found to improve cellulose hydrolysis (Hungate and Stack, 1982; Veldkamp, 1976), alter cell morphology and result in production of cell bound, high molecular weight forms of cellulases (Stack and Hungate, 1984). 3-Phenylpropanoic acid concentration as high as 25mM does not stimulate the growth or rates of cellulose breakdown by *R. flavefaciens* and *B. fibrisolvens* (Stack and Cotta, 1986), whereas this concentration has been found to improve the affinity of *R. albus* for cellulose (Morrison *et al.*, 1990).

1.1.3.9 Enzyme secretion. As an obligatory part of protein secretion, most cellulolytic enzymes are initially synthesized with NH_2-terminal signal sequences ranging in size from 24 to 44 residues composed of a short positively charged NH_2 terminus, a central hydrophobic domain, and a signal peptide cleavage site (Fig. 1.1). However, the signal sequence alone is insufficient to direct extracellular export (Hirst and Welch, 1988). The involvement of additional factors specifically involved in extracellular export by Gram-negative bacteria is suggested by the observation that cellulolytic enzymes can be processed and exported across the inner membrane by *E. coli* into its periplasm but not outside the cell (Hall *et al.*, 1989; Robson and Chambliss, 1986; Din *et al.*, 1990).

In addition, D'Enfert *et al.* (1987; 1989) reported the cloning of genes from *Klebsiella pneumoniae* which allow extracellular export of pullulanase in *E. coli*. But in the absence of these genes, pullulanase is only exported across

the inner membrane. As proposed by Pugsley *et al.* (1990a; 1990b), pullulanase secretion involves at least two distinct steps, the first of which is carried out entirely by the *sec* gene-dependent general export pathway while the second requires the *pul* gene-encoded specific secretion factors. The third and final step in the secretion pathway is the spontaneous release of what appear to be pullulanase micelles from the cell surface.

```
  +          - *  -    *         +    + -    +
M R C L I V C E N L E M L N L S L A K T Y K D Y F K I G A A
```
*C. saccharolyticum xyn*A (Luthi *et al.*, 1990)

```
        + +                                      *
M I T L F R K P F V A G L A I S L L V G G G I G N V A A
```
Bacillus sp. strain C-125 *xyn*A (Hamamoto *et al.*, 1989)

```
  *   + +   +                                    +
M N L R K L R L L F V M C I G L T L I L T A V P A H A
```
*B. pumilus xyn*A (Fukusaki *et al.*, 1984)

```
    +   + + *
M F K F K K N F L V G L S A A L M S I S L F S A T A S A
```
B. subtilis xylanase gene (Paice *et al.*, 1986)

```
  +         +                          +
M R T A M A K S L G A A A F L G A A L F A H T L A A
```
P. fluorescens subsp. *cellulosa xyl*A (Hall *et al.*, 1989)

```
  + +   +   + +       +                          +   -
M K K F K I R K L M A R V L A L A L V F S T F F M V S K V D
  *
A N A
```
*B. fibrisolvens xyn*A (Mannarelli *et al.*, 1990)

```
  + + *     +       +
M K R N L F R I V S R V V L I A F I A S I S L V G A M S
```
*C. saccharolyticum cel*B (Saul *et al.*, 1989)

```
      + + * +
M V S K K Q K F L T V I L V I V L A I V I V G G V F G I S F
  +   +
V K G R
```
*B. fibrisolvens cel*A (Hazlewood *et al.*, 1990)

Fig 1.1 Signal peptides of cellulolytic and xylanolytic enzymes Positively changed amino acids are indicated by + above their single letters code; negatively changed amino acids are indicated by -; polar amino acids are marked by *.

```
  + + + +     +                                  -   *
M K K R R S S K V I L S L A I V V A L L A A V E P N A A L A
```
B. lantus celB (Jorgensen and Hansen, 1990)

```
  *     *   *   + +     +
M Q T V N T Q P H R I F R V L L P A V F S S L L L S S L T V
S A
```
E. carotovora celS (Saarilahti *et al.*, 1990)

```
  + +                                        +     +
M R K F Y S F A I I I S L L V T G L F I H T P K A E A A
```
C. stercorarium celZ (Jauris *et al.*, 1990)

```
  *   + +       +                                *
M N I K K T A V K S A L A V A A A A A A L T T N V S A
```
F. succinogenes 1,3-1,4-glucanase gene (Teather and Erfle, 1990)

```
      + +                                                *
M S T R R T A A A L L A A A A V A V G G L T A L T T T A A Q
A
```
C. fimi cenA (Wong *et al.*, 1986)

```
    + + + + +       +                          *
M M R R R K R S D M K S I S I F I T C L L I A N L T M G G L
L P S P A S A
```
B. subtilis DLG *egl* (Robson and Chambliss, 1987)

```
        +
M S I F R T A S T L A L A T A L A L A A G P A F S
```
X. campestris pv. *campestris eng* (Gough *et al.*, 1990)

```
  + + +                                    *       *   *
M K K R L L V S F L V L S I I V G L L S F Q S L G N Y N S G
  +                 *
L K I G A W V G T Q P S E S
```
C. thermocellum celH (Yague *et al.*, 1990)

Fig 1.1-continued

Recently, Huang and Schell (1990) reported that extracellular export of the endoglucanase (EGL) of *Pseudomonas solanacearum* occurs by a two-step process. The primary precursor EGL has a 45 residue leader sequence but only the first 19 residues of the leader sequence are removed by signal peptidase (Huang *et al.*, 1989). The resultant intermediate precursor is a transient fatty acylated lipoprotein and is located on the periplasmic side

of the inner membrane of *P. solanacearum* (Huang and Schell, 1990). Although *E. coli* could synthesize primary precursor EGL, modify it with palmitate, and removed the first 19 residues of the leader sequence during export across the inner membrane, only *P. solanacearum* could export the intermediate precursor across the outer membrane and remove the remaining 26 residues of the leader sequence producing the native, extracellular EGL (Huang and Schell, 1990).

1.2 Genetic manipulation of rumen bacteria. Plasmids are commonly found in naturally occurring bacterial populations. The functions they encode frequently confer specific competitive advantage upon the host organism. The special features of the rumen environment, such as rapid turnover, high population density and varied input of organic materials, might be expected to promote the development of plasmid-encoded functions (Hazlewood and Teather, 1988).

1.2.1 Plasmids in rumen bacteria. The first report of plasmids in rumen bacteria was made by Teather (1982). Supercoiled plasmid DNA was isolated from a type strain of *B. fibrisolvens* (ATCC 19177). Analysis of the plasmid DNA by sucrose density gradient centrifugation indicated a *Mr* of 250 megadaltons (Teather, 1982). However, no functions were assigned to this large plasmid. Subsequent studies have demonstrated smaller plasmids in a number of strains of *Butyrivibrio*. Mann *et al.* (1985; 1986) described the isolation and restriction mapping of a 2.8kb cryptic plasmid (pOM1) from *B. fibrisolvens* strain 49. With the object of producing a shuttle vector capable of replication in *B.*

fibrisolvens and *E. coli*, a series of hybrids were made, and stably maintained in *E. coli*, but no evidence is available to suggest that these plasmids can be transformed into *Butyrivibrio* spp. A comprehensive survey (Orpin *et al.*, 1986b) was conducted to evaluate the distribution of plasmids in 150 strains of *B. fibrisolvens* isolated on a non-selective habitat-simulating medium from different ruminants. Up to 28% of the isolates contained plasmids, of which the majority contained 2-4 plasmid bands ranging from 1.8 kb to about 40 kb in size. A similar study was demonstrated with 157 strains of *S. ruminantium* isolated from varied ruminants (Orpin *et. al.*, 1986b). The majority of tested strains contained plasmid DNA in the size range of 1 kb to greater than 50 kb with as many as 10 bands of plasmid DNA. The genus *Ruminococcus* has also been examined for the presence of plasmids. The small cryptic plasmid in 6 out of 7 strains of *R. albus* and in all 3 strains of *R. flavefaciens* was isolated by using mutanolysin to effect cell lysis (Kelly and Asmundson, 1986; Asmundson and Kelly, 1987).

1.2.2 Gene transter in rumen bacteria. To date the knowledge of *in vivo* transfer of genetic material in the rumen is still very limited. However many researchers have paid attention to developing vectors and DNA transfer mechanisms for the application of recombinant DNA techniques to this group of microbes.

The interspecies DNA transfer from *E. coli* to *B. fibrisolvens* was achieved by using the broad host-range

conjugative plasmid RP4 (Teather, 1985). PEG-induced uptake of DNA by sphaeroplasts prepared from *B. fibrisolvens* has also been reported, but the method was extremely inefficient due to poor regeneration frequencies and the transformants were unstable (Teather, 1985; Hazlewood and Teather, 1988). Transformation has been investigated in *S. ruminantium* (Orpin *et al.*, 1986a) and the development of a sphaeroplast-based transformation system was also demonstrated (Kamio and Takahashi, 1980). However gene transfer between *Selenomonas* and other organisms by transduction and conjugation is relatively poor. The development of protoplast transformation systems has also been described in *B. ruminicola* (Cheng, 1973) and *Bifidobacterium* (Brigidi *et al.*, 1986).

Various bacteriophages isolated from rumen bacteria may have potential for transferring genes both *in vivo* and *in vitro*. Phages have been reported in *Bacteroides longum* (Sgorbati *et al.*, 1983), while 2% of *S. ruminantium* isolates were shown to be lysogenic when treated with mitomycin C (Hazlewood *et al.*, 1983b). Lysogenic phages have also been described in *S. bovis* (Iverson and Millis, 1976a; 1976b; Tarakanov, 1976). However phage transduction in rumen bacteria has not been demonstrated (Hazlewood and Teather, 1988).

1.3 Importance of *B. fibrisolvens* in rumen. *Butyrivibrio* are common inhabitants of not only the bovine rumen, but are probably present in the rumen of all ruminants. They have been isolated from the rumen of cattle, sheep, Zebu cattle, goats, Alaskan reindeer, Svalbard reindeer, and bison

(Bryant and Small, 1956; Dehority, 1966; Dehority and Grubb, 1976; Margherita and Hungate, 1963; Orpin *et al.*, 1985; Varel and Dehority, 1989), as well as from the hindgut of sheep (Orskov *et al.*, 1970; Mann and Orskov, 1973; Lewis and Dehority, 1985) and from pig caecum (Robinson *et al.*, 1981). The production of large amounts of n-butyric acid from the fermentation of carbohydrates is the major fermentative characteristic that can be used in the identification of the genus *Butyrivibrio* (Hespell and Bryant, 1981). The "classic" appearance of *Butyrivibrio* is a small, motile rod (2-5μm in length and 0.4-0.6μm in diameter) with tapered ends, found singly, in pairs and in chains (Stewart and Bryant, 1988). Motility is by means of single polar or subpolar flagellum (Hespell and Bryant, 1981). *Butyrivibrio* strains have been classified as Gram-negative cells due to their staining by conventional procedures (Shane *et al.*, 1969; Bryant, 1959; Hungate, 1966). However Hewett *et al.* (1976) and Sharpe *et al.* (1975) have isolated from *B. fibrisolvens* the typical components of Gram-positive bacterial cell walls, lipoteichoic acid and glycerol teichoic acid. Subsequently, electron microscopic studies have revealed a Gram-positive morphological type of *B. fibrisolvens* cell wall (Cheng and Costerton, 1977), but the peptidoglycan layer is rather thin (12-18nm) when compared with that of most Gram-positive bacteria (30 to 50 nm). Thus *Butyrivibrio* strains are structurally Gram-positive bacteria, but due to their thin cell wall they stain in a Gram-negative pattern (Hespell and Bryant, 1981). Furthermore, a study of the fatty acid and fatty aldehyde composition of *Butyrivibrio* strains show all of the isolates

lacked hydroxy fatty acids, supporting the view that they are physiologically Gram-positive organisms (Miyagawa, 1982). The pattern of antibiotic sensitivity of *Butyrivibrio* also suggests that this bacterium is physiologically Gram-positive (Stewart and Bryant, 1988).

B. fibrisolvens isolates ferment a wide range of sugars with considerable variation from strain to strain (Bryant and Small, 1956; Hungate, 1966). Shane *et al.* (1969) have placed 19 *Butyrivibrio* isolates into two groups (acetate - utilizers and producers) based on their fermentative patterns. These two groups can be differentiated by their growth responses to added volatile fatty acids (Roche *et al.*, 1973). A later study by Van der Toorn and Van Gylswyk (1985) also found strains of these two biotypes and a third biotype represented by propionate-producing isolates. Apart from the variation in fermentation patterns, *B. fibrisolvens* show substantial variations in antigenic properties and in their content of lipoteichoic acids (Bryant, 1984). DNA hybridization and G + C base composition were used to examine 39 *B. fibrisolvens* strains for DNA relatedness (Mannarelli, 1988). *B. fibrisolvens* strains H17c and 49 were shown to be 96% related and the G + C content was 42% for both strains. The DNA hybridization results indicate that the strains presently classified in the species *B. fibrisolvens* actually comprise a number of distinct species and possibly several genera (Mannarelli, 1988).

1.3.1 Lipolytic activity. Lipids in the ruminant diet are derived from forage crops and from supplements added to

the diet in the form of crushed cereal grains. The lipids of forages constitute about 6-7% of the dry weight of leaf tissue and consist largely of glycolipids and phospholipids (Shorland, 1963). Rumen bacteria, especially *B. fibrisolvens*, with potential for degrading different lipid components of the diet have been isolated from rumen contents (Harfoot and Hazlewood, 1988; Latham *et al.*, 1972; Hazlewood and Dawson, 1975; 1977; 1979). The initial step in the transformation of dietary acyl lipids entering the rumen is the hydrolysis of ester linkages by microbial lipolytic enzymes. This step is essential for the biohydrogenation of unsaturated fatty acids (Harfoot and Hazlewood, 1988). *B. fibrisolvens* is a bacterial species known to be able to biohydrogenate the unsaturated fatty acids (Polan *et al.*, 1964; Kepler *et al.*, 1966; Kepler and Tove, 1967). Kemp and Lander (1984) divided the hydrogenating bacteria into two groups (A and B) based on the pattern of end product of hydrogenation and on the isomerization carried out. *B. fibrisolvens* belongs to group A which hydrogenates linoleic and α-linoleic acids to trans-11-octadecenoic acid (Kemp *et al.*, 1975). Members of group B are capable of hydrogenating a wide range of octadecenoic acids, including cis-9 (oleic) and trans-11 (trans-vaccenic) acids, as well as linoleic acid to stearic acid. However, the complete hydrogenation of α-linoleic and linoleic acids can take place only in the presence of members of both group A and group B (Harfoot and Hazlewood, 1988).

1.3.2 Proteolytic activity. Between 30% and 50% of the bacterial isolates from rumen fluid have proteolytic activity towards extracellular protein (Prins *et al.*, 1983). Among the saccharolytic rumen bacteria *B. amylophilus*, *B. ruminicola* and *B. fibrisolvens* are found to be the major proteolytic species (Wallace and Cotta, 1988). *B. fibrisolvens* can be the predominant proteolytic organism isolated from some animals (Blackburn and Hobson, 1962; Fulghum and Moore, 1963; Hazlewood *et al.*, 1983a; Wallace *et al.*, 1987), and is probably enriched when more resistant types of protein are present in the diet (Wallace *et al.*, 1987). *B. fibrisolvens* can grow in a medium containing protein as the sole source of nitrogen (Wallace and Brammall, 1985; Cotta and Hespell, 1986). High concentrations (20 gl^{-1}) of amino acids or peptides in the medium severely repressed activity, while amino acids at 1 gl^{-1} caused threefold stimulation relative to that expressed with casein (Cotta and Hespell, 1986). Fractionation of supernatant fluids showed that most proteolytic activity is extracellular (Wallace and Brammall, 1985; Cotta and Hespell, 1986). Experiments with proteinase inhibitors showed predominantly serine-protease activity in most strains of *B. fibrisolvens* (Wallace and Brammall, 1985; Cotta and Hespell, 1986).

1.3.3 Amylolytic activity. A number of ruminal bacteria, including *B. fibrisolvens*, exhibit the ability to utilize starch as a growth substrate (Russell and Hespell, 1981; Marounek and Bartos, 1986). Of the amylolytic activities in the ruminal microflora of the high arctic

Svalbard reindeer, the dominant starch-fermenting bacteria during winter is *B. fibrisolvens* (Orpin *et al.*, 1985). As reported by Cotta (1988), the amylolytic activities of 10 selected ruminal bacteria appear to be regulated. For *B. fibrisolvens* 49 and A38, amylase was produced in medium containing maltose or starch as the sole carbon source, but the activity was greatly reduced in glucose-grown cultures (Cotta, 1988). It is interesting to note that the distribution of amylolytic activity was affected by the carbohydrate provided for growth. In starch-medium, the amylase activity of *B. fibrisolvens* 49 was largely associated with cell pellets. However, most of the activity was found in the extracellular fluid fraction of maltose containing medium (Cotta, 1988). Digestion of amylase by extracellular amylolytic activities produced by *B. fibrisolvens* 49 and A38 resulted in significant accumulation of maltotriose (Cotta, 1988). This saccharifying-type activity of amylase has also been described for an α-amylase from *B. fibrisolvens* H17c (Rumbak *et al.*, 1991) and for a few α-amylases from some ruminal bacteria (Walker and Hope, 1964).

1.3.4 Cellulolytic and xylanolytic activity. *B. fibrisolvens* has been shown to ferment cellodextrins (Russell, 1985), and to produce a range of polysaccharide depolymerases and glycoside hydrolases (Howard *et al.*, 1960; Williams and Withers, 1982a; 1982b; Wojciechowicz *et al.*, 1982; Williams *et al.*, 1984; Heinrichova *et al.*, 1985). However, only a few *B. fibrisolvens* have been identified as cellulolytic strains (Dohority, 1966; Hobson and Wallace,

1982). In the rumen of high-arctic Svalbard reindeer, *B. fibrisolvens* was the major cellulolytic bacteria, which represented 66 and 52% of the cellulolytic polulation in summer and winter, respectively (Orpin *et al.*, 1985). Margherita and Hungate (1963) found that *B. fibrisolvens* was the most abundant cellulolytic bacterium in semi-starved zebu cattle in Kenya. This suggests *B. fibrisolvens* may be more resilient than other cellulolytic rumen bacteria, and therefore more able to survive the cycles of nutritional abundance and starvation of the host animal than other cellulolytic species (Orpin *et al.*, 1985).

B. fibrisolvens is the most important rumen bacterium involved in digestion of hemicellulose. Hemicellulose in plant cell wall is more extensively solubilized by *B. fibrisolvens* than is cellulose (Dehority and Scott, 1967; Morris and Van Gylswyk, 1980). Van der Linden *et al.*, (1984) observed no significant changes in the concentration of xylanolytic bacteria in sheep fed on diets containing from 0 to 39% grain. However, *B. fibrisolvens* - like rods were the major hemicellulolytic organisms. *B. fibrisolvens* strains are able to grow on media containing xylan as the only added carbohydrate source (Hobson and Purdom, 1961) and xylanase activity was predominantly extracellular and xylobiase activity mostly associated with the cells (Hespell *et al.*, 1987).

Aim of this thesis. *B. fibrisolvens* H17c is an important rumen bacterium, which is very versatile in its ability to degrade different polysaccharides such as cellulose, xylan,

and starch. A few studies have been done on the physiological assays of crude cellulolytic and xylanolytic activities in this organism, but little was known about the number of enzymes involved or characterization of single enzymes. Recombinant DNA technology provides a powerful tool for studying single genes and their products at the molecular level. Knowledge of the genes encoding cellulolytic and xylanolytic enzymes may contribute to an understanding of gene regulation in this anaerobic rumen organism thus opening the way to manipulation of expression. The approach of this study was therefore the screening of a *B. fibrisolvens* H17c genebank, which was established by Dr. E. Rumbak in *E. coli*, for the genes involved in cellulose and hemicellulose degradation, as well as the characterization of extracellular cellulase and xylanase.

Chapter 2

Cloning, sequencing and analysis of expression of a *Butyrivibrio fibrisolvens* gene encoding a β-glucosidase

Chapter 2

Cloning, sequencing and analysis of expression of a *Butyrivibrio fibrisolvens* gene encoding a β-glucosidase

2.0 Summary

The cloning, expression in *E. coli* and the determination of the nucleotide sequence of a 3.74 kb DNA segment containing a β-glucosidase gene (*bgl*A) from *B. fibrisolvens* H17c has been completed. The *B. fibrisolvens bgl*A open reading frame (ORF) of 2490 bp encoded a β-glucosidase of 830 amino acid residues with a calculated MW of 91,800. In *E. coli* cells the β-glucosidase was localized in the cytoplasm and these cells produced an additional protein with an apparent M_r of approximately 94,000. The *bgl*A gene was expressed from its own regulatory region in *E. coli* and a single mRNA initiation point was identified upstream of the *bgl*A ORF and adjacent to a promoter consensus sequence.

The complete amino acids sequence (830 residues) showed 41% identity with the β-glucosidase from *R. albus* and < 15% identity to the fungal and bacterial groups of β-glucosidase. Four extensive regions (comprising about 70% of BglA)of homology were found among the β-glucosidase from *B. fibrisolvens, R. albus, Kluyveromyces fragilis, C. thermocellum* and *Saccharomycopsis fibuligera.*

The C-terminal homologous region contains an amino acid sequence very similar to the active site of β-glucosidase A3

from *Aspergillus wentii*. The *B. fibrisolvens* β-glucosidase hydrolyzed cellobiose to a limited extent, cellotriose to cellobiose and glucose, and cellotetraose and cellopentaose to predominantly glucose.

2.1 Introduction

Enzymes capable of hydrolyzing cellobiose and cellodextrins are important components of microbial cellulase systems. They convert the cellobiose and cellodextrins formed during the enzymatic degradation of cellulose to fermentable glucose. β-glucosidase may therefore be the rate-limiting enzyme in cellulase preparations obtained from culture filtrates (Coughlan, 1985; Christopher *et al.*, 1991). Localization of β-glucosidase in the cells seems to be advantageous for efficient breakdown of cellulose, since glucose does not accumulate outside of the cells and can be converted to the other compounds by Embden-Meyerhof-Parnas pathway in the cells (Joyer and Baldwin, 1966).

β-Glucosidases from bacteria such as *B. succinogenes* (Groleau and Forsberg, 1981), *R. albus* (Ohmiya *et al.*, 1985), *R. flavefaciens* (Pettipher and Latham, 1979), *C. thermocellum* (Ait *et al.*, 1982) and *Acetovibrio cellulolyticus* (Mackenzie and Bilous, 1982) have been found to be cell-associated. However, enzymes from the fungi *Talaromyces emersonii* (Mchale and Coughlan, 1982), *T. viride* (Berghem and Petterson, 1974) and *T. koningii* (Wood and McCrae, 1982b) were secreted from the cells into the culture supernatant. Thus, bacterial β-glucosidases seem to be retained in or on the cells, whereas fungal β-glucosidases are released from the cells.

With the patterns of specificity observed, *Bacillus polymyxa* synthesizes two different β-glucosidases. β-Glucosidase A is

intracellular and is particularly active on cellobiose. This enzyme is similar to a β-glucosidase from *Agrobacterium* sp., which also showed a high specific activity and a high affinity for cellobiose (Wakarchuk *et al.*,1986). The other enzyme, which is secreted, displayed higher activity for the artificial substrate pNPG than for cellobiose. This preference for aryl-β-glucosides has been observed in the β-glucosidases of *Clostridium stercorarium* (Bronnenmeier and Staudenbauer, 1988), *C. thermocellum* (Ait *et al.*, 1982) and *Caldocellum saccharolyticum* (Patchett *et al.*, 1987).

A number of β-glucosidase genes have been cloned and expressed in *E. coli* and *Saccharomyces cerevisiae* (González-Candelas *et al.*, 1989; Schnetz *et al.*, 1987; Wakarchuk *et al.*, 1988; Kohchi and Toh-e, 1985; Love *et al.*, 1988; Machida *et al.*, 1988; Raynal *et al.*, 1987). The comparative analysis of sequence data allows important information to be obtained concerning the evolutionary relatedness of the genes, the localization of the active site along the primary structure of the enzyme and the relationship between structure and function. This chapter deals with the nucleotide sequence of a β-glucosidase, the activity of the encoded enzyme and an investigation of the structural similarities to other β-glucosidases.

2.2 Materials and Methods

2.2.1 *Bacterial strains and plasmids*. The bacterial strains and plasmids used in this study are listed in Appendix C. The cloning vector pEB1 is an *E. coli/B. subtilis* shuttle vector with positive selection for inserted DNA fragments in *E. coli*. pEB1 was constructed by replacing the pBR322 fragment of pLP1202 (Robson and Chambliss, 1986; Ostroff and Pene, 1983) with pEcoR252, which is a derivative of pEcoR251 lacking the *Pst*I site in the β-lactamase gene (P. Janssen, personal communication). The structure of pEcoR251 (a gift from M. Zabeau, Plant Genetic Systems, Gent, Belgium) is similar to other plasmids utilizing inactivation of the *Eco*RI endonuclease gene as a selective marker (e.g. Cheng and Modrich, 1983) and has been described elsewhere (Zappe *et al.*, 1986).

2.2.2 *Media and buffers*. All media,buffers and solutions not described in the text are in Appendix A.

2.2.3 *Growth conditions*. *B. fibrisolvens* H17c was grown in M10 medium (Caldwell and Bryant, 1966) as described in Appendix A. *E. coli* strains were grown in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982) and ampicillin (100 μg ml^{-1}) was used as the selective marker for transformants.

2.2.4 *Preparation of DNA*. Chromosomal DNA from *B. fibrisolvens* was obtained by the method of Berger *et al.* (1989). *B. fibrisolvens* chromosomal DNA was prepared from 500 ml cultures which were harvested, resuspended in 4 ml

Solution A [Tris-HCl (10mM) pH8.0, sucrose (25%, w/v)] containing lysozyme (5 mg/ml) and agitated gently at 37°C for 30 min. Two ml of ice cold Solution B [EDTA(0.5M),pH 8.0] were added and the mixture was kept on ice for 5 min. Four ml of Solution C [Tris-HCl (10mM), pH7.5; Na_2 EDTA, (1mM); SDS (2%, w/v)], containing proteinase K (5 mg/ml) were added. The mixture was kept at 20°C for 10 min prior to centrifugation at 15,000 x g for 30 min. The DNA was purified by CsCl-ethidium bromide density gradient centrifugation, N-butanol extraction, and isopropanol precipitation (Maniatis *et al.*, 1982). Plasmid DNA was prepared by an alkaline lysis procedure (Ish-Horowicz and Burke, 1981).

2.2.5 *Construction and screening of a B. fibrisolvens H17c genomic library.* *B. fibrisolvens* chromosomal DNA was partially digested by *Sau*3A endonuclease and fractionated on a sucrose density gradient (Maniatis *et al.*, 1982). The fragments ranging from 4-10 kb in length were pooled and ligated with pEB1 which had been digested with *Bgl*II endonuclease. The ligated DNA was used to transform competent *E. coli* LK111 cells (Dagert and Ehrlich, 1979). Transformants were selected on LB agar containing ampicillin (100 $\mu g\ ml^{-1}$). Colonies were lifted off the plates on Whatman No. 1 filter paper discs and saturated with 1 ml of 4-methylumbelliferyl-β-D-cellobioside (MUC, Sigma) at a concentration of 0.02% (w/v) in 0.1 N sodium acetate buffer, pH 5.0. The discs were incubated for 10 min at 37°C and examined for fluorescent halos under UV light (254 nm).

Plasmids were analysed by standard procedures (Maniatis *et al.*, 1982).

2.2.6 *Southern blot analysis.* *B. fibrisolvens* chromosomal DNA and pLS215 were digested to completion with *Eco*RV restriction endonuclease, fractionated by electrophoresis in 0.8% (w/v) agarose gels in Tris-acetate buffer, and transferred to Hybond N (Amersham, UK) (Smith and Summers, 1980). pLS215, nick-translated with [α-^{32}P]dCTP (Rigby *et al.*, 1977) was used as a hybridization probe. These methods are described in Appendix B.

2.2.7 *Nucleotide sequencing.* DNA fragments were subcloned in Bluescript vectors (Stratagene, San Diego, California) and Exonuclease III was used to generate two sets of overlapping deletions of opposite polarity (Henikoff, 1984). Sequencing was done by the chain termination method of Sanger *et al.* (1977) using a Sequenase Kit (United States Biochemical Corporation, Cleveland, Ohio) (Appendix B). The nucleotide and deduced amino acid sequences were analyzed using an IBM XT Computer and DNA tools or Genepro (version 3.1) programmes.

2.2.8 *Primer extension.* mRNA from *E. coli* C600 (pLS215) was extracted by the method of Aiba *et al.* (1981). The cells were grown in 100 ml of 2 YT medium containing 100 µg/ml Ampicillin. At A_{600} = 0.3 the cells were harvested resuspended in 3 ml of a solution of 20 mM sodium acetate (pH 5.5), 0.5% SDS, and 1 mM EDTA. After addition of 3ml of phenol (equilibrated in 20 mM sodium acetate, pH 5.5), the

mixture was incubated at 60°C for 5 min with gentle shaking. After centrifugation the aqueous phase was re-extracted by phenol. The RNA was precipitated by adding 3 volumes of ethanol to the aqueous phase and chilled at -70°C for 30 min. The RNA precipitate was collected by centrifugation and redissolved in 3 ml of the same acetate/SDS buffer. The ethanol precipitate was dissolved in 1 ml of distilled water. The RNA concentration was determined by measuring the optical density at 260 nm. A synthetic 17-oligomer (5' -GCTGTAACCCTTTCACC-3') complementary to nucleotide positions 235 to 251 (Fig. 2.3) was labelled at the 5' end with [γ-^{32}P]ATP (2,000 Ci mmol^{-1}) using T4 polynucleotide kinase. The labelled primer (1 pmol) was hybridized to 5 mg of mRNA isolated from *E. coli* C600 (pLS215) in TE buffer (10 mM Tris-HCl, pH 8.3; 0.35 mM EDTA). After ethanol precipitation, the samples were incubated in 25 µl of 50 mM Tris-HCl pH 8.3, 8 mM $MgCl_2$, 2 mM DTT, 1 mM dNTP's, and 34 U reverse transcriptase at 42°C for 60 min. A DNA sequencing reaction was carried out using the same oligonucleotide as a primer and electrophoresed alongside the RNA primer extended products on a 6% polyacrylamide-7 M urea sequencing gel.

2.2.9 Tn*phoA* *mutagenesis*. Transposon mutagenesis was performed as described by Manoil and Beckwith (1985). *E. coli* CC118 cells (5 ml) containing pLS215 were grown to early stationary phase and infected by phage λ :Tn*phoA* at a multiplicity of infection of approximately 1.0 and incubated at 30°C for 15 min. The mixture was transferred to 50 ml LB broth and incubated for a further 4 hours at 30°C. The cells were concentrated and selected on LB agar supplemented

with Ap (100 µg/ml), Km (250 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (80 µg/ml). After incubation at 30°C for two to three days, plasmid DNA prepared from blue alkaline phosphatase-positive colonies was mapped and sequenced. Nucleotide sequencing was used to determine the exact position and orientation of insertions. The primer (5'-AAACGGCGAGCACCG-3') was complementary to the DNA sequence corresponding to nucleotide positions 126-140 of the *pho*A gene and was used to sequence across the junction of the fusions between the *pho*A and pLS215.

2.2.10 *In vitro transcription and translation.* A procaryotic DNA directed *in vitro* transcription and translation kit (No. N380; Amersham International, Amersham, England) was used as specified by the manufacturers.

2.2.11 *Cell fractionation.* Periplasmic extracts were prepared from early stationary phase *E. coli* C600(pLS215) cultures (200 ml) by the osmotic shock procedure of Willis *et al.* (1974). Cytoplasmic fractions were prepared from osmotically shocked cells by sonication on ice (10 s bursts for a total of 100 s). The preparation was clarified by centrifugation for 15 min at 27,000 xg at 4°C and samples were stored at -20°C.

2.2.12 *Enzyme assays*

2.2.12.1 *Cellulase and hemicellulase assays.* The enzyme activities were determined using 3,5-dinitrosalicylic acid

(DNS; Miller, 1959) with the following modifications: the assay mixture contained 50 µl of diluted enzyme solution and 50 µl of the substrate CMC (0.5%, w/v; Sigma No. C4888; DS 0.7), lichenan (1%, w/v; Sigma No. L-6133), laminarin (1%, w/v; Sigma No. L9634), or oat spelt xylan (0.5%, w/v; Sigma No. X0376) in 50 mM PC buffer (pH 5.0). The reaction mixtures were incubated at 42°C for 30 min and the reactions were stopped by the addition of 150 µl DNS solution. The samples were then boiled at 100°C for 5 min, centrifuged at 12,000 x g for 3 min, diluted with distilled water and the absorbance at 540 nm measured. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmole reducing sugars per min.

Enzyme activity was measured against p-nitrophenyl-β-D-cellobioside (pNPC; 3.4 mM), p-nitrophenyl-β-D-glucopyranoside (pNPG; 6 mM), p-nitrophenyl-β-D-xylopyranoside (pNPX; 6mM), and salicin (2-[hydroxymethyl] phenyl-β-D-glucopyranoside; 1%, w/v) in PC buffer (pH 5.0) by the method of Deshpande *et al.* (1984). The assay mixture containing appropriately diluted enzyme (0.25 ml) and substrate (0.25 ml) was vortexed and incubated at 42°C for 30 min. The assay was terminated by the addition of 0.5 ml of 14% Na_2CO_3, diluted with 1 ml H_20 and the absorbance at 405 nm was measured. One unit of enzyme activity was expressed as the amount of enzyme releasing 1 µmole p-nitrophenol per min.

2.2.12.2 *β-Galactosidase enzyme assays.* β-Galactosidase activity was assayed according to the method of Pardee *et*

al. (1959) as described by Miller (1972). For the assay 100 µl of cell extract (supernatant, periplasmic, or cytoplasmic) was diluted with an equal volume of Z-buffer (Appendix A) and equilibrated at 28°C. The reaction was initiated by the addition of 40 µl of the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG; 13 mM) in sodium phosphate buffer (0.25M, pH 7.0). The mixture was incubated at 28°C for 5 min and the reaction was stopped with 140 µl Na_2CO_3(14%, w/v) and diluted with water (1.2 ml) before measuring OD_{420} of the released *o*-nitrophenol. One unit of enzyme was defined as the amount of enzyme that produced 1 nmole *o*-nitrophenol per min.

2.2.12.3 *β-Lactamase enzyme assays.* β-Lactamase activity was assayed according to the method of Sykes and Nordstrom (1972). The reaction mixture contained 1 ml starch solution (0.2%, w/v), 1 ml Ap substrate (0.1 mM sodium-Ap) and 0.9 ml buffer (0.1 M potassium phosphate buffer, pH 5.9). Both the starch solution and the substrate were made in the above buffer. The reaction was equilibrated at 30°C for 5 min and initiated by the addition of 0.1 ml appropriately diluted sample. The reaction was stopped after 20 min incubation at 30°C with the addition of 1 ml trichloroacetic acid (TCA) (1%, w/v) and the OD_{620} was measured. One unit of β-lactamase activity was defined as the amount of enzyme that hydrolyzes Ap at the rate of 1 µmole per min.

2.2.13 *Analysis of hydrolysis products of cellooligosaccharides by HPLC.* Hydrolysis of cellobiose, cellotriose, cellotetraose and cellopentaose (obtained from

AECI, SA) was determined by incubating the cell extracts (20 µl) with 200 µl of the dextrin (10 mg ml^{-1} in PC buffer) at 42°C. Samples were taken at various times and analyzed in a high-pressure liquid chromatography (HPLC) system equipped with a model 156 refractive index detector (Beckman). Separation was achieved on a C18 column (Waters Associates, Milford, MA). The column was held at room temperature and filtered water was used as an eluant at a flow rate of 1.5 ml min^{-1}.

2.2.14 ***Protein determination and polyacrylamide gel electrophoresis.*** Protein concentrations were determined by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard (Appendix B). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) (Appendix B).

2.3 Results

2.3.1 *Cloning and expression of a β-glucosidase gene from B. fibrisolvens in E. coli*. A MUC -positive *E. coli* LK111 transformant was isolated on LB agar plates containing ampicillin. The *E. coli* cells contained a recombinant plasmid which was designated pLS215. *E. coli* C600(pLS215) cells also showed activity towards 4-methylumbelliferyl-β-D-glucoside (MUG) but no carboxymethyl cellulase activity.

The hybrid plasmid was analyzed with the restriction endonucleases *Eco*RI, *Eco*RV, *Bgl*II, *Hind*III, *Stu*I, *Bam*HI and *Pst*I (Fig. 2.1). The DNA insert was estimated to be 3.7 kb and had a unique *Bam*HI site. To localize the *bgl*A gene on the cloned DNA, a series of derivatives were generated by deleting various segments of pLS215 with restriction enzymes or subcloning the cloned *bgl*A DNA into Bluescript SK. When either the 1.8 kb *Stu*I, 1.7 kb *Bgl*II-*Bam*HI and 1.6 kb *Pst*I fragments were subcloned into Bluescript SK, no β-glucosidase activity was detected. Removing the 0.9 kb *Eco*RI fragment which contains the lambda P_L promoter did not inactivate the β-glucosidase gene. It was therefore concluded that the *bgl*A gene was expressed from a regulatory region present in the insert. Deletion of the *Bgl*II-*Bam*HI fragment led to a *bgl*$^-$ phenotype, suggesting that *Bam*HI lay in a region essential for β-glucosidase expression.

The origin of the cloned fragment on pLS215 was determined by Southern blotting with [^{32}P]-labelled pLS215 as a probe against *B. fibrisolvens* chromosomal DNA digested with *Eco*RV

endonuclease. The insert DNA on pLS215 contained four *Eco*RV restriction sites (Fig. 2.1) and the plasmid hybridized to five bands of *Eco*RV-digested chromosomal DNA. The three which represented the internal fragments of pLS215 had the predicted sizes of 0.6, 1.1 and 1.4 kb (Fig. 2.2).

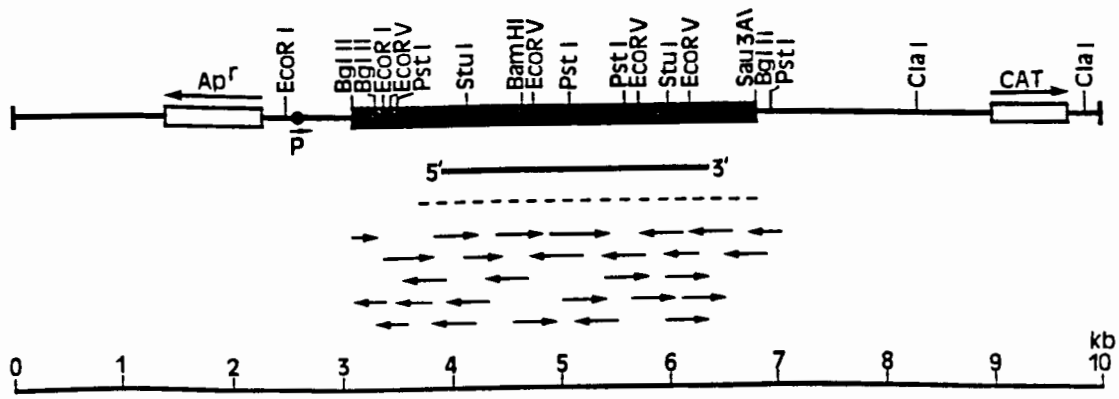

Fig. 2.1 Restriction map and sequencing strategy of plasmid pLS215. Single and bold lines and open boxes represent vector DNA, *B. fibrisolvens* insert DNA and antibiotic resistance genes, respectively. The circle and arrow beneath denote the position and direction of the lambda rightward promoter. The extent of the *bgl*A transcript is indicated by the solid line below the restriction map. The broken line indicates the extent of the nucleotide sequence shown in Fig. 2.3. The arrows denote the direction and extent of sequence determination.

2.3.2 *Nucleotide sequencing.* The nucleotide sequence of the 3.74 kb DNA fragment contained an open reading frame (ORF) encoding 830 amino acids with a calculated MW of 91,800 (Fig. 2.3).

The G+C content of the nucleotides within the ORF (46.1%) was higher than that within the upstream regulatory region (31.9%) or the region of 106 bp immediately downstream of the ORF (27.2%). The average G+C content of the *B. fibrisolvens* genome is 42% (Mannarelli, 1988).

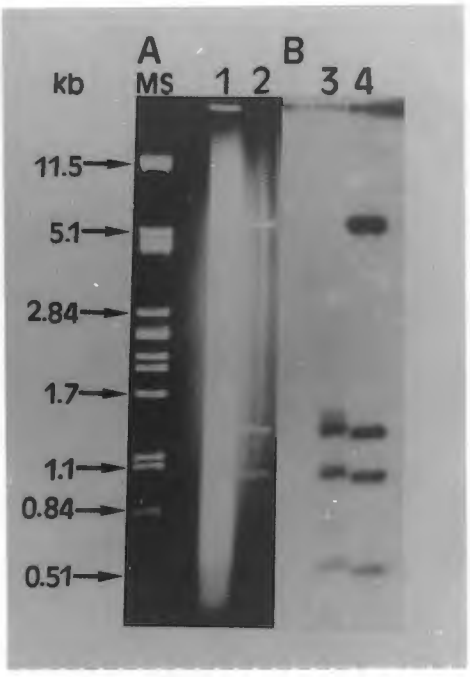

Fig. 2.2 Southern blot analysis of complete digest of genomic DNA from *B. fibrisolvens*. Panel A, Ethidium bromide-stained agarose gel of *Eco*RV digested *B. fibrisolvens* (lane 1) or pLS215 DNA (lane 2). Panel B, Autoradiogram of the gel blotted onto Hybond N probed with nick-translated pLS215. Lane 3, *Eco*RV digested *B. fibrisolvens* DNA; lane 4, *Eco*RV digested pLS215 DNA. Molecular weight markers (MS) are lambda DNA digested with restriction endonuclease *Pst*I.

The region upstream of the ORF, between nucleotides 55 and 84, contained a promoter sequence which consisted of a TTCAAA - 35 region of a TTTTAT -10 region separated by 17 bp (Fig. 2.3). This promoter sequence resembled the consensus sequence of the σ^{70} RNA polymerase recognition site of *E. coli* (Hawley and McClure, 1983; Cowing *et al.*, 1985). Primer extension experiments indicated that this was a functional promoter sequence in *E. coli* and a transcriptional initiation point was detected 7 bp downstream of the -10 region (Fig. 2.4). No other transcriptional initiation point could be detected in the region upstream of the *bgl*A gene.

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AACAAACAGAATTGTTGGAAACTTTTTTATATGCAATATTACTTTAACACAGAATGTCTG      60

GTGATGGTATATGTTTTTTCTTCCTGAGTTATATATTTCAAATCTTAGATGTGCTATGCT     120
                                        -35
TTTATGGCAGAAACATGTTATATACAAGGTAAAGATAAGTAGGATAACGAGGTATTTATA     180
 -10                                          RBS
ATGGAGAAATGGGCAAGAATCAAATATACACCAAATCTTCCGCTTGGAGAGAATGGTGAA     240
M  E  K  W  A  R  I  K  Y  T  P  N  L  P  L  G  E  N  G  E

AGGGTTACAGCGAGTCAGAAGCACATTGAGCTTTCATGCGAGGCAGCATGTGAGGGAATG     300
R  V  T  A  S  Q  K  H  I  E  L  S  C  E  A  A  C  E  G  M

GTACTTCTCAAGAATGACAGAAACGTTCTTCCTATCAGAAAGGGCACAAGAGTAGCCCTC     360
V  L  L  K  N  D  R  N  V  L  P  I  R  K  G  T  R  V  A  L

TTTGGAAAGGGAGTATTTGACTATGTAAAAGGCGGCGGTGGTAGCGGAGATGTAACAGTT     420
F  G  K  G  V  F  D  Y  V  K  G  G  G  G  S  G  D  V  T  V

CCTTACATCAGAAACCTCTACGAAGGCCTTTCTCAGTACACATCAGACATTTCAATTTAC     480
P  Y  I  R  N  L  Y  E  G  L  S  Q  Y  T  S  D  I  S  I  Y

GACAAATCTGTCAGATTCTATCAGGAATATGTAGCAGACCAGTACAGACTTGGAATTGCA     540
D  K  S  V  R  F  Y  Q  E  Y  V  A  D  Q  Y  R  L  G  I  A

CCAGGCATGATCAAAGAGCCGGCTCTTCCGGAAGATATTCTTGCAGATGCAGCAGCCTAT     600
P  G  M  I  K  E  P  A  L  P  E  D  I  L  A  D  A  A  A  Y

GCAGATACTGCAATCATCGCAATCAGCAGATTCTCCGGAGAAGGCTGGGACAGAAAGGTT     660
A  D  T  A  I  I  A  I  S  R  F  S  G  E  G  W  D  R  K  V

GCAGGCGTTGACAGAGAAATCAAGTGCGAAGCCAAGGACCTCGTAGAGCAGGGCAACAAG     720
A  G  V  D  R  E  I  K  C  E  A  K  D  L  V  E  Q  G  N  K

ATATTTGATCATGGTGATTTCTACCTCACAAATGCTGAGAAGAAGATGGTCAAGATGGTA     780
I  F  D  H  G  D  F  Y  L  T  N  A  E  K  K  M  V  K  M  V

AAAGAGAACTTCTCAAGCGTCATTGTAGTCATGAATGTCGGAGGAGTCGTAGACACAACA     840
K  E  N  F  S  S  V  I  V  V  M  N  V  G  G  V  V  D  T  T

TGGTTTAAAAAGGATGACCAGATTTCATCAGTCCTCATGGCATGGCAGGGTGGAATTGAA     900
W  F  K  K  D  D  Q  I  S  S  V  L  M  A  W  Q  G  G  I  E

GGCGGACTTGCCGCAGCCAGGATCCTTCTTGGCAAGGTTAATCCTTCAGGTAAGCTCTCA     960
G  G  L  A  A  A  R  I  L  L  G  K  V  N  P  S  G  K  L  S

GATACATTCGCAGCAAGGCTTGAAGACTATCCTTCAACAGAGGGCTTCCACGAAGATGAT    1020
D  T  F  A  A  R  L  E  D  Y  P  S  T  E  G  F  H  E  D  D

GACTACGTGGATTACACAGAAGATATCTACGTTGGCTATAGATATTTCGAGACCATTCCC    1080
D  Y  V  D  Y  T  E  D  I  Y  V  G  Y  R  Y  F  E  T  I  P

GGGGCAAAAGAGAAAGTTAACTACCCCTTTGGCTATGGCCTTTCCTATACAACTTTCCTG    1140
G  A  K  E  K  V  N  Y  P  F  G  Y  G  L  S  Y  T  T  F  L
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```
CTTGAAGACTATAAGGCAGAGCCTTTTGTGGCTTCAGCAGCAGACGAGGTCGGTAAATCT  1200
L  E  D  Y  K  A  E  P  F  V  A  S  A  A  D  E  V  G  K  S

GATAGCGACCTTGCAGATGCAATCGTAGCCTCAGTTACAGTCACAAACATTGGCAAGATT  1260
D  S  D  L  A  D  A  I  V  A  S  V  T  V  T  N  I  G  K  I

CCGGGCAAAGAGGTTGTTCAGCTCTACTACAGCGCTCCTCAGGGCAAGCTCGGTAAGCCT  1320
P  G  K  E  V  V  Q  L  Y  Y  S  A  P  Q  G  K  L  G  K  P

GCTAAAGTCCTTGGCGGCTATGCCAAGACAAGGCTACTGCAGCCGGGAGAGAGCCAGAGA  1380
A  K  V  L  G  G  Y  A  K  T  R  L  L  Q  P  G  E  S  Q  R

GTGACAATTGCTCTTTATATGGAGGATATGGCATCTTACGACGACCTTGGCAAGGTTAAA  1440
V  T  I  A  L  Y  M  E  D  M  A  S  Y  D  D  L  G  K  V  K

AAGGCTGCCTGGCTCCTTGAAAAAGGTGAATATCATTTCTTCCTTGGAACATCAGTAAGA  1500
K  A  A  W  L  L  E  K  G  E  Y  H  F  F  L  G  T  S  V  R

GACACAAGGCTTCTTGATTACACCTATGAACTTTCTAAGAACATAATAGTTGAACAGGTC  1560
D  T  R  L  L  D  Y  T  Y  E  L  S  K  N  I  I  V  E  Q  V

TCAAACAAGCTCGTTCCAACATCTCTTCCCAAGAGAATGCTTGCTGATGGCACATATGAG  1620
S  N  K  L  V  P  T  S  L  P  K  R  M  L  A  D  G  T  Y  E

GAACTTCCTCAGACAGAACCTGTAGATACTTATGCAACAATCTTCCCAAGACCTAAGAAC  1680
E  L  P  Q  T  E  P  V  D  T  Y  A  T  I  F  P  R  P  K  N

TGGAAAGAAACAATTGAGCACGACGTATTAAAGACTCCTGTAGTTCGTCCACAGGACAGA  1740
W  K  E  T  I  E  H  D  V  L  K  T  P  V  V  R  P  Q  D  R

TTCCAGCTCTTTTTGCCACCTAAGGAAGGTGACCCTAAGAAATTTATCGAAGTTGCAGAA  1800
F  Q  L  F  L  P  P  K  E  G  D  P  K  K  F  I  E  V  A  E

TGCAAGGTGACACTTGAAGACTTTATTGCACAGCTATCTAACGAGCAGCTTGCAAGCCTT  1860
C  K  V  T  K  E  D  F  I  A  Q  L  S  N  E  Q  L  A  S  L

CTTGGAGGACAGCCAAATGTCGGAATGGCTAACACCTTTGGATACGGCAACCTTCCTGAG  1920
L  G  G  Q  P  N  V  G  M  A  N  T  F  G  Y  G  N  L  P  E

GTTGGAGTTCCTAATGCCCAGACCTGTGATGGTCCTGCAGGTGTCCGTATTGCACCGGAA  1980
V  G  V  P  N  A  Q  T  C  D  G  P  A  G  V  R  I  A  P  E

GTTGGTGTTGTGACTACAGCATTCCCATGTTCAACCCTTCTTGCATGCACATGGAATGAA  2040
V  G  V  V  T  T  A  F  P  C  S  T  L  L  A  C  T  W  N  E

GATATCTGCTACGAAGTCGGAGTTGCAGGCGGAGAAGAGGCCAAGGAGTGCAATTTTGGT  2100
D  I  C  Y  E  V  G  V  A  G  G  E  E  A  K  E  C  N  F  G

GCATGGCTTACTCCTGCTGTTAACATCCATAGAAGCCCTCTTTGCGGCAGAAACTTTGAG  2160
A  W  L  T  P  A  V  N  I  H  R  S  P  L  C  G  R  N  F  E

TACTACTCCGAAGATCCATTCCTTGCAGGTAAACAGGCAGCAGCTATGGTTCGTGGTATC  2220
Y  Y  S  E  D  P  F  L  A  G  K  Q  A  A  A  M  V  R  G  I
```

```
CAGAGCAACAACATAATTGCTACACCTAAACATTTTGCCCTCAACAACAAGGAATCCAAT  2280
Q  S  N  N  I  I  A  T  P  K  H  F  A  L  N  N  K  E  S  N

AGAAAAGGCAGCGATTCACGTGCTTCTGAGCGTGCGATCAGAGAAATATATTTAAAGGCC  2340
R  K  G  S  D  S  R  A  S  E  R  A  I  R  E  I  Y  L  K  A

TTTGAAATCATTGTTAAAGAGCAGAGCCCTGGAGCATCATGTCTTCAATACAATATAGTT  2400
F  E  I  I  V  K  E  Q  S  P  G  A  S  C  L  Q  Y  N  I  V

AACGGTCAGAGATCATCCGAATCTCACGACCTCCTCACAGGAATCCTCCGCGATGAGTGG  2460
N  G  Q  R  S  S  E  S  H  D  L  L  T  G  I  L  R  D  E  W

GGCTTTGAAGGTGTTGTAGTCAGCGACTGGTGGGGCTTTGGTGAGCATTACAAGGAAGTC  2520
G  F  E  G  V  V  V  S  D  W  W  G  F  G  E  H  Y  K  E  V

CTTGCAGGCAACGATATCAAGATGGGCTGTGGCTATACAGAACAGCTCCTTGAAGCAATT  2580
L  A  G  N  D  I  K  M  G  C  G  Y  T  E  Q  L  L  E  A  I

GATAAGAAAGCTCTTAAGAGAAAAGATTTGGAAAAGAGGCAGAGCGAGTCCTCAAGATGC  2640
D  K  K  A  L  K  R  K  D  L  E  K  R  Q  S  E  S  S  R  C

TTCTCAAACTCGACTAAGCTCAAAGCCGCTTAGAATAAACGTTTAAGTCATAAAAAAGTA  2700
F  S  N  S  T  K  L  K  A  A  *

ATGTATTGTTCATAAACTATCAATATTTTTCTGACAATTTGTCGTATAATATAAATATGA  2760

TATAGTTGTTGGGAATATCTTCTGAGAATTGTATCAGGAGAAAGTTGTAGAACTACAACT  2820

CTCGGAGGTATGTCTATGAACAAAAAAGCTATTGTTGGTATTTTTATGTCCATTTTGATG  2880
                  M  N  K  K  A  I  V  G  I  F  M  S  I  L  M
                                 TnphoA-1   TnphoA-2
GCAGGGCTCGTTGGATGTGCCGGTAGCAGTGATGCCCAGGCAGGAGATGACCTCAAGCCG  2940
A  G  L  V  G  C  A  G  S  S  D  A  Q  A  G  D  D  L  K  P
                                                    TnphoA-3
GTTATTTATCTTTATCCACAGGAAGATAATACCGAGATTTCAGTAAGCCTTGATTATAAC  3000
V  I  Y  L  Y  P  Q  E  D  N  T  E  I  S  V  S  L  D  Y  N

GGAAATCTGGTTGACCTGATTCCTGAGTTTAATGCAGATAAGACATGGAATGTTACAGCT  3060
G  N  L  V  D  L  I  P  E  F  N  A  D  K  T  W  N  V  T  A

AACAAAGATGGCAAGATTACCTTTGAAGGACAGACTTATGACTATCTGTTTTGGGAAGGC  3120
N  K  D  G  K  I  T  F  E  G  Q  T  Y  D  Y  L  F  W  E  G

GATC                                                           3124
D
```

Fig. 2.3 Nucleotide sequence of the *B. fibrisolvens bgl*A structural gene and flanking regions. Open circles indicate transcriptional start points for the *bgl*A gene and the associated arrow indicates the direction of transcription. Consensus -10 and -35 regions of the *bgl*A promoter are underlined and the RBS is boxed. Inverted repeat sequences are shown by converging arrows. The signal peptide of the downstream ORF is underlined.

A possible ribosome binding site (AACGAGG) was situated 8 bp upstream of the ATG putative initiation codon. The stop codon of the *bgl*A gene is followed by an inverted repeat sequence and a region encoding a stretch of 5 U residues (Fig. 2.3). The potential stem/loop structure that could be formed in the resultant mRNA message had a ΔG = -1.21 Kcal/mol (calculated according to Salser, 1977) and consisted mainly of A and U residues. It is therefore unlikely that the structure would function as a rho-independent terminator in *E. coli*.

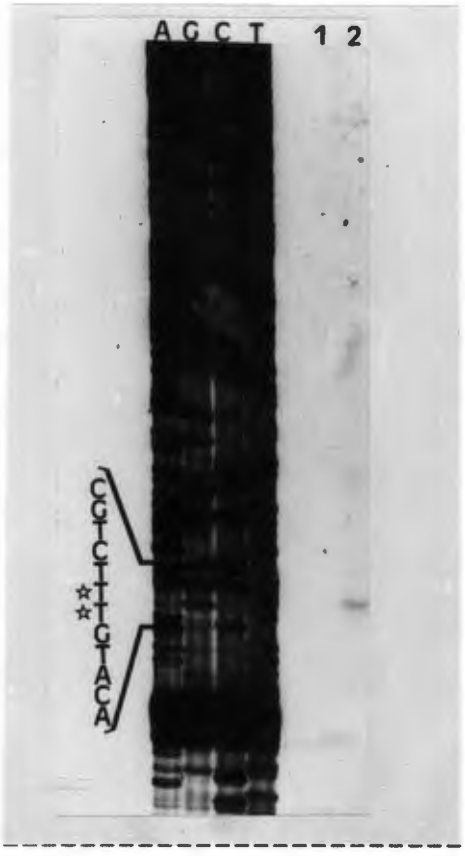

Fig. 2.4 Primer extension analysis of the 5' end of *bgl*A mRNA. An oligonucleotide complementary to nucleotide positions 235 to 251 (Fig. 2.3) was hybridized to RNA from *E. coli* C600(pLS215) cells (lane 2) and a control without RNA (lane 1). A sequencing reaction was carried out using the oligonucleotide as a primer with DNA from pLS215.

The presence of a signal peptide at the amino terminal end of the *bgl*A encoded protein was investigated by Tn*pho*A mutagenesis. Since the alkaline phosphatase moiety on Tn*pho*A lacks its own signal peptide, it is inactive unless secreted to the periplasmic space by insertion of Tn*pho*A downstream of a functional signal peptide (Manoil and Beckwith, 1985). No Tn*pho*A insertions resulting in a $PhoA^+$ phenotype could be isolated when Tn*pho*A transposed into the *bgl*A gene. However, $PhoA^+$ *E. coli* CC118(pLS215) colonies were isolated when Tn*pho*A insertions were located in an unidentified ORF found, in the same reading frame, 166 bp downstream of the *bgl*A gene (Fig. 2.3). Analysis of the deduced amino acid showed the presence of a signal peptide characteristic of extracellular enzymes produced by Gram-positive bacteria (Perlman and Halvorsen, 1983). A short stretch of 3 positively charged amino acids was followed by a hydrophobic domain of 21 amino acids. These results confirmed that the *bgl*A gene product does not have an amino terminal signal peptide.

2.3.3 *Sequence comparison with other β-glucosidases.* The deduced amino acid sequence of the *B. fibrisolvens* β-glucosidase was compared with the sequences of the bacterial and fungal β-glucosidases using BESTFIT programme from the Genetics Computer Group sequence analysis package (Devereux *et al.*, 1984). The highest homology (41% identity) existed between *B. fibrisolvens* β-glucosidase and β-glucosidase of *R. albus* (Ohmiya *et al.*, 1990) over 830 amino acid residues. If amino acid replacement by conservative substitution was taken into account, the overall protein

similarity increased to 62%. The alignment of peptide sequences showed four extensive homologous regions which shared 34, 48, 52, 46% identity respectively (Fig. 2.5 and Table 2.1). In contrast, the sequence comparison of *B. fibrisolvens* BglA (over 830 residues) with β-glucosidases of *Kluyveromyces fragilis* (Raynal *et al.*, 1987), *Saccharomycopsis fibuligera* Bgl1 and Bgl2 (Machida *et al.*, 1988), and *C. thermocellum* (Gräbnitz *et al.*, 1989) showed 13, 16, 16 and 12% identity respectively. Although there was low overall identity between these enzymes, the four conserved regions which comprise about 70% of *B. fibrisolvens* β-glucosidase were present among the above 6 β-glucosidases (Table 2.1 and Fig. 2.6).

The region II and III corresponded to the B and C homologous regions between the β-glucosidases of *C. thermocellum* and *K. fragilis* identified by Gräbnitz *et al.* (1989). The conserved region IV (corresponding to region A of *C. thermocellum* β-glucosidase) at the C-terminal of *B. fibrisolvens* BglA (residues 538-779) shared > 33% identity with all enzymes of this family (Table 2.1). This block was further inspected for conserved residues identified as the active site of *Aspergillus wentii* β-glucosidase A3 (Legler *et al.*, 1979; Bause and Legler, 1980). The conserved aspartic acid and flanking residues were found within region IV between amino acids 762-787 (Fig. 2.5 and Fig. 2.7).

```
Region I

Bfi    30  ELSCEAACEGMVLLKNDRNVLPIRKGTRVALFGKGVFDYVKGGGGSGD-V
           *   *   ** *** *:  ***: *     :**:  :** *:* ***: *
Ral    11  EKAAEVNAEGAVLLVNN-GVLPLDKNAVTQVFGRIQLDYYKSGTGSGGMV

Bfi    79  TVPYIRNLYEGLSQYTSDISIYDKSVRFYQEYVADQYRLGIAPGMIKEPA
            *: :  : :** :   :  : :  :: * :***::   : : *   **
Ral    60  NVAKVTGITDGLIE--AGAKLNEDVLKAYKDYVAEH-PYDYGEGWGGEPW

Region II

Bfi   177  QGNKIFDHGDFYLTNAEKKMVKMVKENFSSVIVVMNVGGVVDTTWFKKDD
           : :     * : **::** ::: *::*** :::::*** ::*  ::   *
Ral   139  EQDNSCKAGSYLLTDGEKAILRKVRDNFSKMVILLNVGNIIDMGFI---D

Bfi   227  QIS--SVLMAWQGGIEGGLAAARILLGKVNPSGKLSDTFAARLEDYPSTE
           ::*   *:  ****: ** : **:*** * *:*** **:*  : ****
Ral   186  EFSPDAVMYVWQGGMTGGTGTARVLLGEVSPCGKLPDTIAYDITDYPSDK

Bfi   275  GFHEDDDYVDYTEDIYVGYRYFETIPGAKEKVNYPFGYGLSYTTFLLEDY
            **: *  : * ***:******:*:  **::* :********* *  *
Ral   236  NFHNRDVDI-YAEDIFVGYRYFDTF--AKDRVRFPFGYGLSYTQF--EIS

Region III

Bfi   346  DAIVASVTVTNIGKIPGKEVVQLYYSAPQGKLGKPAKVLGGYAKTRLLQP
           *::*    * ***  :******:*  **  ****:*:** *: **::* *
Ral   288  DGVVITAKVKNIGSAAGKEVVQVYLEAPNCKLGKAARVLCGFEKTKVLAP

Bfi   396  GESQRVTIALYMEDMASYDDLGKVKKA-AWLLEKGEYHFFLGTSVRDTRL
            * * :** :   *:***** *    * **: * *** *: *  **  :
Ral   338  NEEQTLTIEVTERDIASYDDSGITGNAFAWVEEAGEYTFYAGSDVRSAKE

Region IV

Bfi   538  VAECKVTLEDFIAQLSNEQLASLLGGQ----PNVGMANTFGYG----NLP
           **  * **:***** :::* :*: *:    * *  :   ::*    :*
Ral   459  VAHGKNTLDEFIAQLDDNDLNCLVRGEGMCSPKVTPGTAAAFGGVAKHLE

Bfi   630  GGEEAKECNFGAWLTPAVNIHRSPLCGRNFEYYSEDPFLAGKQAAAMVRG
            * * :    : :* *:;**** ** ******:****** *  *** : *
Ral   557  LGLEMRANKVDCLLGPGMNIHRHPLNGRNFEYFSEDPFLTGTMAAAELEG

Bfi   680  IQSNNIIATPKHFALNNKESNRKGSDSRASERAIREIYLKAFEIIVKEQS
           ::*  : :* ***  ** * **   ** *****:******:*** *:
Ral   607  LHSVGVEGTIKHFCANNQETNRHFIDSVASERALREIYLKGFEIAVRKSK

Bfi   730  PGASCLQYNIVNGQRSSESHDLLTGILRD|EWGFEGVVVSD|WWGFGEHY
           :      *  *** :  :* ** * *** |:***:*  : *|**:
Ral   657  ARSVMTTYGKVNGLWTAGSFDLNTMILRK|QWGFDGFTMTD|WWANINDR
```

Fig. 2.5 Homologous regions of the β-glucosidase of *B. fibrisolvens* (Bfi) and *R. albus* (Ral). Homology was maximized by introducing gaps denoted by a dash(-). Residues that are identical (*) or that represent conservative substitutions (:) are indicated. Numbers on the left refer to the first amino acid in each line. Boxed sequences represent the putative active site.

Table2.1 Comparison of amino acid sequences of β-glucosidase within conserved regions I, II, III, and IV

sequence comparison	Identity(%)[a] Region I	Region II	Region III	Region IV
B. fibrisolvens vs				
R. albus	34	48	52	46
K. fragilis	25	38	49	41
C. thermocellum	35	36	40	42
C. pelliculosa	27	33	14	41
S. fibuligera(Bgl1)	23	37	32	33
S. fibuligera(Bgl2)	29	33	35	35
R. albus vs				
K. fragilis	26	35	43	41
C. thermocellum	29	36	33	40
C. pelliculosa	34	37	23	34
S. fibuligera(Bgl1)	32	30	30	31
S. fibuligera(Bgl2)	26	30	31	33
K. fragilis vs				
C. thermocellum	27	43	56	45
C. pelliculosa	24	32	24	36
S. fibuligera(Bgl1)	31	29	38	33
S. fibuligera(Bgl2)	37	32	43	35
C. thermocellum vs				
C. pelliculosa	30	25	26	36
S. fibuligera(Bgl1)	30	36	38	40
S. fibuligera(Bgl2)	28	31	41	39
C. pelliculosa vs				
S. fibuligera(Bgl1)	40	49	34	54
S. fibuligera(Bgl2)	40	49	28	54
S. fibuligera(Bgl1) vs				
S. fibuligera(Bgl2)	83	83	85	87

[a]Identity is the number of all identical amino acid residues in each pairwise comparison, expressed as a percentage of the total number of amino acid positions compared.

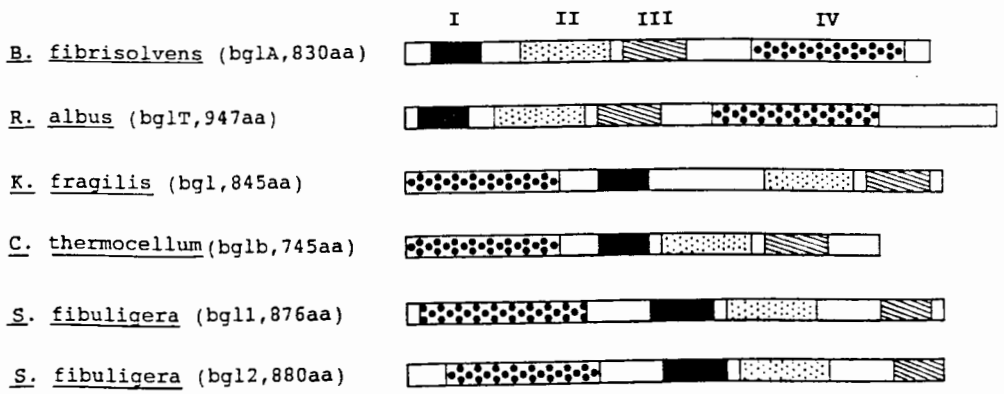

Fig. 2.6 Homologous domains of *B. fibrisolvens* β-glucosidase and other type II β-glucosidases. Domains showing significant similarity are indicated by the same pattern.

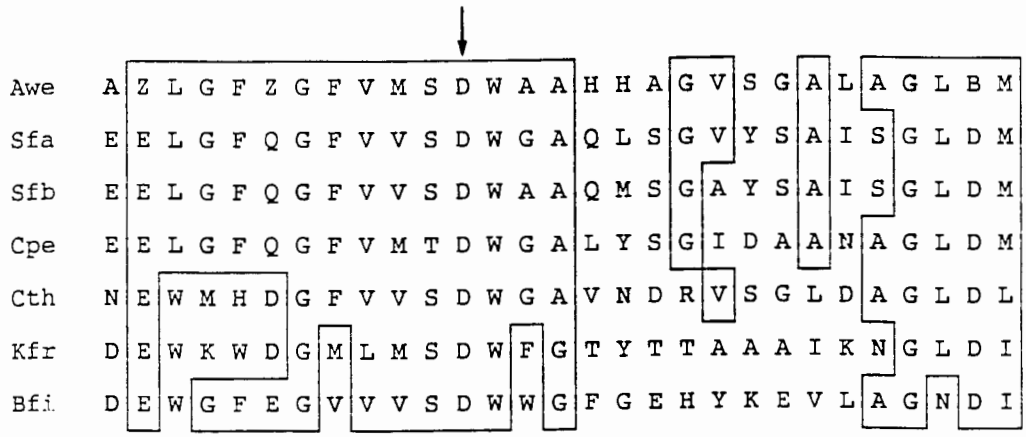

Fig. 2.7 Similarity of β-glucosidases to the active site of β-glucosidase A3 of *Aspergillis wentii*. Boxes indicate identical amino acid positions or those corresponding to conservative amino acid substitution. The arrow indicates the aspartic acid residue of *A. wentii* β-glucosidase. Abbreviations: Awe, *A. wentii* β-glucosidase A3; Sfa, *S. fibuligera* β-glucosidase 1; Sfb, *S. fibuligera* β-glucosidase 2; Cpe, *Candida pelliculosa* β-glucosidase (Kohchi and Toh-e, 1985); Cth, *C. thermocellum* β-glucosidase B; Kfr, *K. fragilis* β-glucosidase; Bfi, *B. fibrisolvens* β-glucosidase.

The homology between the amino acid sequence of the *B. fibrisolvens* β-glucosidase and the bacterial β-glucosidases from *E. coli* (Schnetz *et al.*, 1987), *Bacillus polymyxa* (Gonzalez-Candelas, 1990), *Caldocellum saccharolyticum* (Love

et al., 1988) and an *Agrobacterium* spp. (Wakarchuk *et al.*, 1988) was also determined. The *B. fibrisolvens* enzyme showed < 15% identity with these bacterial β-glucosidases which showed significant similarity when compared with one another.

2.3.4 *Localization of the activity in E.coli C600 (pLS215).* Experiments on the localization of the *B. fibrisolvens* β-glucosidase in *E. coli* cells showed that 95.8% of the enzyme activity was present in cytoplasmic fractions and only low levels of activity were detected in periplasmic fractions and the supernatant medium (Table 2.2). β-Galactosidase, a cytoplasmic enzyme and β-lactamase, a periplasmic enzyme, were used as controls. This is consistent with the absence of a signal sequence in the gene.

Table 2.2 Distribution of β-glucosidase in *E. coli* C600 (pLS215) cells

Fraction	Total activity (Units)		
	β-Glucosidase (%)	β-Galactosidase (%)	β-Lactamase (%)
Supernatant	1 (4)	79 (<1)	110 (30)
Periplasmic	<1 (<1)	47 (<1)	231 (63)
Cytoplasmic	25 (96)	34,000 (99)	26 (7)

2.3.5 *Analysis of proteins encoded by pLS215 in vitro and in vivo.* SDS-PAGE analysis of *in vitro* translation products of pLS215 showed the presence of, amongst others, a protein

with an apparent M_r of approximately 94,000 (Fig. 2.8A, lane 2). The apparent M_r of this protein is in agreement with a predicted M_r of 91,800 encoded by the *bgl*A gene. The vector pEB1 did not encode any proteins with apparent M_r > 30,000 (Fig. 2.8A, lane 1).

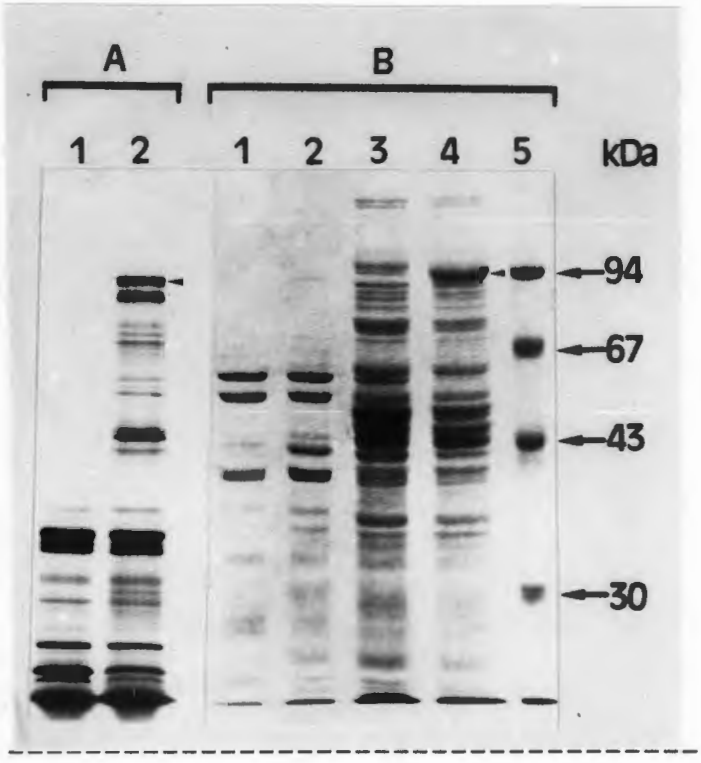

Fig. 2.8 SDS-PAGE analysis of proteins encoded by pLS215 and pEB1 *in vitro* (A) and *in vivo* (B). (A) Lane 1, pEB1; lane 2, pLS215. (B) lanes 1 and 2, periplasmic fractions of *E. coli* K514(pEB1) and K514(pLS215), respectively; lanes 3 and 4, cytoplasmic fractions of *E. coli* K514(pEB1) and K514(pLS215), respectively; lane 5, molecular weight standards: 94 kDa, rabbit muscle phosphorylase b; 67 kDa, bovine serum albumin; 30 kDa, bovine carbonic anhydrase. The arrow head indicates the position of the *bgl*A gene product.

The cytoplasmic fraction of *E. coli* C600(pLS215) cells (Fig. 2.8B, lane 4) showed the presence of a protein with an apparent M_r 94,000. This protein was absent in the periplasmic fraction of this strain and in periplasmic and

cytoplasmic fractions of *E. coli* C600(pEB1) cells (Fig. 2.8B, lanes 2, 1, and 3 respectively).

2.3.6 *Enzyme activity.* The *B. fibrisolvens* β-glucosidase cloned in *E. coli* had a pH optimum of 5.0 and activity decreased rapidly at pH values above and below pH 5.0 (Fig. 2.9A). The optimum temperature for activity of the β-glucosidase was 42°C (Fig. 2.9B). Cell free extracts were assayed with different glucosidic substrates (Table 2.3). The enzyme encoded by *bgl*A was active on aryl-glucosides such as p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-cellobioside, whereas p-nitrophenyl-β-D-xyloside was a poor substrate. Salicin was also a substrate but lichenan did not appear to be hydrolyzed. Hydrolysis of laminarin was also observed, whereas the enzyme was practically inactive on oat spelt xylan and carboxymethylcellulose.

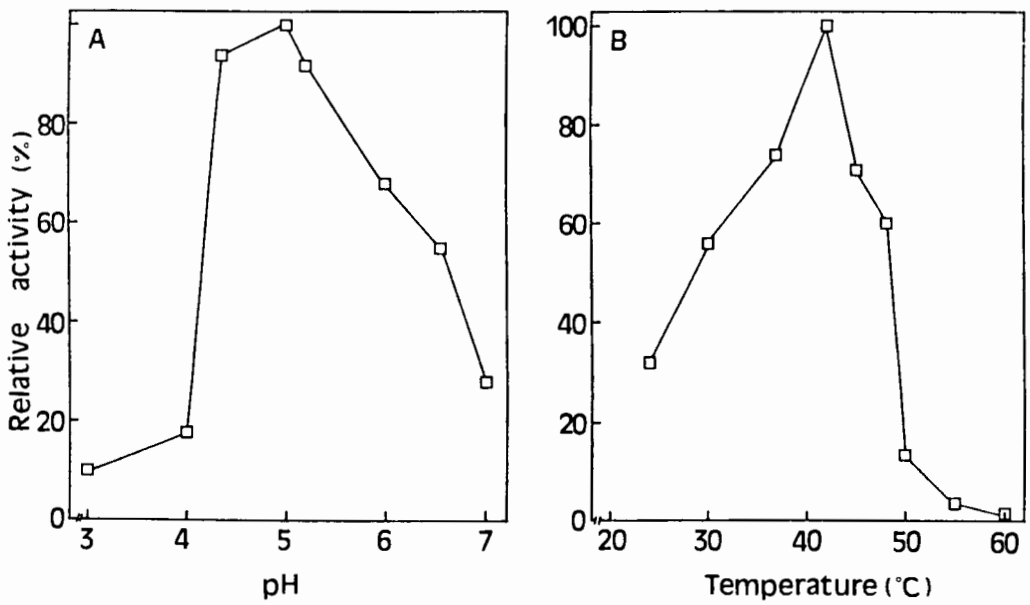

Fig. 2.9 pH activity (A) and temperature activity (B) profiles of the β-glucosidase from cell free extract of *E. coli* C600 (pLS215).

Table 2.3 Substrate specificity of β-glucosidase of cell free extract from *E. coli* C600(pLS215)

Substrate	Linkage	Specific activity (U/mg protein)
pNPG	β-1-4	1.28
pNPC	β-1-4	0.58
pNPX	β-1-4	0.23
Salicin	β-1-4	0.01
Lichenan	β-1-4\β-1-3	<0.01
Laminarin	β-1-4	0.06
Oat spelt xylan	β-1-4	<0.01
CMC	β-1-4	<0.01

The activity of the cloned β-glucosidase against various cellodextrins was examined by high pressure liquid chromatography (Fig. 2.10). The β-glucosidase was able to hydrolyze cellobiose to a limited extent. The enzyme hydrolyzed cellotriose to cellobiose and glucose. Cellotetraose and cellopentaose were hydrolyzed predominantly to glucose.

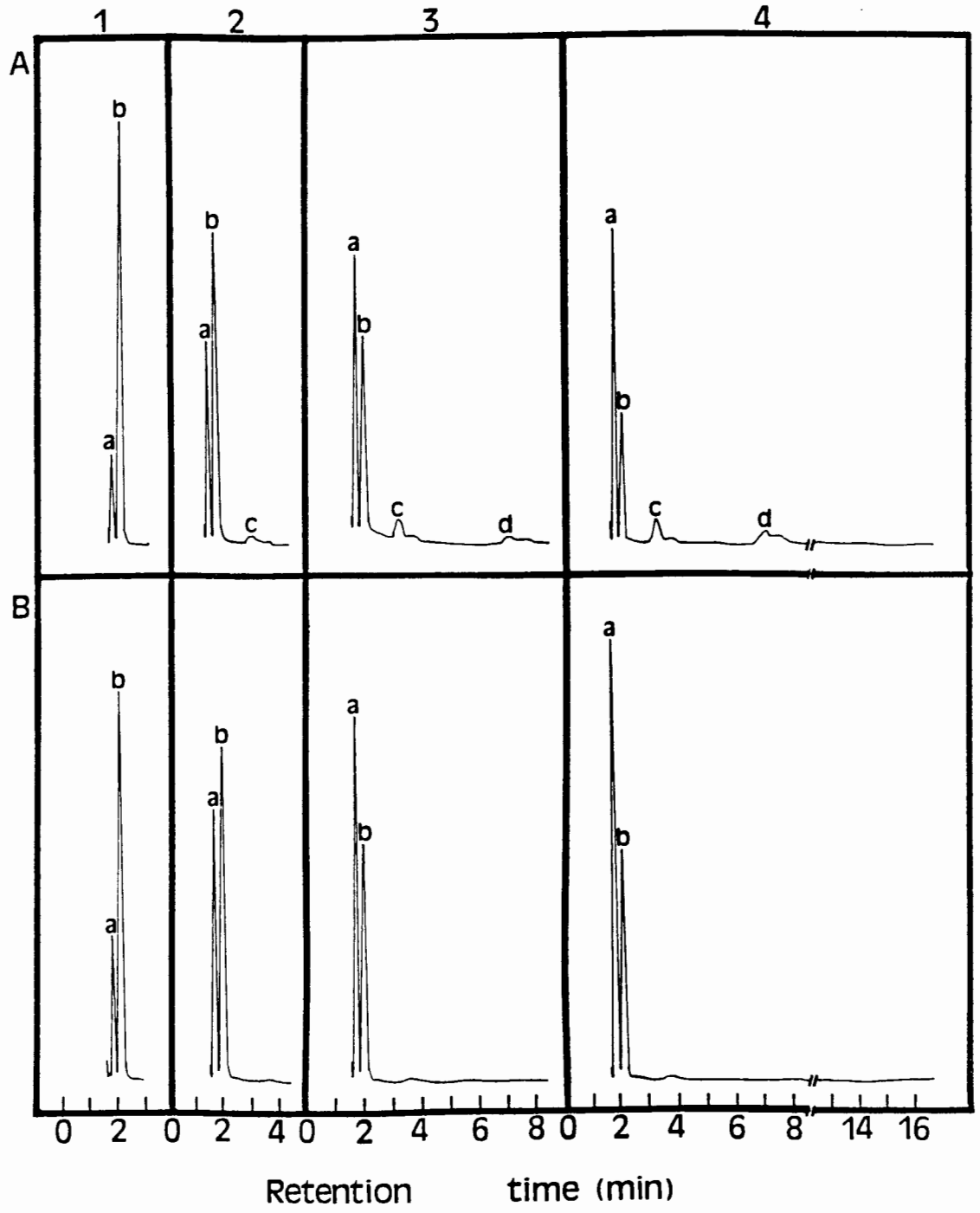

Fig. 2.10 High-pressure liquid chromatograms of the hydrolysis products of cellulooligosaccharides by the cloned β-glucosidase. (A) and (B) Cell extracts were incubated at 42°C for 60 min (A) and 90 min (B) with 200 μg of cellulooligosaccharides in 0.05 M phosphate-citrate buffer (pH 5.0). Substrates used were: panel 1, cellobiose; panel 2, cellotriose; panel 3, cellotetraose; panel 4, cellopentaose. Peaks represent: a, glucose; b, cellobiose; c, cellotriose; d, cellotetraose; e, cellopentaose.

2.4 Discussion

This is the first β-glucosidase gene cloned from *B. fibrisolvens*, and it was expressed in *E. coli*. Southern hybridization with genomic DNA confirmed that the cloned fragment was derived from *B. fibrisolvens*. The results of *in vitro* and *in vivo* transcription-translation of insert DNA are consistent with the proposal that a single gene encodes a 94 kilodalton (kDa) protein. On the basis of observed homologies, Gonzales-Candelas *et al.* (1990) classified two groups of microbial β-glucosidases : type I, including most bacterial enzymes (M_r<60kDa), and type II, including enzymes from different yeast species and one from *C. thermocellum* (M_r>80kDa). Based on molecular weight and sequence comparisons the 94 kDa *B. fibrisolvens* BglA can be placed among the type II β-glucosidases.

Although the protein encoded by *bgl*A of *B. fibrisolvens* was able to hydrolyze cellobiose it had higher activity on cellodextrins and on artificial arylglucosides, such as p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-cellobioside. Despite its activity on cellodextrins, the action of this enzyme is different from cellodextrinases which show no activity on cellobiose and release predominantly cellobiose from cellodextrins (Huang and Forsberg, 1987; Rasmussen *et al.*, 1988; Wang and Thomson, 1990). With respect to its substrate specificity, the enzyme of *B. fibrisolvens* is similar to the enzymes isolated from *C. thermocellum* (Aït *et al.*, 1982) and from *Aspergillus fumigatus* (Rudick and Elbein, 1973). It also resembles the

β-glucosidase of *R. albus* (Ohmiya *et al.*, 1985) although the latter showed a higher activity on cellobiose.

The N-terminal amino acid sequence of BglA of *B. fibrisolvens* does not contain a leader peptide , a characteristic of most secreted proteins (Pugsley and Schwartz, 1985). This was confirmed by the Tn*phoA* experiment. Localization experiments also showed that in *E. coli* C600(pLS215) cell extracts 96% of the β-glucosidase activity occurred in the cytoplasmic fraction. This localization is in keeping with most bacterial β-glucosidases analyzed. Thus BglA is likely to be located intracellularly in *B. fibrisolvens*.

In *C. thermocellum*, the finding of a cellobiose phosphorylase and a cellodextrin phosphorylase active on cellotriose, cellotetraose, and cellopentaose suggested that cellodextrins could be phosphorylated inside the bacterium with the formation of glucose-1-phosphate (Alexander, 1967; Alexander, 1968). In addition, *R. flavefaciens* also contains a cellodextrin uptake system similar to the one of *C. thermocellum* (Helaszek and White, 1991). According to the location of BglA enzyme and its extreme activity on cellodextrins, it is possible that *B. fibrisolvens* also has a cellodextrin uptake system.

To date, the primary structures have been determined for two β-glucosidases produced by anaerobic rumen bacteria (Lin *et al.*, 1990; Ohmiya *et al.*, 1990). Extensive homology between BglT of *R. albus* and BglA of *B. fibrisolvens* suggests a

common ancestral gene and strong selection pressure for the retention of conserved sequences. Gräbnitz *et al.*, (1989) reported three homologous regions identified between *C. thermocellum* BglB and fungal β-glucosidases, indicating the possibility of a genetic exchange between thermophilic anaerobic bacteria and yeasts. As Table 2.1 shows, the highly conserved region of β-glucosidases among a variety of species may have resulted from the convergent evolution of structures having a common function.

Legler *et al.*, (1979) and Base and Legler (1980) have shown that the β-glucosidase inhibitors D-glucal and conduritol B epoxide bind specifically to an aspartic acid residue in the enzymes and reported the amino acid sequence encompassing this putative active site of *A. wentii* β-glucosidase A3. The sequence around the inhibitor-binding aspartic acid residues is highly conserved among the homologous β-glucosidases. It is unfortunate that more of the amino acid sequence of β-glucosidase A3 is not available so that a better comparison could be made with the putative active region. The investigation of these active sites could be facilitated by a combination of the identification of essential amino acids through the use of site-specific mutagenesis of the cloned genes and the use of active site inhibitors.

Chapter 3

Cloning, sequencing and expression of a gene encoding a 73 kDa xylanase enzyme from the rumen anaerobe *Butyrivibrio fibrisolvens* H17c

Chapter 3

Cloning, sequencing and expression of a gene encoding a 73 kDa xylanase enzyme from the rumen anaerobe *Butyrivibrio fibrisolvens* H17c

3.0 Summary

The cloning, expression and nucleotide sequence of a 3 kb DNA segment on pLS206 containing a xylanase gene (*xyn*B) from *B. fibrisolvens* H17c was investigated. The open reading frame (ORF) of 1,905 bp encoded a xylanase of 635 amino acid residues (M_r 73,156). The putative ribosome binding site (GGAGG) was upstream of an ATG start codon. At least 850 bp at the 3' end of the gene could be deleted without loss of xylanase activity. In *E. coli* C600 (pLS206) cells the xylanase was localized in the cytoplasm. The primary structure of the xylanase showed a significant level of identity with a cellobiohydrolase/endoglucanase of *C. saccharolyticum*, as well as with the xylanases of the alkalophilic *Bacillus* sp. strain C-125, *B. fibrisolvens* strain 49, and *P. fluorescens* subspecies *cellulosa*.

3.1 Introduction

The xylanolytic systems of microorganisms, important components of enzymatic lignocellulose biodegradation, have received considerable attention in the past decade. Xylan degradation by specific xylanases may be improved when more is known about the induction and secretion of these enzymes. Alternatively, these goals may be achieved by mutagenesis or genetic engineering, the latter offering the opportunity to produce single xylanolytic enzymes selected for specific applications. The genetic manipulation of anaerobic bacteria and ruminal organisms to increase the production of xylan-degrading enzymes is currently being investigated as a complementary means of improving the digestion of plant materials for increased milk and beef production (Armstrong and Gilbert, 1985; Forsberg *et al.*, 1986; Patterson, 1989).

Xylanases from various microorganisms including fungi and yeasts have been isolated and characterized (Biely and Vrsanka, 1988; Desphande *et al.*, 1986; Desphande and Sadana, 1989; Gruninger and Fiechter, 1986; McCarthy, 1987; Mountfort and Asher, 1989; Tan *et al.*, 1987). Cloning a gene into a non-xylanolytic host facilitates enzyme isolation for further analysis and applications such as the development of immobilized enzyme system. Knowledge of the structure of genes and their regulatory elements could allow specific alterations to be made to obtain high level expression in the original strain or in a foreign host. Luthi *et al.* (1990a) reported the cloning and sequencing of

a gene (*xynA*) from *Caldocellum saccharolyticum* that has xylanase/xylosidase activity, of a separate gene that does not show activity against xylan but does have xylosidase activity, and a third gene, which codes for an acetyl esterase. When the *xynA* was subcloned into a temperature - inducible expression vector, the enzyme was overexpressed and made up to 20% of total cell protein (Luthi *et al.*, 1990b). The xylanase gene of *Streptomyces flavogriseus* was cloned and expressed in an *E. coli* lysogenic vector which could be induced to lyse the cell and allowed efficient release of cloned enzyme activity into the extracellular environment (Srivastava *et al.*, 1991).

The location in *E. coli* of cloned bacterial xylanases carrying signal sequences seems to depend on whether the donor bacterium is Gram-positive or Gram-negative. Genes from *B. succinogenes* (Sipat *et al.*, 1987) and *Aeromonas* sp. strain 212 (Kudo *et al.*, 1985), which are Gram-negative bacteria, coded for xylanases located in the periplasm of *E. coli*. Genes from *B. subtilis* (Bernier *et al.*, 1983a), *B. pumilus* (Panbangred *et al.*, 1983), *C. saccharolyticum* (Luthi *et al.*, 1990b), which are Gram-positive bacteria, produced enzymes located in the cytoplasm. Mannarelli *et al.* (1990) reported a *B. fibrisolvens* gene coding for xylanase activity which is located in the cytoplasm. This is very interesting because although *B. fibrisolvens* stains as a Gram-negative bacterium its cell wall structure is more similar to that of Gram-positive bacteria. The classification of this species is currently in a state of flux (Mannarelli, 1988), but the

above comparison would support it being classed among Gram-positive bacteria.

This chapter describes the cloning and sequencing of a xylanase gene from *B. fibrisolvens* H17c in *E. coli*. As Mannarelli *et al.* (1990) have reported the cloning of a different xylanase gene from *B. fibrisolvens* strain 49 which appears to be closely related to strain H17c, the xylanase gene from strain H17c was termed *xynB*. Comparison of the deduced amino acid sequence with various previously reported sequences showed good similarity between the *B. fibrisolvens* XynB enzyme and the catalytic domains of two xylanases and one cellobiohydrolase/endoglucanase.

3.2 Materials and Methods

3.2.1 *Bacterial strains and plasmids.* The bacteria used are listed in Appendix C. The plasmids used were the *E. coli/B. subtilis* shuttle vector pEB1 (Chapter 2.2.1) and the M13-derived Bluescript plasmid (Stratagene; San Diego).

3.2.2 *Media, buffers, and growth conditions.* All media and buffers not described in the text are given in Appendix A. Growth conditions were as described in Chapter 2.2.3.

3.2.3 *Preparation of DNA.* *B. fibrisolvens* chromosomal DNA was prepared according to the method described in Chapter 2.2.4. Plasmid DNA was isolated by the alkaline hydrolysis method of Ish-Horowicz and Burke (1981).

3.2.4 *Screening a B. fibrisolvens H17c genomic library.* Plasmid DNA from the *B. fibrisolvens* H17c genomic library described previously (Chapter 2.2.5) was used to transform *E. coli* C600 competent cells, and the transformants were selected on LB agar containing ampicillin (100 µg/ml) and 0.1% (w/v) oat spelt xylan (Sigma Chemical Co.). After 20 h of incubation at 37°C, the colonies were lifted off the plates with Whatman No. 1 filter paper discs and the plates were stained with 0.1% (w/v) Congo red, followed by destaining with 1 M NaCl (Teather and Wood, 1982). The xylanase positive clone was identified by a clear zone around the colony.

3.2.5 *Restriction mapping and subcloning procedure.* The recombinant plasmid, pLS206, was characterized by restriction endonuclease digestion. The fragment sizes were determined by agarose gel (0.7%, w/v) electrophoresis as described by Maniatis *et al.* (1982). The desired fragments were eluted from low melting point agarose (SeaPlague, FMC Corporation) and subcloned into the Bluescript vector (Maniatis *et al.*, 1982). Each subclone was transformed into *E. coli* JM103, spread on LB agar containing ampicillin and xylan, and incubated overnight at 37°C to test for xylanase activity as before.

3.2.6 *Southern blot analysis.* Chromosomal DNA from *B. fibrisolvens* was digested with restriction endonuclease, and resolved by electrophoresis in an agarose gel (0.8%, w/v) in Tris-acetate buffer. The DNA was transferred to Hybond-N membrane (Amersham) as described by Reed and Mann (1985). A 2.3 kb *Bgl* I - *Bgl* II DNA fragment was eluted from a *Bgl* I - *Bgl* II digestion mixture of pLS206 and nick-translated with [α-^{32} p] dCTP was used as a hybridization probe (Rigby *et al.*, 1977). These methods are described in Appendix B.

3.2.7 *Nucleotide sequencing.* The templates for nucleotide sequencing were created by unidirectional overlapping deletions using exonuclease III (Henikoff, 1984) (Appendix B). The nucleotide sequence of both strands was determined using the dideoxy chain termination method (Sanger *et al.*, 1977) (Appendix B).

3.2.8 ***In vitro transcription and translation.*** Protein synthesis was analysed using a prokaryotic DNA-directed *in vitro* transcription and translation kit (Amersham) according to the manufacturer's specifications. The translated proteins were separated by 10% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% (w/v) stacking gel (Laemmli, 1970). BDH molecular mass standards were used as markers.

3.2.9 ***Localization of xylanase.*** *E. coli* C600 (pLS206) and C600 (pLS383) were grown to early stationary phase, cells were harvested by centrifugation at 6,500 x g for 10 min at 4°C and subjected to osmotic shock for release of the periplasmic fraction (Willis *et al.*, 1974). After the periplasmic fraction had been removed, the cells were disrupted by sonication on ice (ten 10 s bursts with 10 s intervals). The cytoplasmic fraction was clarified by centrifugation for 15 min at 27,000 x g at 4°C. The enzymes, β-galactosidase and β-lactamase were used as cytoplasmic and periplasmic markers, respectively.

3.2.10 ***Enzyme assays.*** The activity of xylanase was determined by the procedure described in Chapter 2 (2.2.1.2) except incubation was at 55°C for 15 min. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmole xylose equivalent per min. p-Nitrophenyl-β-D- cellobiosidase (pNPCase) activity was determined by the method of Deshpande *et al.* (1984). The assays for β-galactosidase and β-lactamase were performed according to Pardee *et al.* (1959) and Sykes and Nordstrom (1972) (Chapter

2.2.12), respectively. Units of β-galactosidase were expressed as nmole *o* - nitrophenol released per min and for β-lactamase as µmole ampicillin hydrolyzed.

3.3 Results

3.3.1 ***Cloning, restriction endonuclease mapping and identification of xynB gene product.*** A gene library of *B. fibrisolvens,* previously constructed in the vector pEB1 was screened for the expression of xylanase. Ten batches of pooled genomic DNA were transformed into *E. coli* C600. Of ca. 20,000 tranformants only one colony showed xylanase activity. The hybrid plasmid isolated from this clone, designated pLS206, contained a 3.8 kb insert and was analyzed with the restriction endonucleases *Bgl*II, *Cla*I, *Eco*RV, *Bgl*I, and *Pst*I (Fig. 3.1).

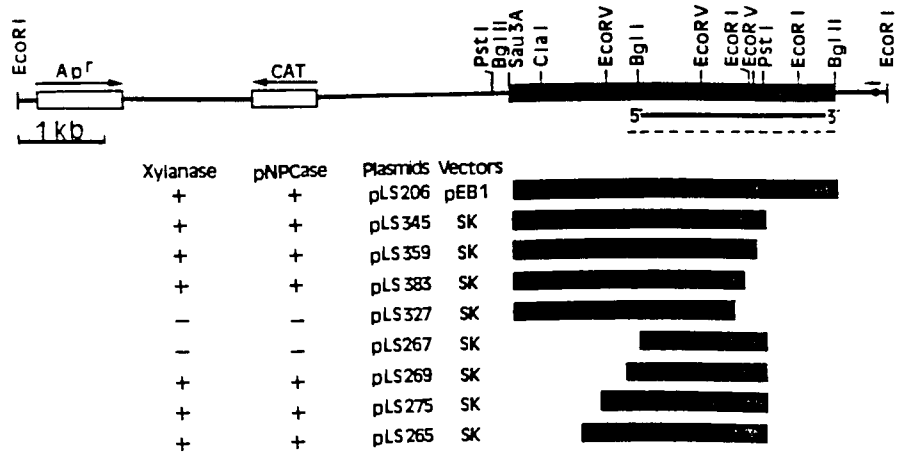

Fig. 3.1 Restriction map of pLS206 and shortening strategy in Bluescript SK. Single and bold lines and open boxes represent vector DNA, *B. fibrisolvens* insert DNA and antibiotic resistance genes, respectively. The circle and arrow above denote the position and direction of the lambda rightward promoter. The extent of the *xyn*B transcript is indicated by the solid line below the restriction map. The broken line indicates the extent of the nucleotide sequence shown in Fig. 3.4. + and - denote enzyme activity or lack thereof exhibited by the various subclones. pNPCase, p-nitrophenyl-β-D- cellobiosidase.

The origin of the cloned fragment in pLS206 was determined by hybridization of [α - ^{32}P] dCTP labelled 2.3 kb *Bgl*I - *Bgl*II fragment to restriction endonuclease digests of *B. fibrisolvens* chromosomal DNA (Fig. 3.2). *Bgl*I/ *Pst*I endonuclease digestion of pLS206 produced one internal insert fragment (1.5 kb) which hybridized to an identically sized *B. fibrisolvens* DNA fragment (Fig. 3.2 lane 2). Digestion of pLS206 with *Pst*I/ *Cla*I endonuclease produced a 2.6 kb internal insert fragment. This fragment was bound to the same size fragment of *B. fibrisolvens* chromosomal DNA (Fig. 3.2, lane 3). The *Pst*I site in pLS206 insert resulted in a second hybridizing band (Fig. 3.2, lane 3).

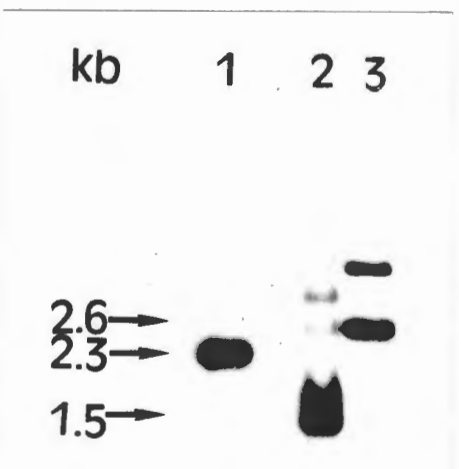

Fig. 3.2 Southern blot analysis of the 2.3 kb *Bgl*I - *Bgl*II fragment against complete digests of genomic DNA from *B. fibrisolvens*. Lane 1, 2.3 kb *Bgl*I - *Bgl*II fragment; lane 2, *B. fibrisolvens* DNA digested with *Bgl*I and *Pst*I; lane 3, *B. fibrisolvens* DNA digested with *Cla*I and *Pst*I.

A 3.0 kb *Pst*I fragment was subcloned into Bluescript SK in both orientations. A serial deletion analysis of the insert

identified a 1.3 kb region between an *Eco*RI and an *Eco*RV site as being essential for enzyme activity. All deleted clones which exhibited xylanase activity (0.31 U/mg protein) also showed pNPCase activity (0.081 U/mg protein) (Fig. 3.1). Analysis of plasmid-encoded proteins *in vitro* showed that the insert of pLS206 directed the synthesis of a protein having an apparent mobility of approximately 74,000 (Fig. 3.3). The shortened clones pLS345, pLS383 and pLS269 produced proteins with molecular masses of approximately 60,000, 43,000 and 56,000 respectively. It was therefore apparent that although the shortened clones produced active xylanase enzyme, parts of the C-terminus had been removed.

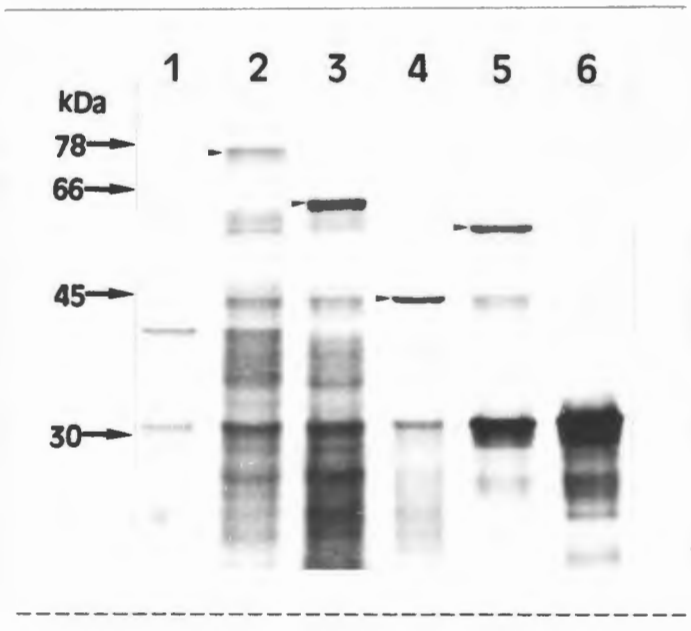

Fig. 3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of ^{35}S-labelled proteins synthesized *in vitro*. Lane 1, pEB1; 2, pLS206; 3, pLS345; 4, pLS383; 5, pLS269; 6, bluescript SK. The small arrow heads indicate the positions of the xylanase enzyme encoded by the various plasmids.

We had expected that the proteins encoded by pLS345 and pLS269 would be the same size as they contain the same 3' end and later mucleotide sequencing showed that both carried the intact 5' end of the *xyn*B gene (Fig. 3.1). On the basis of these data both should encode a protein of 486 amino acids (ca. 56,000). While pLS269 encoded a protein of the expected size, pLS345 directed the synthesis *in vitro* of a protein with an apparent molecular mass of 60,000. We conclude that this is due to readthrough into the vector.

The localisation experiments revealed that 89% to 94% of the xylanase activity was present in the cytoplasm of *E. coli* (Table 3.1). Control experiments showed 98% to 99% of the β-galactosidase activity was located in the cytoplasm and 81% of the total β-lactamase activity was present in the periplasm.

Table 3.1 Distribution of xylanase in E. coli C600 harbouring plasmids

Fraction	Total activity (Units)		
	xylanase (%)	β-Galactosidase (%)	β-Lactamase (%)
pLS206			
Supernatant	6.7 (3)	40.0 (<1)	4.6 (1)
Periplasmic	16.5 (8)	862.0 (2)	250.0 (81)
Cytoplasmic	177.0 (89)	43611.0 (98)	54.2 (18)
pLS383			
Supernatant	8.4 (3)	260.0 (<1)	30.1 (6)
Periplasmic	7.6 (3)	182.0 (<1)	396.2 (81)
Cytoplasmic	250.0 (94)	74500.0 (99)	66.4 (13)

3.3.2 *DNA sequence of xyn*B. The nucleotide sequence of *xyn*B and the deduced amino acid sequence are shown in Fig. 3.4. A 1,905 bp open reading frame (ORF) starting at ATG (position 241) and terminating at TAG (position 2,148) was found which was capable of coding a peptide of 635 amino acid residues with a calculated molecular mass of 73,156 daltons. A ribosome binding site (RBS), GGAGG, was situated 4 bp upstream of the presumptive translational start codon. At 37 bp downstream of the *xyn*B TAG stop codon there is an inverted repeat of 10 nucleotides (position 2186-2208). The transcript would have the potential to form a stem-loop structure but as its ΔG = -2.9 Kcal/mol (Salser, 1977) it is unlikely to act as a rho-independent transcription terminator. The typical hairpin loop transcription terminus has been found at the 3' end of several bacterial cellulase genes (Greenberg *et al.*, 1987; Grepinet and Beguin, 1986; Mackay *et al.*, 1986), suggesting that most of these sequences are monocistronic. The termination of transcription of the *B. fibrisolvens* xylanase gene could be dependent on an additional factor like the *rho* protein. These complex forms of transcription terminations are not well known (Platt and Bear, 1983). The G + C base composition of the *xyn*B coding sequence is 41.7 mol % which compares well with the average G + C content of *B. fibrisolvens* which is 42 mol % (Mannarelli, 1988). The XynB C-terminus deletions synthesized by plasmids pLS345, pLS359, and pLS327 showed no apparent change in enzymatic activity, indicating that the last 250 amino acids could be removed without loss of activity. However, removing the last 289 amino acids (pLS327) abolished activity (Fig. 3.1).

Deleting 71 bp (positions 1 to 71; pLS269) from the upstream region did not affect the expression of *xyn*B, but when 185 bp (positions 1 to 185) were removed, the resulting clone (pLS267) showed no xylanase or pNPCase activity (Fig. 3.1). This indicates that the upstream region must play a role in the expression of *xyn*B, although no obvious *E. coli*-like -35 or -10 consensus sequences could be detected.

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ATTGTAGAATAATCTCCATTTCCTTTTTTACATGTACTTCAAACATTCTTTTGCTTTCAT     60

TTATATAGGTAGGTATTTGCGCTTAACAGAGCGCTTTGATGGCCTTCAAATGGCTTTAGT    120
           └►pLS269
CAATATTCAAGCAGAAATGCGCAACTATAAGTATTATCTTTTCACCGGTTCGTATATCAT    180

ATGGTAACATAAGGCAATTTAAATGCCTACATCTAACGATAAAAAAGCCTTGGAGGCAAT    240
   └►pLS267                                          R B S
ATGAATCTTAAAACAGCTTACGAACCATATTTTAAAATCGGTGCTGCAATTTCCAGATGG    300
M  N  L  K  T  A  Y  E  P  Y  F  K  I  G  A  A  I  S  R  W

AATCTGCATACACCGGCTCATACTAAGCTTTTGGCTGAACAGTTTAACAGCTTTACCTGT    360
N  L  H  T  P  A  H  T  K  L  L  A  E  Q  F  N  S  F  T  C

GAAAACGACATGAAGCCTATGTACTACCTGGACAGAGAAGCTAACAAAAAAGATCCCGAG    420
E  N  D  M  K  P  M  Y  Y  L  D  R  E  A  N  K  K  D  P  E

AAATACAATCTTTCTCCGGCTCTTACCTTTGAAAACGCCATTCCCTATCTTGAATTTGCC    480
K  Y  N  L  S  P  A  L  T  F  E  N  A  I  P  Y  L  E  F  A

AAAGATAATAAAATTGCCATGAGAGGACACACTCTGGTTTGGCACAACCAGACTCCAAAG    540
K  D  N  K  I  A  M  R  G  H  T  L  V  W  H  N  Q  T  P  K

TGGTTTTTCTGCGAAAGATATAACGAGAACTTTCCCATGGCTGATCGGGAGACTATCCTT    600
W  F  F  C  E  R  Y  N  E  N  F  P  M  A  D  R  E  T  I  L

GCAAGACTTGAAAGCTATATTCACGGAGTCCTTGATTTCGTACAGACAAATTACCCGGGA    660
A  R  L  E  S  Y  I  H  G  V  D  L  F  V  Q  T  N  Y  P  G

ATAATTTATGCCTGGGATGTTGTAAATGAAATAGTAGATGAGGGCGCATTTAGAAAATCC    720
I  I  Y  A  W  D  V  V  N  E  I  V  D  E  G  A  F  R  K  S

ATCTGGACAGAAACTGTCGGAGAGGATTTCTTTATCAAAGCTTTTGAATTTGCCAGAAAA    780
I  W  T  E  T  V  G  E  D  F  F  I  K  A  F  E  F  A  R  K

TATGCGGCTCCGGAGGTATCTCTTTTCTACAACGACTATGAAACAGCCCAGCCCTGGAAA    840
Y  A  A  P  E  V  S  L  F  Y  N  D  Y  E  T  A  Q  P  W  K

AGAGATTTCATTTTAGAAAAAGTACTAGGGCCTCTTATTGACAAAAAACTGATTGATGGA    900
R  D  F  I  L  E  K  V  L  G  P  L  I  D  K  K  L  I  D  G

ATGGGAATGCAGTCCCATCTTCTCATGGATCATCCTGATATCAGTGAGTATCGTACAGCA    960
M  G  M  Q  S  H  L  L  M  D  H  P  D  I  S  E  Y  R  T  A

CTCGAAATGTACGGATCAACCGGACTTCAGATTCACATAACAGAGCTTGATATGCACAAC   1020
L  E  M  Y  G  S  T  G  L  Q  I  H  I  T  E  L  D  M  H  N
```

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GCTGATCCAAGCGAAGAATCAATGCACGCTTTAGCGACAAGGTATCAGGAATTTTTCCAA  1080
A  D  P  S  E  E  S  M  H  A  L  A  T  R  Y  Q  E  F  F  Q

ACATACCTTGATGCCAAAAAATCAGGCAAAGCTAATATCACAAGTGTAACCTTTTGGAAT  1140
T  Y  L  D  A  K  K  S  G  K  A  N  I  T  S  V  T  F  W  N

CTCCTGGATGAGAACAGCTGGCTCTCAGGTTTTAGACGTGAAACGAGCTACCCACTTGTA  1200
L  L  D  E  N  S  W  L  S  G  F  R  R  E  T  S  Y  P  L  V

TTTAAGGGAAAATGCGAAGCCAAAGAAGCTTACTACGCAGTTCTAAAAGCTGCTGTATCA  1260
F  K  G  K  C  E  A  K  E  A  Y  Y  A  V  L  K  A  A  V  S

GATGATTCAATTGACAAATGGGTTCCTGATTATAGCGAGGAAGATTATAAGCTTCAGGGA  1320
D  D  S  I  D  K  W  V  P  D  Y  S  E  E  D  Y  K  L  Q  G
          pLS327
ATGCCAACGCCTGATATAAAGCGCTTTAGAGAGAACATCTGGCAAGAAAATGAATATAAC  1380
M  P  T  P  D  I  K  R  F  R  E  N  I  W  Q  E  N  E  Y  N

TACGAAGCTTCCTATGGTTTTATTCCTAATCTTTTTGCTTATCTTCACAATGATGACGTC  1440
Y  E  A  S  Y  G  F  I  P  N  L  F  A  Y  L  H  N  D  D  V
       pLS383
AAGCGTGATTGTATGCTGGTCATACCAGGAGGTGGCTATTGCATGTGCTGCTCTCACGAG  1500
K  R  D  C  M  L  V  I  P  G  G  G  Y  C  M  C  C  S  H  E

GGCGAGCTTGCAGCTATGGAATTCTACAACCGCGGAATGAATGCCTTTGTACTGAGCTAC  1560
G  E  L  A  A  M  E  F  Y  N  R  G  M  N  A  F  V  L  S  Y

ACTACAGATATCACTATGTCTGTCCCACTTCATAAACAACCTCTTGAAGATATATCAAGA  1620
T  T  D  I  T  M  S  V  P  L  H  K  Q  P  L  E  D  I  S  R
                        pLS359
GCAGTAAGATTTATCAGAAAGAATGCTTCCAAATACAATATAGATGGCAAAAAGCTCGTT  1680
A  V  R  F  I  R  K  N  A  S  K  Y  N  I  D  G  K  K  L  V

ATCATGGGCTTTTCTGCAGGTTCTCACGTATGTGGAAGTCTTGCTGTCCATTTTGATGAC  1740
I  M  G  F  S  A  G  S  H  V  C  G  S  L  A  V  H  F  D  D
                              pLS345
GTTAAAGATAATAATCCTGAATATGCTGATATTTCAGGTAGGCCCGATGGTGTCATACTA  1800
V  K  D  N  N  P  E  Y  A  D  I  S  G  R  P  D  G  V  I  L

TCCTATCCTGTTATAACAACAGGTAGGTATACTCACGCTGACTCTGTCAGAACACTTTTA  1860
S  Y  P  V  I  T  T  G  R  Y  T  H  A  D  S  V  R  T  L  L

GGAGCTAATCCTACAGATGAAGAGCTTACATACTTCTCACTTGAGAAGCAGGTTAAAGAT  1920
G  A  N  P  T  D  E  E  L  T  Y  F  S  L  E  K  Q  V  K  D

AATACTCCACCCTGCTTTATCTGGCAGACCGAGGAGGACAGCGTAGTTCCTGTAGAGAAC  1980
N  T  P  P  C  F  I  W  Q  T  E  E  D  S  V  V  P  V  E  N

AGCTACCTATTTGCCAATGCTCTCAGAGAAAAGAAAATTCCTTTTGCCCACTACGTATTT  2040
S  Y  L  F  A  N  A  L  R  E  K  K  I  P  F  A  H  Y  V  F

CCAAGAGGCTTCCACGGACTTACAGTAGCCAATGATGAATTCTTCTCAGGCTGGTCAGGC  2100
P  R  G  F  H  G  L  T  V  A  N  D  E  F  F  S  G  W  S  G

GGTGAATACTCCATGGAGCAGACAATGCGCGCACGTTTCGCCGTATAGGAAGGAAAAGGA  2160
G  E  Y  S  M  E  Q  T  M  R  A  R  F  A  V  *

GTAAATGTCTCCGAAAAAAGAAGGGAAGAACTTACACAGCAAGTTCTTTAGTGGCAAGGT  2220
```

Fig. 3.4 Nucleotide sequence of the *B. fibrisolvens xyn*B structural gene and flanking regions. The vertical lines and associated arrows indicate the position and direction of the various deletions. The putative ribosome-binding sequence (RBS) is boxed. An inverted repeat sequence is shown by converging arrows.

3.3.3 ***Sequence comparison with other cellulolytic organisms.*** The predicted amino acid sequence of the xylanase was compared with sequences in the GenEMBL data base using TFASTA and BESTFIT programmes from the Genetics Computer Group sequence analysis package (Devereux *et al.*, 1984). Homology was found between XynB enzyme and the cellobiohydrolase/ endocellulase (CelB) of *C. saccharolyticum* (Saul *et al.*, 1989) as well as the xylanases of the alkaliphilic *Bacillus* sp. strain C-125 (Hamamoto *et al.*, 1987) and *P. fluorescens* subspecies *cellulosa* (Hall *et al.*, 1989). The alignment of the deduced amino acid sequences of the xylanases from *B. fibrisolvens* strains H17c and 49, *Bacillus* sp. strain C-125 and the CelB of *C. saccharolyticum* is shown in Fig. 3.5. The degree of amino acid identity between XynB and CelB was 40% and between XynB and the *Bacillus* xylanase was 38%. When amino acid replacement by conserved amino acids was taken into account, the similarity increased to 60% and 61% respectively. The degree of identity between the xylanases of *B. fibrisolvens* strains H17c and 49 was 32%. There was a region of extended homology between the N-terminal region of XynB, which comprises about 50% of the protein and the C-terminal region of XynA from *P. fluorescens*. There were 25% identical residues in these regions.

3.3.4 ***Codon usage.*** Choice among synonymous codons in both prokaryotic and eukaryotic genes is clearly non-random, although the choice does not affect the nature of the protein synthesized. Codon-choice patterns of microorganisms are known to reflect their G+C genome content

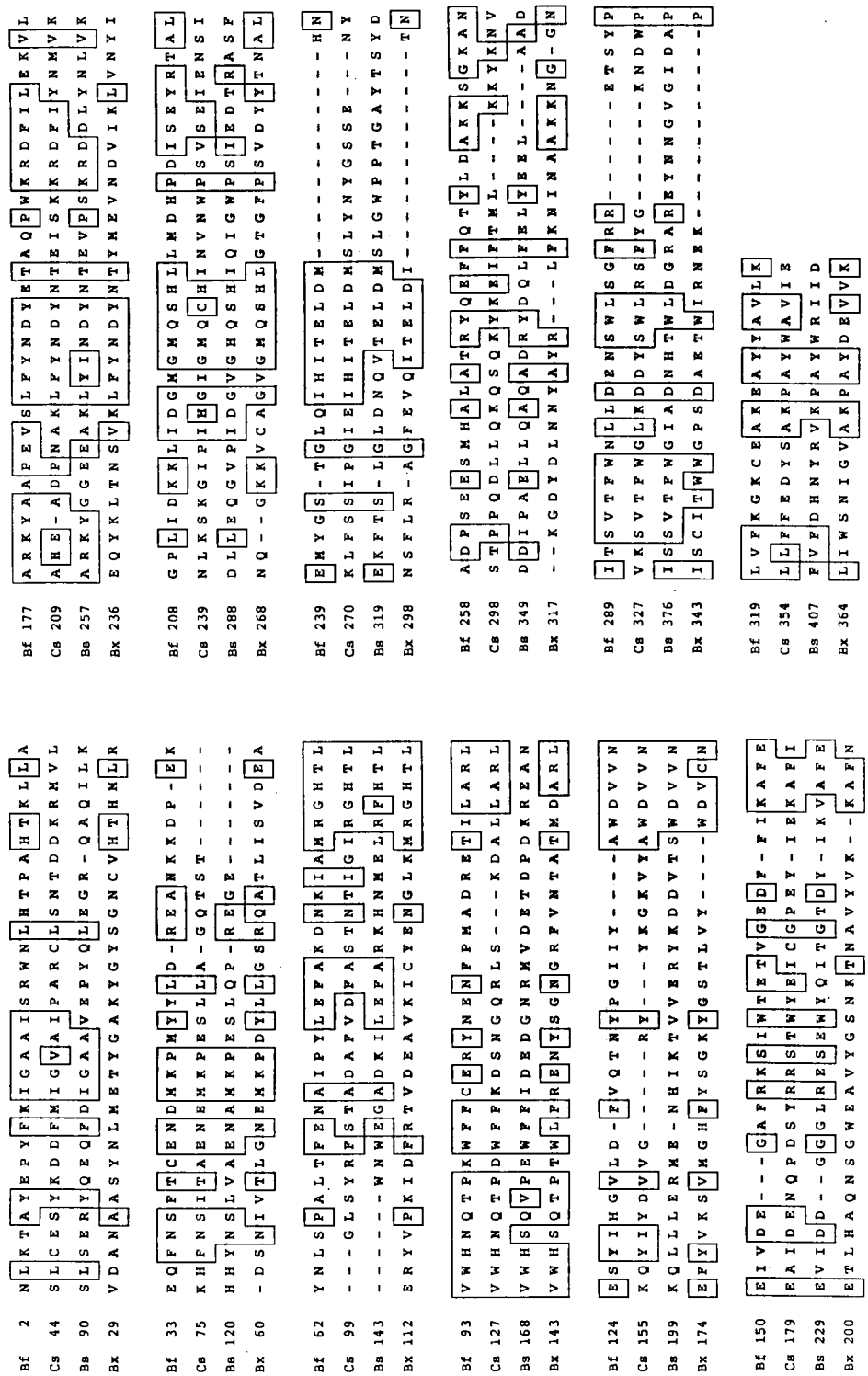

Fig. 3.5 Amino acid sequence alignments of the xylanases from *B. fibrisolvens* H17c (Bf), *C. saccharolyticum* (Cs), *Bacillus* sp. strain C-125 (Bs) and *B. fibrisolvens* 49 (Bx). The amino acids are identified by the single letter code and regions of identical residues are boxed. The alignment was maximised by introducing gaps denoted by a dash (-). Numbers on the left refer to the first amino acid in each line.

(Nichols *et al.*, 1981; Yanofsky and vanCleemput, 1982; Kagawa *et al.*, 1984; Muto *et al.*, 1984). In addition, codon usage by bacteria also reflects their requirement for speed of translation (Klump and Maeder, 1991). In contrast to eucaryotes, whose choice of codon is based on a requirement for accuracy, bacteria appear, to some extent, to have sacrificed precision for speed. The preferred codons are usually of intermediate stability and the frequency of G or C in the position III serves to counterbalance the average local GC frequencies in positions I and II. A similar proposal was made by Grantham *et al.*(1981).

In the eight genes of *B. fibrisolvens* analyzed, quartet codons are rich in G+C in positions I and II, while duet codons are poor (Table 3.2). Therefore, in agreement with the above proposal, quartet codons favour A or T in position III while the duets and the one trio (isoleucine) show no preference. This compare well with the codon-anticodon pairs of intermediate energy that are favoured in the highly expressed *E. coli* genes (Grantham *et al.*, 1981).

Codons of strongest pairing energy always have a CC, CG, GC or GG doublet in positions I and II (Claverie, 1971). These doublets are found in the quartet codons of proline, arginine, alanine and glycine. In agreement with the above proposal all the genes analyzed showed a preference for A or T in position III, except for the glycine codon GGC in *bgl*A. Interestingly, the codon for proline preferred by *E. coli* is CCG, which releases the most energy upon pairing with its anticodon (Grantham *et al.*, 1981). This is not found among

the genes from *B. fibrisolvens* analyzed where CCT is the preferred codon. Apart from methionine and tryptophan, which are each encoded by one codon, there are 13 codons which end in G. Among these only AAG, CAG and GAG are preferred codons for lysine, glutamine and glutamic acid respectively. Thus, in these cases *B. fibrisolvens* again tends to prefer codons of intermediate energy.

Table 3.2 Synonymous codon usage in genes from *B. fibrisolvens*[a]

Amino acid	Codon	Usage[b]							
		*xyn*B	*ced*1	*end*1	*bgl*A	*xyn*A	*cel*A	*amy*1	*glg*B
Arg[c]									
	CGG	1	0	0	0	0	0	0	0
	CGA	0	1	0	0	0	0	0	0
	CGT	4	1	3	5	4	0	2	2
	CGC	3	1	0	1	1	2	0	3
	AGG	3	1	4	6	1	1	2	5
	AGA	16	10	13	23	9	8	19	12
Leu[c]									
	CTG	7	7	2	2	4	0	5	5
	CTA	4	3	0	2	1	0	0	1
	CTT	26	28	14	41	18	21	42	16
	CTC	6	4	2	18	0	0	6	13
	TTG	1	6	1	2	2	4	7	5
	TTA	3	9	3	2	3	7	3	3
Ser[c]									
	TCG	0	0	0	1	1	0	1	0
	TCA	11	4	16	19	12	7	23	9
	TCT	7	12	12	9	5	5	20	4
	TCC	7	4	6	6	2	2	4	3
	AGT	3	6	3	1	0	2	8	5
	AGC	9	5	9	13	5	4	23	7
Thr									
	ACG	2	0	1	0	2	0	0	1
	ACA	18	10	23	29	21	8	45	16
	ACT	9	8	10	7	3	8	23	5
	ACC	5	1	1	5	4	3	5	4
Pro									
	CCG	4	4	6	6	0	1	5	8
	CCA	9	7	10	9	9	4	7	10
	CCT	12	6	17	20	5	7	16	10
	CCC	6	1	2	3	0	0	1	2
Ala									
	GCG	2	4	3	2	0	1	7	3
	GCA	9	14	15	39	17	12	38	11
	GCT	24	24	13	15	6	17	31	17
	GCC	13	6	5	13	5	2	9	8

Table 3.2-continued

Amino acid	Codon	Usage[b]							
		*xyn*B	*ced*1	*end*1	*bgl*A	*xyn*A	*cel*A	*amy*1	*glg*B
Gly									
	GGG	1	1	1	1	2	0	3	1
	GGA	15	27	13	22	11	7	45	20
	GGT	9	7	9	19	10	9	19	17
	GGC	9	9	10	30	6	7	25	18
Val									
	GTG	0	7	2	5	4	5	1	2
	GTA	16	13	21	16	17	8	28	15
	GTT	9	17	24	26	9	15	26	12
	GTC	8	0	0	16	2	5	5	2
Lys									
	AAG	11	24	19	38	16	24	28	37
	AAA	27	16	6	23	15	12	11	17
Asn									
	AAT	20	21	26	12	23	16	37	17
	AAC	14	7	17	21	11	11	28	11
Gln									
	CAG	11	10	13	25	9	7	20	10
	CAA	3	0	0	1	2	4	1	3
His									
	CAT	6	9	5	5	6	6	8	15
	CAC	12	2	3	4	1	2	4	4
Glu									
	GAA	3	8	2	5	11	16	47	19
	GAG	6	4	7	8	7	27	28	21
Asp									
	GAT	32	32	31	26	16	34	68	32
	GAC	9	9	12	24	1	6	14	18
Tyr									
	TAT	18	24	19	19	16	14	44	24
	TAC	19	5	9	20	14	5	14	20
Cys									
	TGT	3	8	2	5	3	2	2	3
	TGC	6	4	7	8	2	2	4	5
Phe									
	TTT	26	21	12	16	12	5	17	24
	TTC	12	6	8	15	6	5	14	15
Ile									
	ATA	9	3	7	6	2	4	7	11
	ATT	13	13	13	18	10	15	27	12
Met									
	ATG	20	12	12	13	11	11	19	20
Trp									
	TGG	12	8	13	11	8	10	19	18

[a] *Xyn*B, xylanase (Lin and Thomson, 1991); *ced*1, cellodextrinase, (Berger *et al.*, 1990); *end*1, endoglucanase (Berger *et al.*, 1989); *bgl*A, β-glucosidase (Lin *et al.*, 1990); *xyn*A, xyalnase (Mannarelli *et al.*, 1990); *cel*A, endoglucanase (Hazlewood *et al.*, 1990); *amy*1, α-amylase (Rumbak *et al.*, 1991); *glg*B, glucose glycosylation enzyme (Rumbak, personal communication).

The frequency of codon usage of *xyn*B was compared with E. coli high and low expression genes and with the same *B. fibrisolvens* genes used in the above analysis using CORRESPOND program from GCG sequence analysis package (Devereux *et al.*, 1984). The frequency of codon usage of all these *B. fibrisolvens* genes is closely related to that of low expression genes of *E. coli* (Table 3.3). Correlation (distance squared value <3.4) in codon frequencies is found between the *xyn*B and the other seven genes from *B. fibrisolvens*. The *ced*1, *amy*1, and *glg*B genes show different codon frequencies compared with *bgl*A although those were cloned from the same strains H17c, whereas the codon usage of *ced*1 corresponds to that of *xyn*A cloned from a different strain, 49. However, the D-values between *xyn*A from strain 49 and five genes from strain H17c are <1.7 suggesting that the two strains have a similar selection of codons.

Table 3.3 Correspondence analysis on frequencies by the usage of 64 codons

Gene	Distance squared value[a]									
	1	2	3	4	5	6	7	8	9	10
1.Ecohigh	0.0	3.4	5.2	8.0	6.5	5.2	7.7	7.0	7.3	6.0
2.Ecolow		0.0	2.1	2.8	2.9	2.5	2.8	2.4	3.0	1.8
3.*Xyn*B			0.0	1.9	1.2	1.2	1.7	1.5	3.3	3.1
4.*Ced*1				0.0	1.2	4.1	1.4	3.1	0.9	1.2
5.*End*1					0.0	2.7	1.3	3.0	0.3	0.6
6.*Bgl*A						0.0	3.6	2.4	2.9	2.9
7.*Xyn*A							0.0	3.3	1.3	1.2
8.*Cel*A								0.0	2.7	3.0
9.*Amy*1									0.0	0.8
10.*Glg*B										0.0

[a] $d^2(i_1 i_2) = \sum_{j=1}^{64} (f_{i_1 j} - f_{i_2 j})$, where $f_{i_1 j}$ and $f_{i_2 j}$ are the frequencies of codon j in mRNA i_1 and i_2. The square of the distance between two mRNAs is the sum of the squares of their differences in the use of each of the 64 codons.

3.4 Discussion

The xylanase encoded by pLS206 was approximately 74 kDa in size. However at least 850 bp at the 3' end of the gene could be deleted without loss of enzymatic activity. The catalytic function of some prokaryotic cellulases is vested in a clearly defined independent catalytic domain which may account for less than half of the total gene (Beguin, 1990; Gilbert *et al.*, 1990; Gough *et al.*, 1990; Yague *et al.*, 1990). In such cases, a substantial non-essential portion of the gene may be deleted without affecting the catalytic function of the encoded protein. To determine whether the C-terminal region of XynB might be a binding domain it was scanned for the features common to substrate binding domains of endoglucanases. These include a short segment (10 to 30 residues) rich in proline and hydroxy amino acids separating the catalytic and binding domains, and direct repeats in the C-terminal region of the latter (Beguin, 1990). None of these features was found in the deduced amino acid sequence of XynB. In addition a computer search using the non-catalytic domain of XynB failed to reveal similarities with the non-catalytic regions of the cellulases and xylanases in the GenEMBL database. Therefore the function of the non-catalytic C-terminus region remains to be determined. It was of interest to note that the xylanase Z of *C. thermocellum*, which has a central region of 60 amino acids containing a duplicated segment of 24 amino acids that can be deleted without loss of activity (Grepinet *et al.*, 1988), showed no significant homology to the C-terminus region of XynB.

Although the size of the deduced amino acid sequence agreed well with the apparent molecular mass of the protein synthesized *in vitro* by pLS206, certain anomalies were observed in the nucleotide sequence. No consensus -35 and -10 promoter sequences were detected and deletion of 185 bp upstream of the *xyn*B gene abolished xylanase activity. Therefore to confirm that the deduced amino acid sequence was correct, the amino acid sequence of the N-terminus of the purified xylanase was determined (Chapter 4.3.2). The first seven amino acids were identical to the deduced sequence. In addition G + C base composition of the *xyn*B gene is 41.7 mol% which compares well with the average G + C content of *B. fibrisolvens* which is 42 mol% (Mannarelli, 1988).

Comparison of the XynB protein sequence of strain H17c with a number of other xylanases and cellulases showed there was significant homology with the cellobiohydrolase/ endocellulase of *C. saccharolyticum,* and the xylanases of the alkalophilic *Bacillus* sp. strain C-125 and *B. fibrisolvens* strain 49. The enzymes had, respectively, 40%, 38% and 32% identical residues compared with XynB. The similarities were predominantly in the N-terminal regions of the enzymes but there was also a region of homology between the N-terminus region of XynB and the C-terminal region of XynA from *P. fluorescens* subspecies cellulosa, both of which contain the catalytic domain.

In order for the XynB enzyme to catalyze xylan degradation it is presumably secreted by *B. fibrisolvens* H17c. However,

it does not have a hydrophobic signal sequence common to many secreted proteins and was located in the cytoplasm in *E. coli*. The xylanase gene cloned by Mannarelli *et al.* (1990) from *B. fibrisolvens* strain 49 does have such a sequence but was not secreted by *E. coli*. Both an endoglucanase and a cellodextrinase cloned from *B. fibrisolvens* H17c (Berger *et al.*, 1989; Berger *et al.*, 1990) were secreted into the *E. coli* periplasm but only the former carried a signal sequence. Therefore it is not possible to conclude how *B. fibrisolvens* secretes proteins. Wang and Thomson (1990) reported the cloning of a cellodextrinase gene from another rumen anaerobe, *R. flavefaciens*, into *E. coli* where the enzyme encoded was secreted into the periplasm without a signal sequence. It will therefore be of interest to address the question of protein secretion by rumen anaerobes which are providing such a wealth of cellulolytic and hemicellulolytic enzymes.

Chapter 4

Purification and properties of an endo-1,4-β-xylanase translated from a *Butyrivibrio fibrisolvens* H17c gene in *Escherchia coli*

Chapter 4

Purification and properties of an endo-1,4-β-xylanase translated from a *Butyrivibrio fibrisolvens* H17c gene in *Escherichia coli*

4.0 Summary

An endoxylanase encoded by the *xyn*B gene of *B. fibrisolvens* expressed in *E. coli* was purified by anion-exchange chromatography, gel filtration, and chromatography on hydroxylapatite. The enzyme, with a molecular weight (M_r) of 72,000 showed maximal xylanase activity between pH 5.4 and pH 6.0 and at a temperature of 55°C. The amino acid sequence of the enzyme at the N-terminus was Asn- Leu- Lys- Thr- Ala- Tyr-. The enzyme displayed activity against xylan, p-nitrophenyl-β-D-cellobioside, and p-nitrophenyl-β-D-xylopyranoside. In a 12 h incubation with oat spelt xylan, the major products were xylose, xylobiose and xylooligosaccharides with the polymerization degree of over 3. The xylanase activity was enhanced in the presence of Na^+, K^+, NO_3^-, and SCN^- but effectively inhibited by Ag^+, Cu^{2+}, Zn^{2+} and Ca^{2+}.

4.1 Introduction

The conversion of lignocellulosic materials to energetic, chemical and nutritional feedstocks is one of the most studied fields of research (Bisaria and Ghose, 1981; Magee and Kosaric, 1985). 15 to 30% of total dry weight of lignocellulose is represented by hemicelluloses, mainly xylan (Dekker and Lindner, 1979). There is considerable economic interest in the enzymatic hydrolysis of lignocellulosic biomass to sugars by a combination of hemicellulases and cellulases.

To ensure optimal enzyme production and facilitate purification, the xylanases characterized so far have generally been isolated from cultures grown in liquid medium. Several studies have been carried out on the production of xylanase using natural substrates. Solka floc was used as carbon source for producing xylanases of *Trichoderma harzianum* (Tan *et al.*, 1985a). With *Schizophyllum radiatum* xylanase production reached maximum yield when the medium contained wheat straw as sole carbon source (Cavazzoni *et al.*, 1989). Among the xylanolytic bacteria, *Clostridium acetobutylicum* (Lee *et al.*, 1987) and *Bacillus stearothermophilus* (Nanmori *et al.*, 1990) can produce xylanase in liquid medium containing oat spelt xylan. In addition, larchwood xylan was used as the sole carbon component in production of xylanases from alkalophilic thermophilic *Bacillus* spp. (Okazaki *et al.*, 1984). There are several reports of xylanase genes cloned in high copy number vectors (Sipat *et al.*, 1987; Yang *et*

al., 1989; Zappe *et al.*, 1987) such as pUC vectors. Such overexpressed xylanases aid enzyme purification.

In the purification procedure of cellulolytic enzymes, adsorption to cellulose and ion-exchange chromatography were the earliest methods used. Molecular sieving, chromatofocussing and affinity to different specific sorbents have also been used (Enari and Niku-Paavola, 1987). Difficulties in purification were found due to the close similarity in physicochemical properties of the hydrolytic enzymes of cellulolytic microorganisms (Enai and Niku-Paavola, 1987). In order to be considered biochemically pure, an enzyme must show a clearly defined enzymatic activity connected with a homogeneous property. The homogeneity of the purified xylanolytic enzymes have been determined by electrophoresis in denaturing conditions (Dobberstein and Emeis, 1989; Cavazzoni *et al.*, 1989), by isoelectric focussing (Nanmori *et al.*, 1990; Lee *et al.*, 1987; Tan *et al.*, 1985a; Mishra *et al.*, 1990), and by immunoelectrophoresis (Zappe *et al.*, 1987). According to these criteria each of the xylanolytic enzyme preparations reported by different groups has been homogeneous.

The existing confusion regarding the properties of xylanolytic enzymes is caused by the difficulties in obtaining pure but fully active enzymes. The application of recombinant DNA techniques provided a new approach to the study of the properties of these enzymes. The cloning of a gene into a non-xylanolytic host enables one to obtain an individual enzyme free from other xylanolytic enzymes. In

this chapter, the purification and characterization of a xylanase encoded by a *B. fibrisolvens* gene cloned in *E. coli* is described.

4.2 Materials and Methods

4.2.1 *Enzyme preparation.* *E. coli* JM103 (pLS206) was grown in 1 l LB medium containing ampicillin (100 µg/ml) and 5 mM $CaCl_2$. The cells were harvested at the early stationary phase and resuspended in 20 ml 50 mM sodium phosphate buffer (pH 6.0). After the pellet was dissolved completely, the cells were sonicated on ice for nine 10 s bursts with 10 s intervals and clarified by centrifugation for 15 min at 27,000 x g at 4°C.

4.2.2 *Enzyme activity assays.* Xylanase activity was assayed by the procedure described in Chapter 3 (3.2.10).

4.2.3 *Purification of xylanase enzyme.* The crude extract was applied to a column (2.6 x 40 cm) of DE-52 (Whatman) that was equilibrated with starting buffer (50 mM sodium phosphate buffer, pH 6.0). After the sample was applied, the column was washed with 1 bed volume of starting buffer, and then 800 ml of a linear buffer gradient from 0 to 500 mM NaCl was applied. The flow rate was 20 ml/h, and 5 ml fractions were collected. The xylanase peak fractions eluting in the buffer gradient were concentrated and desalted by ultrafiltration through a membrane (PM-10; Amicon Corp. Lexington, Mass.). The concentrated enzyme solution was reapplied to a DE-52 column (1.5 x 40 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.8). After washing with 200 ml of starting buffer, 600 ml of a linear buffer gradient from 0 to 500 mM NaCl was applied. The column was eluted at 20 ml/h, with a fraction size of

5 ml. The fraction containing xylanase activity was pooled, concentrated, and applied to a Sephacryl S-200 column (1.5 x 100 cm) equilibrated with 100 mM NaCl in 50 mM sodium phosphate buffer at a flow rate of 12 ml/h, and 3 ml fractions were collected. Active fractions were concentrated and applied to a DNA grade BioGel hydroxylapatite column (1.5 x 20 cm). The column was eluted with a 200 ml linear gradient of sodium phosphate (pH 6.8) from 50 to 500 mM. The flow rate was 15 ml/h and 2 ml fractions were collected. Active fractions were concentrated by ultrafiltration with a membrane (pM-10; Amicon) and the proteins analysed by SDS-PAGE as described in Appendix B.

4.2.4 ***Detection of xylanase activity in gels.*** Proteins were separated by electrophoresis on 10% polyacrylamide gels in the absence of SDS. Xylanase activity was detected by overlaying the gel with a polyacrylamide gel overlay containing 0.1% (w/v) xylan, incubating in the humidity chamber at 37°C for 90 min, staining with 0.1% (w/v) Congo red, destaining with 1 N NaCl and fixing with 5% acetic acid.

4.2.5 ***N-terminal amino acid sequence determination.*** The 72,000 Da protein which showed xylanase activity was subject to N-terminal amino acid sequence analysis using a gas-phase sequencer, coupled with HPLC for identification of the resulting amino acid phenylthiohydantoins.

4.2.6 ***Determination of optimal pH and temperature.*** The effect of pH on xylanase and pNPCase activity was measured over a range of 4 to 10 by using sodium phosphate-citric acid (pH 4 to 7), sodium phosphate (pH 6 to 8) and glycine buffers (pH 8 to 10) under standard assay conditions. The temperature optimum was determined by adding enzyme solutions to substrate prewarmed at temperatures from 40°C to 75°C and assaying at that temperature.

4.2.7 ***Substrate specificity.*** Potential substrates (oat spelt xylan [0.5%, w/v], CMC [0.5%, w/v], lichenan [1%, w/v], laminarin [1%, w/v], salicin [1%, w/v], pNPC [3.4 mM], pNPG [6 mM], or pNPX [6 mM]) in 50 mM PB buffer (pH6) were vortexed and incubated with purified enzyme at 55°C for 15 min. The assays were performed as described in Chapter 2 (2.2.12).

4.2.8 ***Analysis of hydrolysis.*** The purified xylanase was incubated with oat spelt xylan (0.5%, w/v) in 50 mM PB buffer (pH 6.0) at 37°C for 12 h. The reaction was stopped by heating at 100°C for 5 min. The control was performed by terminating the reaction immediately after the enzyme was mixed with the substrate. The insoluble xylan was sedimented at 12,000 xg for 10 min. The hydrolysate was then analyzed by high pressure liquid chromatography using the procedure described in Chapter 2.2.13. For identification of hydrolysis product, xylose and xylobiose were used as standards.

4.2.9 *Effect of chemicals on xylanase activity.* Enzyme (12 U in 50 mM phosphate buffer, pH 6.0) was incubated with no additions, or in the presence of the following compounds: KCl, $AgNO_3$, $FeSO_4$, $CuCl_2$, $ZnSO_4$, $MnSO_4$, $CoCl_2$, $CaCl_2$, $MgSO_4$, NaCl, $NaNO_3$, NaI, NaSCN, or NaF. The residual enzyme activity was determined in a 15 min assay at 55°C.

4.2.10 *Protein estimation.* Protein was determined by the method of Bradford (1976) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) used as standard. The protein concentrations in column fractions were expressed as the A_{280}.

4.3 Results

4.3.1 *Xylanase purification*. A crude preparation of xylan-digesting enzyme was obtained from sonicator-treated cells of *E. coli* JM103(pLS206). The cell-free extract was separated on a DE-52 column. Elution was performed by increasing the concentration of NaCl in PB buffer from 0 to 0.5 M. The enzyme was eluted at an NaCl concentration of approximately 0.1 M (Fig. 4.1). The xylanase-active fraction obtained after DE-52 chromatography was reapplied to the DE-52 column, equilibrated with 50 mM PB (pH 6.8). Enzyme activity was desorbed from the column at 0.13 M NaCl (Fig. 4.2). Active fractions pooled from the anion-exchange chromatography were further purified on Sephacryl S-200 HR column (Fig. 4.3). The single xylanase activity peak corresponded to a protein peak in fractions from 36 to 43. In subsequent purification on Bio-Gel hydroxylapatite, the enzyme was eluted from the column at approximately 0.1 M sodium phosphate (Fig. 4.4).

Enzyme purification (Table 4.1) resulted in a 253-fold purification of xylanase relative to the crude extract and a substantial increase in the specific activity (from 0.31 to 78.4 U/mg protein). Approximately 18% of xylanase activity in the crude enzyme was recovered and the enzyme was shown to have an apparent molecular mass of 72,000 (Fig. 4.5A, lane 6). The purified xylanase was SDS sensitive since attempts to recover activity after SDS-PAGE using the activity gel overlay technique described by Beguin (1983) were unsuccessful. The activity bands were visualized on

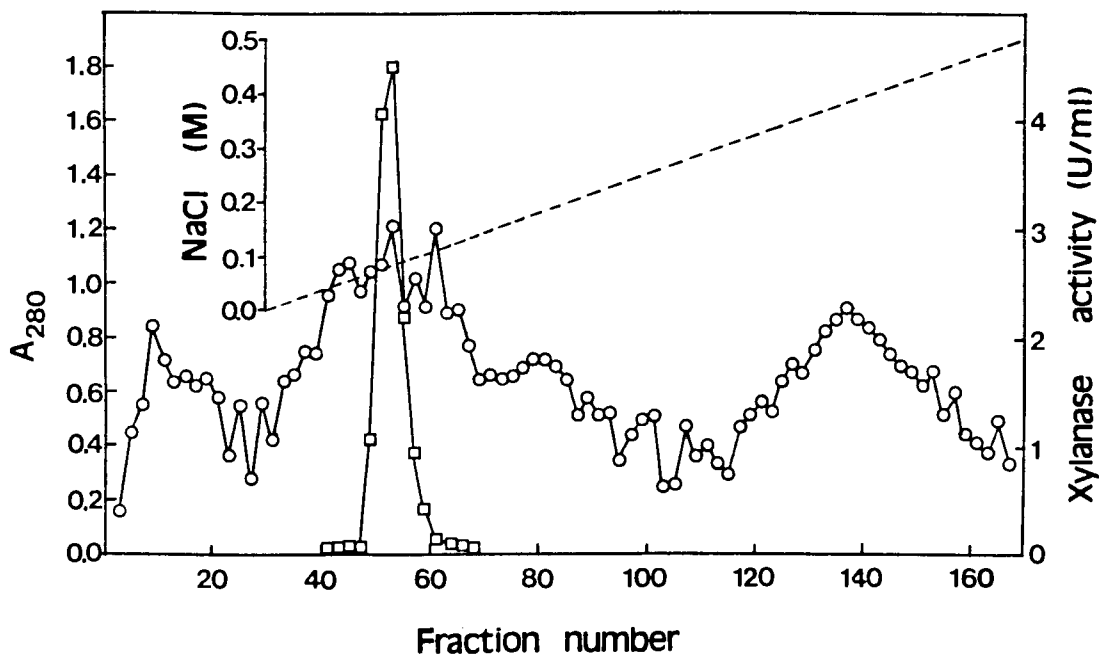

Fig 4.1 Ion-exchange chromatography of cell-free extract on DE-52. Symbols: ❑, xylanase; O, protein; ----, sodium chloride gradient.

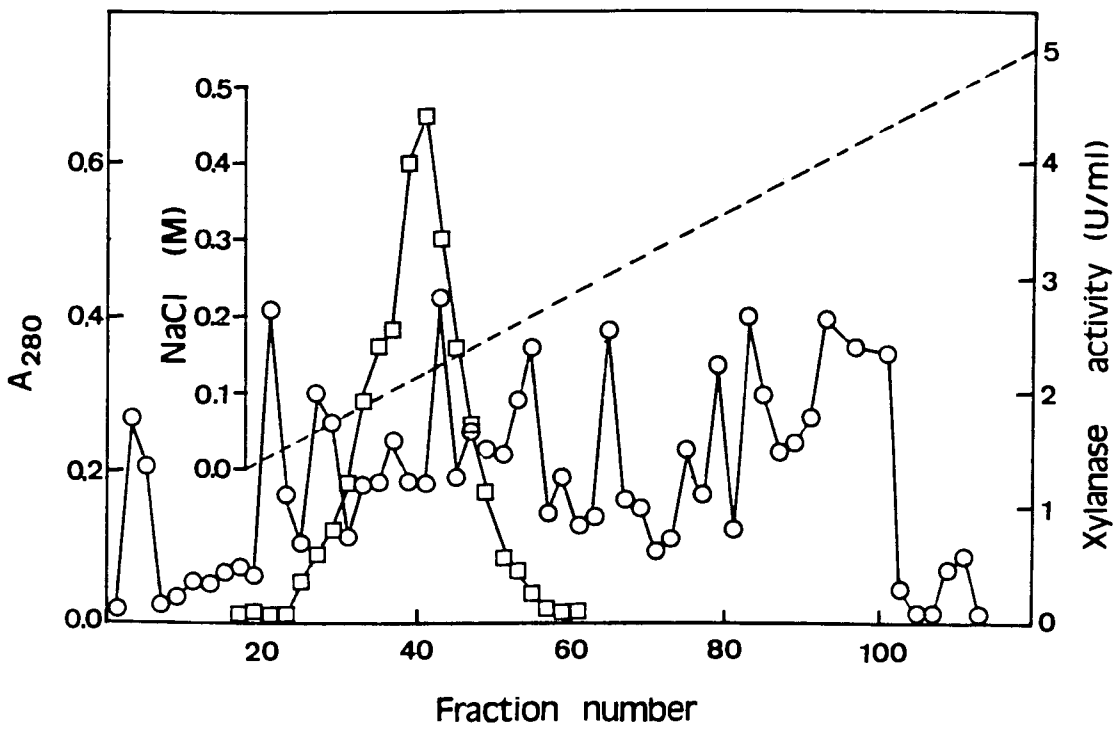

Fig 4.2 Rechromatography of xylanase activity on DE-52 after first DE-52 column chromatography. Symbols: ❑, xylanase; O, proteins; ----, sodium chloride gradient.

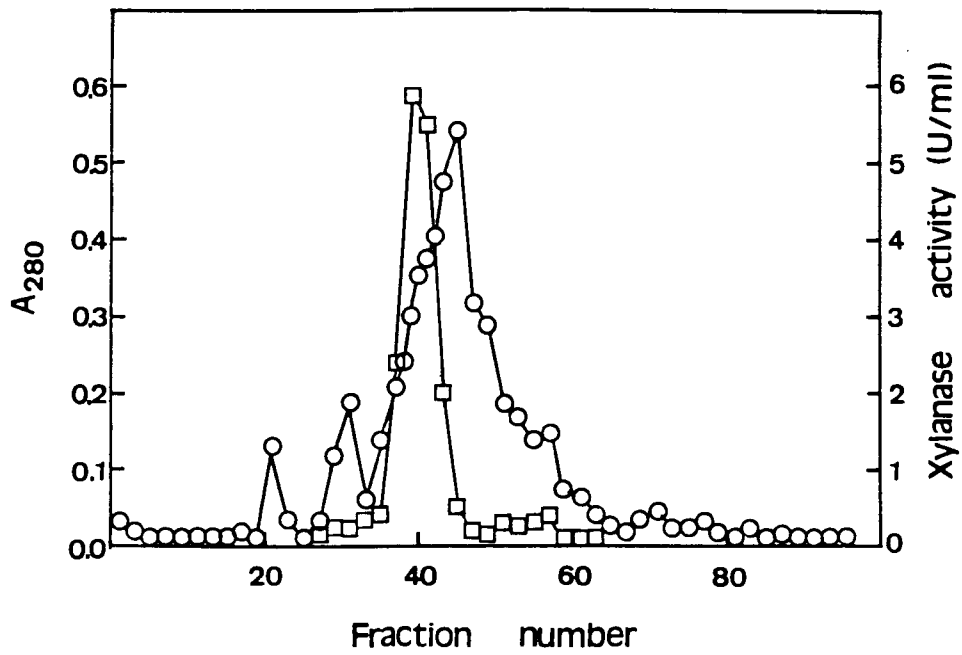

Fig 4.3 Gel filtration of xylanase activity on Sephacryl S-200 after the second DE-52 chromatography. Symbols: ❑, xylanase; O, protein.

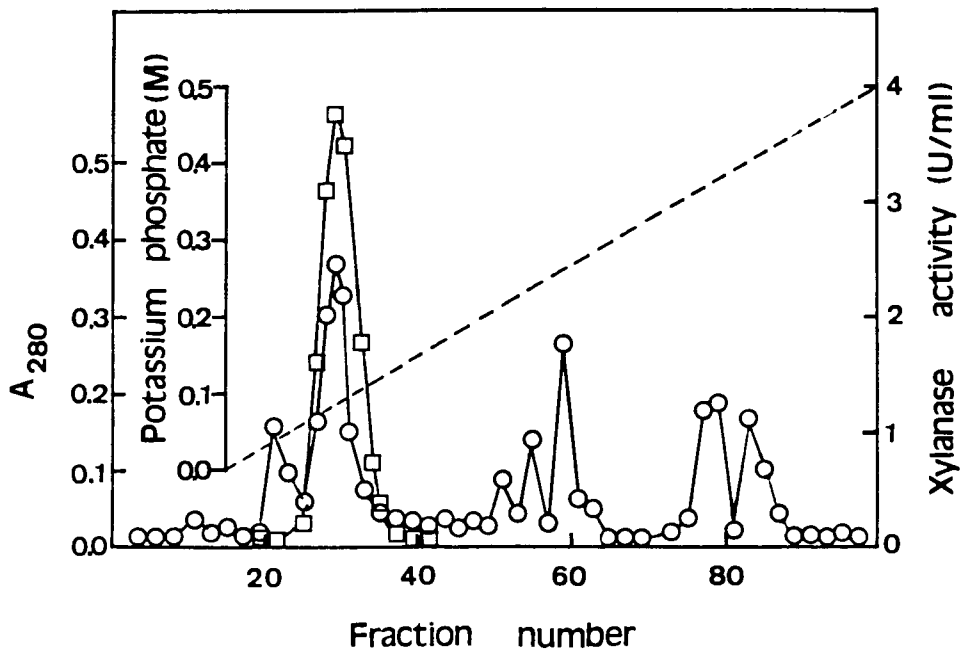

Fig 4.4 Hydroxylaptatite chromatography of xylanase eluted from gel filtration column. Symbols: ❑, xylanase; O, protein; ----, sodium phosphate gradient.

non-denaturing gels using the same technique (Fig. 4.5B, lane 2). Two very faint activities could be discerned corresponding to smaller protein bands.

Table 4.1 Purification of xylanase from cell free extract of *E. coli* JM103(pLS206)

Purification step	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)
Cell free extract	2283.1	0.31	100
DE-52 (I)	695.0	0.86	30
DE-52 (II)	252.6	1.98	11
Sephacryl S-200	90.0	3.46	4
Hydroxylapatite	1.6	78.40	0.07

4.3.2 ***Amino-terminal protein sequence.*** Approximately 50 µg of purified xylanase was subjected to 7 cycles of Edman degradation. The amino terminus of xylanase was determined to be Asn-2. The results of the following 6 cycles of Edman degradation confirmed the amino acid sequence Asn-Leu-Lys-Thr-Ala-Tyr derived from the DNA sequence of the gene (Chapter 3, Fig. 3.4).

4.3.3 ***Temperature and pH optima.*** The optimum temperatures for reactions were examined. Both xylanase and pNPCase exhibited a similar temperature profile, the optimum being around 55°C in 50 mM phosphate buffer (pH 6.0). The activities decreased sharply above 60°C (Fig. 4.6).

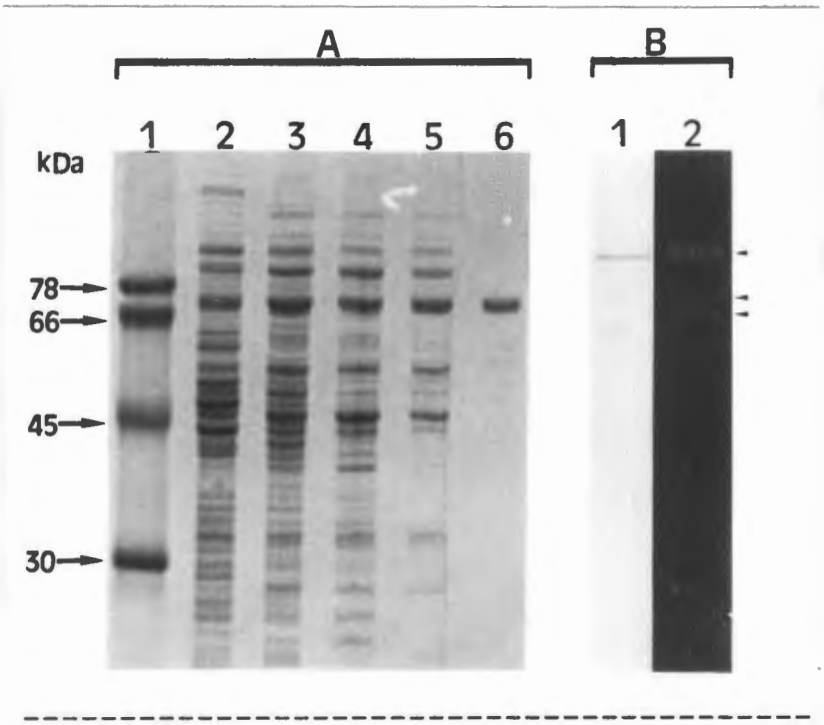

Fig 4.5 SDS-PAGE electrophoresis of proteins isolated from *E. coli* JM103 (pLS206) during the purification of the xylanase (A). Lanes 1, molecular mass standards; 78 kDa, ovotransferrin; 66 kDa, albumin; 45 kDa, ovalbumin and 30 kDa, carbonic anhydrase; 2, cell free extract; 3, pooled xylanase activity from first DE-52 column; 4, pooled xylanase activity from second DE-52 column; 5, eluant from Sephacryl S-200 column; 6, eluant from hydroxylapatite column. Zymogram of xylanase activity after purification of XynB by non-denaturing gel (B). Lane 1, purified xylanase; lane 2, zymogram.

The effect of pH on the activities of xylanase and pNPCase were determined at 55°C in various buffers ranging from pH 4.0 to pH 10.0. The maximum activities being observed between pH 5.4 and pH 6.0 (Fig. 4.7). The activities decrease sharply at lower and higher pH values with < 80% remaining at pH 5.0 and at pH 8.0.

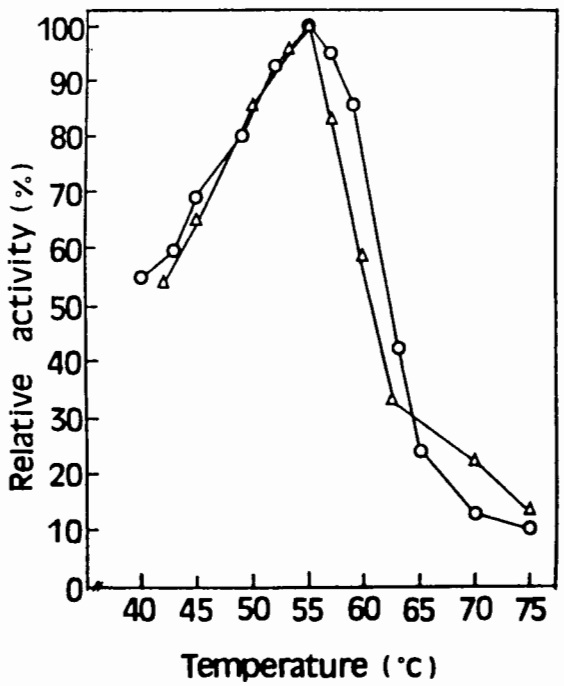

Fig 4.6 Determination of the optimum pH for the xylanase and pNPCase activities of the XynB enzyme. Symbols: ❑ , xylanase; Δ, pNPCase.

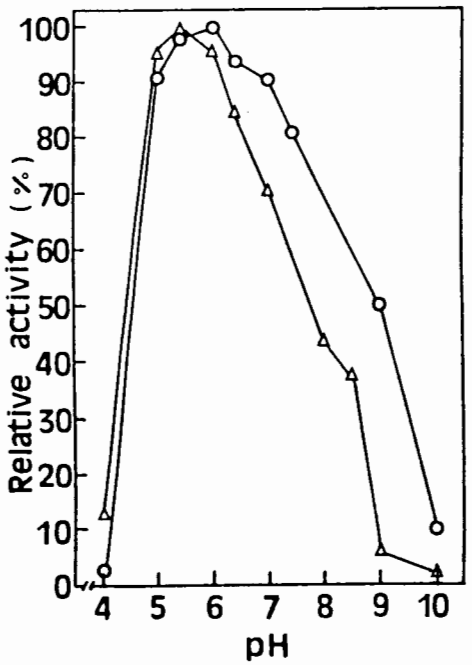

Fig 4.7 Determination of the optimal temperature for the xylanase and pNPCase activities of the XynB enzyme. Symbols: ❑, xylanase; Δ, pNPCase.

4.3.4 ***Activities on different substrates.*** The substrate specificity was tested using a variety of substrates (Table 4.2). When the enzyme was assayed for reducing sugars, it was shown to have strong activity on oat spelt xylan. Low amounts of reducing sugars were detected when CMC was used as substrate, indicating that the enzyme demonstrated low level of endo-1,4-β-D-glucanase activity. The enzyme was unable to degrade lichenan (a β-1-4\β-1-3-alternated glucose polymer), laminarin (a β-1-3-linked glucose polymer), or salicin. It had no β-glucosidase activity but showed some activity on pNPC and pNPX.

Table 4.2 Substrate specificity of xylanase from *E. coli* JM103(pLS206)

Substrate	Linkage	Specific activity (U/mg protein)
Oat spelt xylan[a]	β-1-4	13.30
CMC[a]	β-1-4	0.04
Lichenan[a]	β-1-4\β-1-3	<0.01
Laminarin[a]	β-1-3	<0.01
Salicin[b]	β-1-4	<0.01
pNPC[b]	β-1-4	1.26
pNPG[b]	β-1-4	<0.01
pNPX[b]	β-1-4	0.75

[a] One unit of enzyme activity was expressed as the amount of proteins liberating 1 μmol of reducing sugars per min.

[b] One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min.

To distinguish between endo- and exo-1,4-β-xylanases, appropriate amounts of the enzyme were added to oat spelt

xylan to produce limited hydrolysates which were analyzed by HPLC (Fig. 4.8). The enzyme degraded the substrate to xylose, xylobiose, and higher xylose polymers. The relative amount of xylobiose produced was considered to be too small to be characteristic of exoxylanase activity. Therefore the 72,000 dalton xylanase is probably an endo-1,4-β-xylanase.

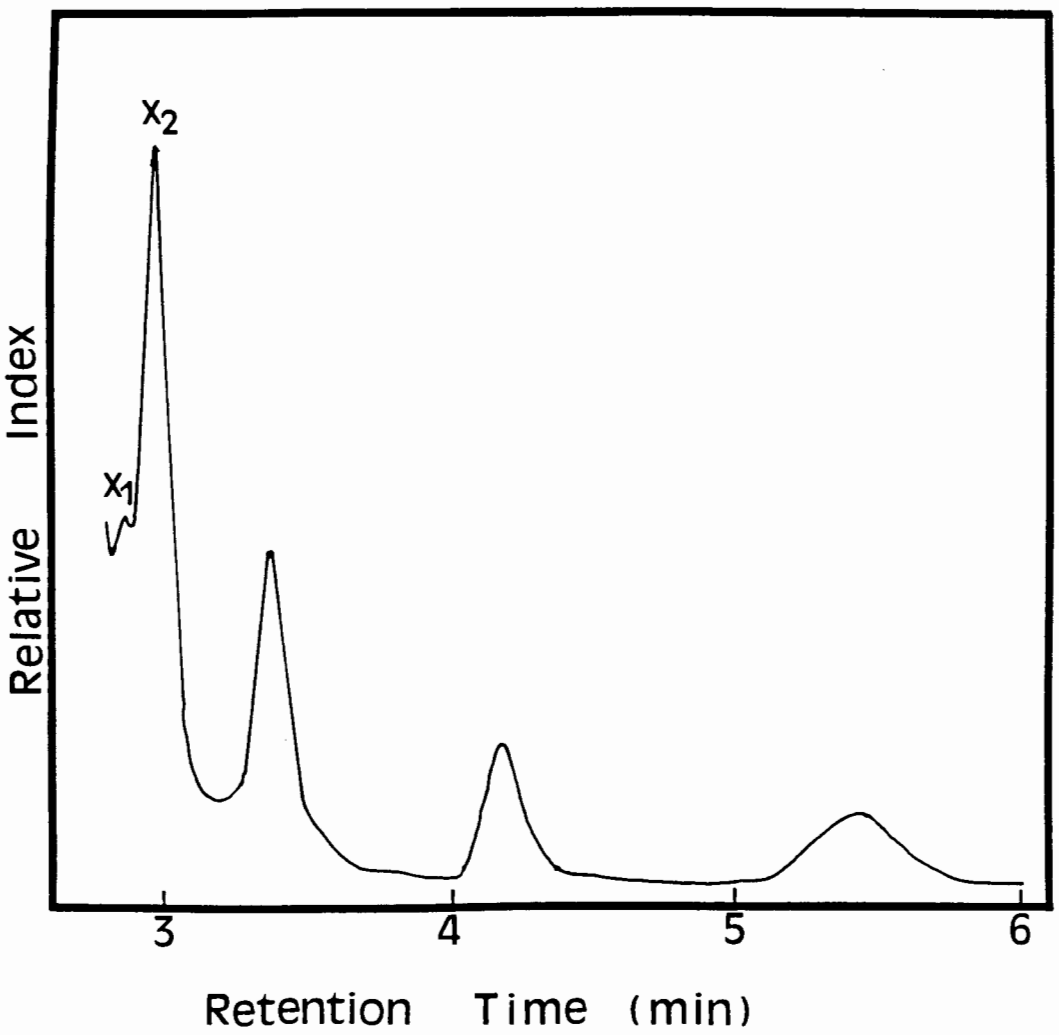

Fig 4.8 HPLC analysis of xylanase activity on oat spelt xylan. Abbreviations: X_1, xylose; X_2, xylobiose.

4.3.5 *The effect of chemicals on the activity of xylanase.* The activity of xylanase was affected by certain chemicals (Table 4.3). While K^+ enhanced the xylanase activity by 16.4%, significant inactivation of the enzyme was observed with Ca^{2+}. At 0.75 mM, Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+} and Mg^{2+} inhibited 100, 98.8, 73.9, 30.8, 24.5, and 11.9% of the xylanase activity, respectively. At 0.05mM the enzyme was completely inhibited by Ag^+.

Table 4.3 Effect of cations on the activity of xylanase from *E. coli* JM103(pLS206)[a]

Cation	Concentration (mM)	Relative activity (%)
Control	-	100.0
$K^+(KCl)$	75.00	116.4
$Ca^{2+}(CaCl_2)$	7.50	18.2
$Cu^{2+}(CuCl_2)$	0.75	0.0
$Zn^{2+}(ZnSO_4)$	0.75	1.2
$Mn^{2+}(MnSO_4)$	0.75	26.1
$Co^{2+}(CoCl_2)$	0.75	69.1
$Fe^{2+}(FeSO_4)$	0.75	75.5
$Mg^{2+}(MgSO_4)$	0.75	89.1
$Ag^+(AgNO_3)$	0.05	0.3

[a] Enzyme activity in absence of added substance(control) was taken as 100% and was equivalent to 11.6 Units/mg protein.

The effect of five anions on xylanase revealed that there was a considerable difference in the efficiency of these anions with respect to the stimulating effect (Table 4.4). Cl^-, NO_3^- and SCN^- all exhibited a stimulatory effect on

the enzyme, although the anion concentrations required for effective stimulation varied. At a concentration of 50 mM F^- showed a slight stimulatory effect but inhibited enzyme activity at higher concentrations. I^- inhibited the enzyme at all concentrations tested.

Table 4.4 Effect of anions on the activity of xylanase from *E. coli* JM103(pLS206)[a]

Anion	Concentration (mM)	Relative activity (%)
Control	-	100.0
NaCl	50	124.7
	100	104.7
	250	92.0
$NaNO_3$	50	131.6
	100	123.3
	250	115.0
NaSCN	50	153.0
	100	165.0
	250	116.7
NaF	50	105.0
	100	87.1
	250	76.0
NaI	50	56.0
	100	36.7
	250	3.3

[a] Enzyme activity in absence of added substance(control) was taken as 100%, and was equivalent to 11.2 Units/mg protein.

4.4 Discussion

As a result of a four-stage purification ending with hydroxylapatite column chromatography, the xylanase was purified 253-fold. Purified xylanase exhibited homogeneity as shown by polyacrylamide gel electrophoresis. The enzyme showed a pH optimum within the most commonly observed range of 4.0 - 6.0 (Paice *et al.*, 1978; Biely *et al.*, 1980b; Uchino and Nabane, 1981). The temperature optimum of *B. fibrisolvens* enzyme at 55°C is marginally lower than xylanases from *B. stearothermophilis* (Nanmori *et al.*, 1990) and *S. roseisclerotícus* (Grabski and Jeffries, 1991), similar to a 1,3-β-D-glucanase of *R. flavefaciens* (Erfle and Teather, 1991), and 16 degrees higher than cellulases from *F. succinogenes*, formerly *B. succinogenes* (Huang *et al.*, 1988; McGavin and Forsberg, 1988; Cavicchioli and Watson, 1991).

The xylanase encoded by pLS206 had a higher M_r than other purified xylanases. The M_r of xylanase as determined by SDS-PAGE was 72,000 which agreed with the MW estimated from the deduced amino acid sequence (73,156). Most xylanases found among fungi, yeasts, and bacteria have M_r values of less than 50,000. The xylanase from the shiitabe mushroom (*Leutinula exodes*) had an apparent M_r of 41,000 (Mishra *et al.*, 1990), whereas 2 xylanases purified from *T. hanzianum* showed M_r of 20,000 and 29,000 (Tan *et al.*, 1985a). *S. lividans* xylanase had an apparent M_r of 43,000 (Morosoli *et al.*, 1986a), the same as that reported for an alkalophilic *Bacillus* species C-125 (Honda *et al.*, 1985a). The xylanases

purified from *C. albidus* (Morosoli *et al.*, 1986b) and *S. racliatum* (Cavazzoni *et al.*, 1989) had an apparent M_r of 48,000 and 25,700, respectively.

When the enzyme reacted with oat spelt xylan some xylose was found in the reaction mixture indicating β-xylosidase activity. Endo-acting xylanases which also produce xylose are found in fungi (Gorbacheva and Rodionova, 1977; John *et al.*, 1979; Takahashi and Kutsum, 1979; Yoshioka *et al.*, 1981), yeasts (Biely *et al.*, 1980; Stüttegn and Sahm, 1982), and bacteria (Okazaki *et al.*, 1984). The xylanase encoded by pLS206 also showed activity towards pNPX. These results indicate that the enzyme first cleaves the substrate to liberate xylooligosaccharides and then some of these oligosaccharides are cleaved to form xylose by the β-xylosidase activity. The accumulation of xylooligosaccharides may be due to feedback inhibition by the end products. According to the report of Gorbacheva and Rodionova (1977b), xylans of various structures and different origin were degraded from 14 to 30% with endo-1,4-β-xylanase from *A. niger* strain 14 but up to 97% after elimination of products formed as a result of hydrolysis of xylans. The elimination of end products thus appears to remove the feedback inhibition.

It is worth noting that the purified xylanase was able to degrade polysaccharides other than xylan. The enzyme could degrade CMC to a limited extent which reflects the nonspecific nature of this enzyme toward β-1,4 linkages. Endoxylanases with activity against cellulose are found in

C. acetobutylicum (Lee *et al.*, 1987). *B. acidocaldarius* (Uchino and Nakane, 1981) and *A. niger* (Fournier *et al.*, 1985).

Microbial cellulases are generally known to require Ca^{2+} for activity and stabilization (Roy *et al.*, 1990; Yoshimatsu *et al.*, 1990). The effect of Ca^{2+} on enzyme activity may be due to changes in electrostatic bonding, which could change the tertiary structure of the enzyme. Among xylanases, neither that of *T. harzianum* (Tan *et al.*, 1985b) or an alkalophilic *Bacillus* spp. (Akiba and Horikoshi, 1988) was greatly inhibited or stimulated by Ca^{2+}. The inhibition of the *B. fibrisolvens* H17c xylanase activity by Ca^{2+} is very unusual and cannot be readily explained. Stimulation by K^{+} cations could arise from interaction of the ions with free carboxyl groups of some amino acid residues in the xylanase molecule or from changes in the gross conformation of the enzyme. A similar stimulating effect by K^{+} was found in an endoglucanase from the alkalophilic *Bacillus* spp. KSM-635 (Yoshimatsu *et al.*, 1990). The enzyme was strongly inhibited by Cu^{2+}, and such inhibition has also been noted with xylanases from *Trichoderma lignorum* (John and Schmidt, 1988) and an alkalophilic *Bacillus* spp. (Akiba and Horikoshi 1988). The *B. fibrisolvens* xylanase was also inhibited by Zn^{2+}, Mn^{2+}, and Co^{2+}. These inhibitory effects of divalent metal ions suggest the involvement of sulfhydryl groups in the active site on the binding domain of the enzyme.

A unique feature of the purified xylanase was the stimulation of enzyme activity by anions. Most of the

enzymes which have been demonstrated to be activated by monovalent anions appear to be of eukaryotic origin (Gorter and Gruber, 1970; Kalish *et al.*, 1974; Webb and Morrow, 1959). Activation of prokaryotic enzymes by anions is comparatively rare. Among the tested anions Cl^-, NO_3^- and SCN^- were found to be stimulatory. Activation by Cl^- and NO_3^- was also observed with a Cl^--stimulated cellobiosidase (Huang *et al.*, 1988) from *B. succinogenes*. The mechanism of anion activation of enzymes is unclear at present, although it has been suggested that chloride may induce a conformational change of a mammalian α-amylase (Levitzki and Steer, 1974).

Chapter 5

An analysis of the extracellular xylanases and cellulases of *Butyrivibrio fibrisolvens* H17c

5.0

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5.0 Summary

The extracellular xylanase and cellulase components of *B. fibrisolvens* H17c were investigated. Two major peaks of enzyme activity were eluted by hydroxylapatite chromatography and designated complex A (C_A) and complex B (C_B). C_A had cellulase activity, degrading both carboxymethylcellulose (CMC) and p-nitrophenyl-β-D-cellobioside (pNPC), and laminarinase activity. C_B showed predominantly xylanase and cellulase activity, with some activity on laminarin. However, the cellulase activity in C_B differed from that in C_A in that it did not degrade pNPC. Although β-glucosidase and β-xylosidase activities were detectable in the concentrated culture supernatant, they were not found in either C_A or C_B. C_B was further purified on a DE-52 column and subjected to gel filtration. The xylanase and CMCase activities were eluted in a single peak with an apparent M_r greater than thyroglobulin (M_r 669,000). CMC zymograms of polyacrylamide gels electrophoresed under non-denaturing conditions indicated the presence of 5 bands with CMCase activity from C_A and 8 from C_B. Xylan zymograms under the same conditions confirmed the absence of xylanase activity in C_A and indicated the presence of 4 bands of activity in C_B. Under mild denaturing conditions the xylanase activity in C_B was found in 11 bands with M_r

ranging from 45 kDa to 180 kDa, and the CMCase activity in 3 bands with M_r ranging from 45 kDa to 60 kDa. This indicates that C_B exists as a multi-subunit protein aggregate of xylanases and cellulases. The pH and temperature optima for CMCase in the complex were pH 6 and 50 to 60°C, and for xylanase pH 5 and 60°C respectively.

5.1 Introduction

B. fibrisolvens is a cellulolytic and xylanolytic rumen anaerobe which is the predominant bacterial species in the rumen of animals surviving under adverse conditions (Orpin *et al.*, 1985). Dehority (1965) has shown that many cellulolytic rumen bacteria, including *B. succinogenes, R. albus* and *R. flavefaciens*, can degrade hemicellulose extensively but only a few strains are capable of growing on these substrates. Unlike these species, *B. fibrisolvens* can grow on xylan as the sole carbon source (Hobson and Purdom, 1961).

Cellulose and hemicellulose are complex substrates and in order to grow on them bacteria synthesise a number of different enzymes. The degradation of cellulose requires the combined activity of endoglucanases, cellobiohydrolases β-glucosidases, and possibly cellodextrinases. In some bacteria many of these enzymes are found in a cell-associated complex called the cellulosome (Lamed and Bayer, 1988). In the case of *C. thermocellum* the cellulosome contains at least 15 polypeptides (Lamed and Bayer, 1983). The extracellular components of *C. thermocellum* also exist as protein aggregates which can be separated by mild SDS treatment (Wu *et al.*, 1988).

Some bacteria appear to secrete their cellulases into the culture fluid. Two enzyme complexes containing 18 endoglucanases have been found in the culture fluid of the rumen anaerobe, *R. flavefaciens* FD-1 (Doerner and White,

1990). A mesophilic *Clostridium* strain C7 also produces an extracellular, aggregated cellulase complex consisting of at least 15 proteins including catalytic and non-catalytic components (Cavedon *et al.*, 1990). Supernatants from cultures of *C. fimi* contain up to 10 components with CMCase activity (Langsford, *et al.*, 1984).

Naturally-occurring cellulose is usually associated with hemicellulose. Unlike cellulose, hemicellulose does not have a homogeneous chemical composition, but the predominant polymer is xylan, composed of xylose with arabinose side chains. Xylan degradation requires the concerted action of a number of enzymes including xylanases and xylosidases, the latter including xylobiase activity. The definition of an enzyme as a xylanase is complicated due to the frequently observed cross-specificity of xylanases and cellulases (Wong *et al.*, 1988). For instance, all 18 endoglucanases in the two extracellular complexes secreted by *R. flavefaciens* FD-1 had xylanase activity. Less is known about the composition of xylanase enzyme complexes and their localization than about cellulase complexes. Morag *et al.* (1990) have found xylanases present in both the cellulosome and the culture supernatant of *C. thermocellum*, the latter being characterised by a lower average M_r. Hespell *et al.* (1987) have shown that *B. fibrisolvens* secretes xylanase while xylobiase activity is cell-associated. This chapter describes the isolation and analysis of xylanases and cellulases from the culture supernatant of this strain, some of which are present in the form of a multienzyme complex.

5.2 Materials and Methods

5.2.1 ***Bacterial strain and culture conditions.*** The rumen anaerobe, *B. fibrisolvens* H17c (Dehority, 1966) was obtained from Dr R B Hespell (Department of Dairy Science, University of Illinois, Urbana). The culture was grown in M10 medium (Caldwell and Bryant, 1966) as described in Appendix A.

5.2.2 ***Enzyme preparation and fractionation.*** *B. fibrisolvens* was inoculated into 200 ml M10 medium, incubated for 2 days and used to inoculate 6 l of M10 medium. After 36 h incubation, the culture was centrifuged at 6500 x g for 30 min at 4°C remove the cells. The culture supernatant was concentrated to approximately 80 ml using a filtration system (Pellicon; Millipore Corp., Bedford, Mass.) equipped with a 10,000 MW cut-off membrane. The concentrated material was then subjected to a stirred ultrafiltration cell with a PM10 membrane (10,000 MW cut-off; Amicon Corp., Lexington, Mass.). The resulting supernatant concentrate (Sc; 10 ml) was maintained aerobically at 4°C for enzyme fractionation.

The concentrate was applied to a column of hydroxylapatite (2.6 cm x 40 cm; Bio-Rad) equilibrated with 50 mM potassium phosphate buffer (pH 6.8). The column was washed with one bed volume (250 ml) of the equilibration buffer and the adsorbed materials eluted with a 800 ml linear gradient of 50 mM to 250 mM phosphate buffer (pH 6.8). Fractions (20 ml) were collected at a flow rate of 40 ml h^{-1}. The fractions from the two activity peaks, C_A and C_B, were

concentrated by ultrafiltration through a PM10 membrane. The concentrate of C_B was passed through a DE-52 chromatography column (1.6 cm x 20 cm; Whatman) equilibrated with 50 mM phosphate buffer, pH 6.8. After the sample was applied, the column was washed with the equilibration buffer (50 ml) and the proteins were eluted with 300 ml of a linear sodium chloride gradient (0 to 0.5 M). The fraction size was 5 ml and the flow rate 60 ml h^{-1}. The fractions containing xylanase activity were combined and concentrated to 1 ml using an Amicon filter with a PM10 membrane. This was applied to a Sepharose CL-6B column (1.6 cm x 80 cm; Pharmacia, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer (pH 6.8) containing 0.1 M sodium chloride. Protein was eluted with the equilibrating buffer (flow rate 20 ml h^{-1}). The following M_r standards were used to calibrate the column: blue dextran, 2,000 kDa; thyroglobulin, 669 kDa; apoferritin, 443 kDa and catalase, 232 kDa (Sigma Chemical Co., St. Louis, Mo.).

5.2.3 ***Enzyme assays.*** The enzyme activities were assayed according to the procedures as described in Chapter 2 (2.2.12).

5.2.4 ***Zymograms for CMCase and xylanase activity.*** Polyacrylamide gel electrophoresis (PAGE) was performed in 7.5% (w/v) gels with or without SDS by the method of Laemmli (1970). Before SDS-PAGE, the samples were kept at room temperature for 1 h for denaturation. CMCase or xylanase activities in gels were detected by using polyacrylamide overlays on both sides of the gel, containing

either CMC or xylan (both at 0.1%, w/v) as substrate. The zymogram was incubated in a humidity chamber at 37°C in contact with the gel. After incubation (12 h for SDS-PAGE, 1 h for non-denaturing PAGE), the gel was soaked in a 0.1% (w/v) solution of Congo red for 15 min. Excess dye was decanted, and the gel destained with 1 M NaCl.

5.2.5 ***Protein determination.*** Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

5.2.6 ***Determination of optimal pH and temperature.*** The effect of pH on enzyme activities was measured from pH 3 to pH 10 using sodium phosphate-citrate buffer (pH 3 to pH 6), sodium phosphate buffer (pH 6 to pH 8) and glycine-NaOH buffer (pH 8.6 to pH 10). The temperature optima were determined by adding enzyme solutions to substrate prewarmed at temperatures ranging from 30°C to 70°C and assaying at that temperature at the optimal pH.

5.3 Results

5.3.1 ***Isolation of extracellular enzyme complexes.*** Two major peaks of enzyme activity were eluted by hydroxylapatite chromatography and designated complex A (C_A) and complex B (C_B) (Fig. 5.1A). C_A had CMCase activity, whereas C_B showed both xylanase and CMCase activity (Fig. 5.1A). When C_B was subsequently applied to a DE-52 column and eluted with a NaCl gradient, one peak of xylanase and CMCase activity was observed (Fig. 5.1B). The active fractions were applied to a Sepharose CL-6B gel filtration column, and the activity eluted between the void volume and thyroglobulin (M_r 669,000) (Fig. 5.1C). It was not possible to do a similar analysis on C_A as the protein concentration was too low. The high A_{280} of C_A shown in Fig. 5.1A was due to a pigment which co-eluted with the complex.

The concentrated supernatants, C_A and C_B were analyzed by non-denaturing PAGE, CMCase and xylanase zymograms (Fig. 5.2). Although a number of proteins found in the supernatant were not present in either C_A or C_B, most of those showing enzymatic activity were. C_A showed at least five CMCase components (Fig. 5.2A, lane 2), none of which had xylanase activity (Fig. 5.2B, lane 2). C_B revealed at least 8 CMCase bands (Fig. 5.2A, lane 3) and four bands with xylanase activity (Fig. 5.2B, lane 3). Only one band showed both xylanase and CMCase activity and it was also detectable in the supernatant.

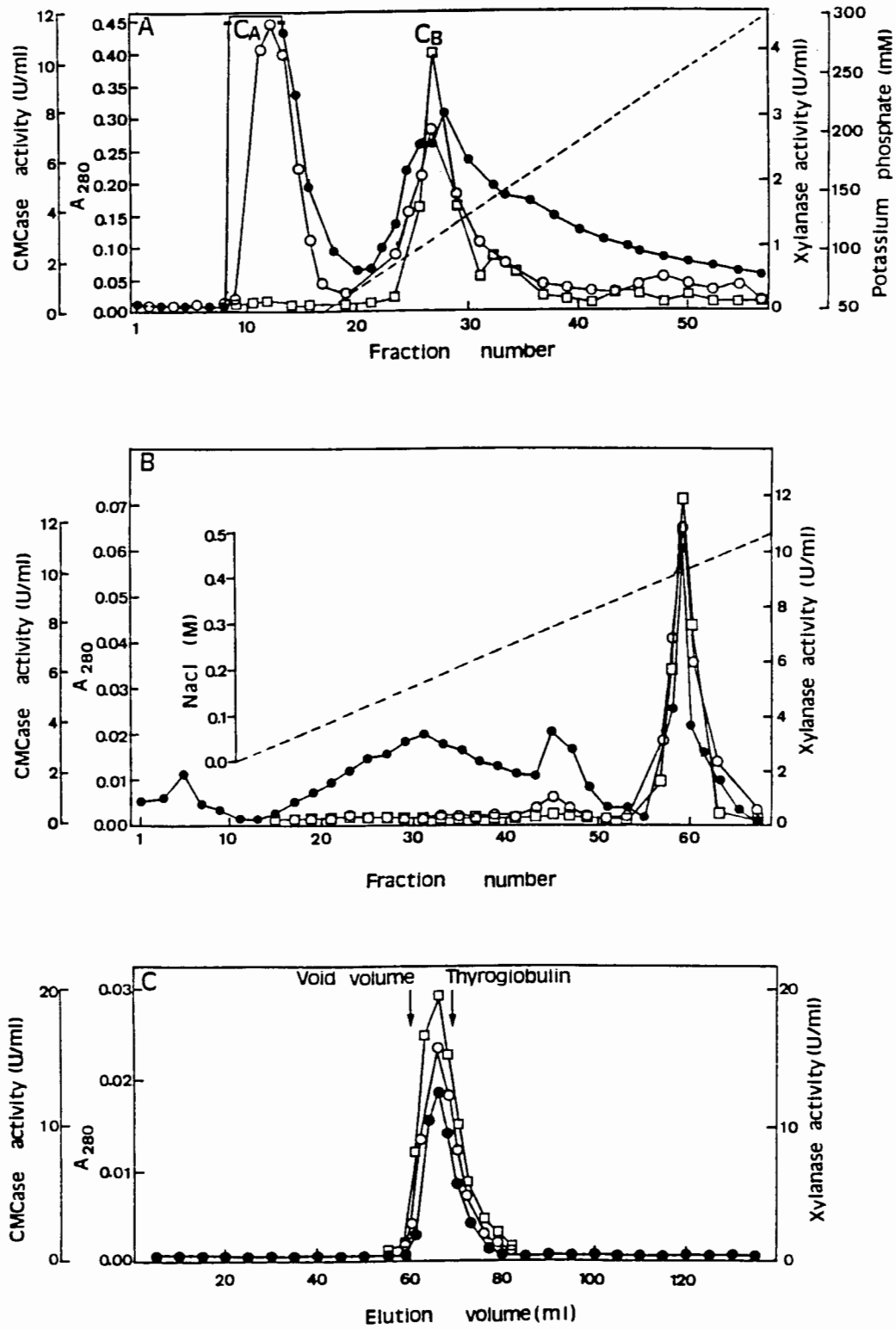


Fig.5.1 (A) Hydroxylapatite chromatography of extracellular culture fluid from *B. fibrisolvens* H17c. (B) Ion-exchange chromatography of complex B (C_B) on DE-52 column. (C) Gel filtration chromatogrphy on CL-6B column of pooled fractions from DE-52 chromatography: ○, CMCase activity; □, xylanase activity; ●, A_{280}; ----, potassium phosphate or sodium chloride buffer gradients.

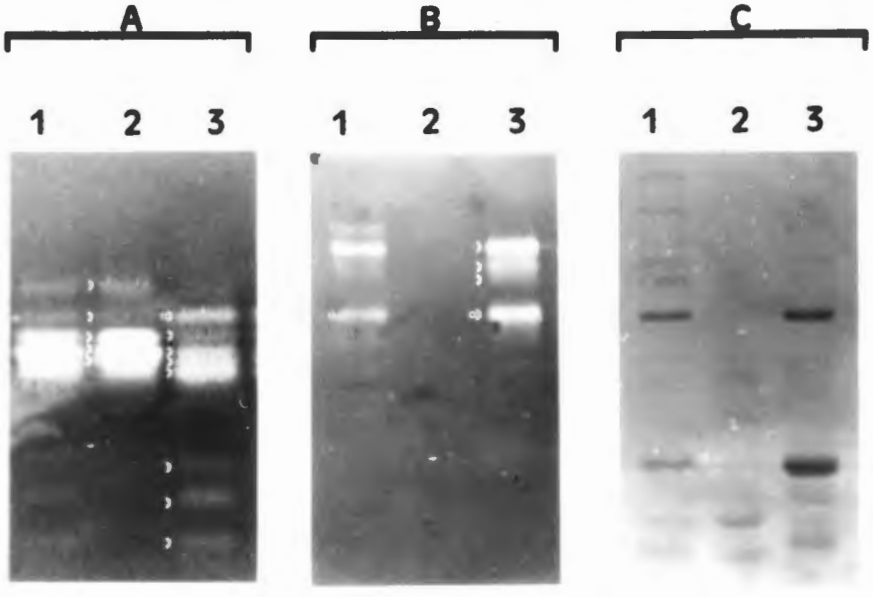


Fig.5.2 Nondenaturing PAGE of enzyme-active hydroxylapatite column fractions. (A) CMCase zymogram; (B) xylanase zymogram; (C) proteins stained with Coomassie brilliant blue R_{250}. Lanes 1, supernatant concentrate; 2, C_A; 3, C_B. Circles represent the bands which showed both CMCase and xylanase activity.

To further analyse the enzyme complexes, C_A and C_B were subjected to mild denaturation by treatment with SDS sample buffer at room temperature before subjecting them to SDS-PAGE and zymograms. In C_A, two bands with CMCase activity were detected (Fig. 5.3A, lane 1) and, unexpectedly, a band with an approximate M_r of 59 kDa exhibited xylanase activity (Fig. 5.3B, lane 1). In C_B, three bands with CMCase activity were detected, and approximately 11 bands exhibited xylanase activity, three of which also had CMCase activity (Fig. 5.3A, lane 2; Fig. 5.3B, lane 2). Many of these enzymes appeared to be extremely active as the proteins were not detectable on Coomassie brilliant blue R_{250}-stained PAGE gels. There was no apparent difference when the xylanase zymogram of the C_B complex was compared with that of the

xylanase-containing fractions from the DE-52 column (Fig. 5.4).

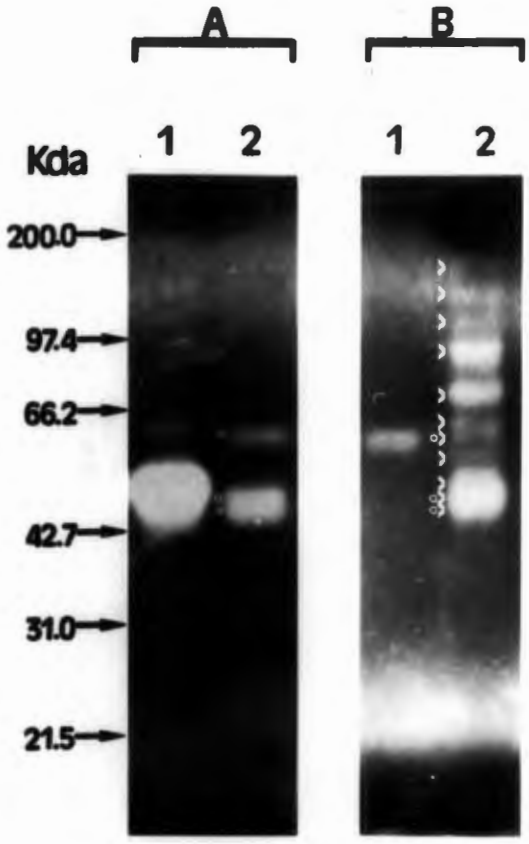


Fig.5.3 SDS-PAGE zymograms of mildly denatured enzyme-active hydroxylapatite column fractions. (A) CMCase; (B) xylanase. Lanes 1, C_A; 2, C_B. Molecular weight markers: β-amylase (Sigma), 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa (Bio-Rad).

5.3.2 ***Substrate specificity of enzyme complexes.*** The culture supernatant and the C_A and C_B complexes were tested for their ability to hydrolyze various carbohydrates and aryl-glucosides (Table 5.1). As expected, C_A had predominantly CMCase activity, and C_B predominantly xylanase activity with some activity on CMC. Both complexes showed

some activity on laminarin but neither contained the β-glucosidase or β-xylosidase activity found in the culture supernatant. The CMCase activity in C_B was different from that in C_A as the latter showed activity on pNPC. The pNPCase activity could be due to the presence of a cellobiohydrolase which, together with the endoglucanases in C_A as indicated by CMCase activity, should be able to degrade crystalline cellulose. However, no activity was found when C_A was incubated with Avicel. To test the Avicel binding-ability of the enzymes in the culture supernatant and in the complexes, the samples were incubated with Avicel (1%, w/v) at 37°C for 30 min and 48 h with occasional mixing. The enzyme activity that bound to Avicel was estimated by subtracting the activity that remained in the supernatant from the total enzyme activity. The results showed that none of the fractions exhibited an affinity for Avicel.

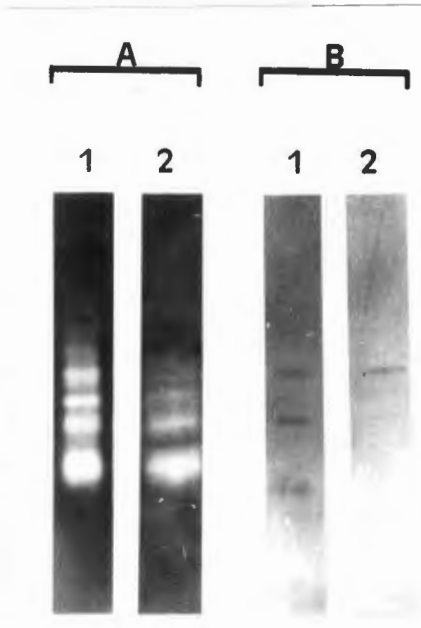


Fig.5.4 SDS-PAGE of the xylanase-active fractions from C_B and DE-52 chromotography. (A) zymogram; (B) proteins stained with Coomassie briliant blue R_{250}. Lanes 1, C_B; 2, pooled fractions from DE-52 column.

Table 5.1 Enzyme activities of Sc, C_A, and C_B complexes

Enzyme	Complex		
	Sc[c]	C_A	C_B
Xylanase[a]	144.4	<0.1	244.6
CMCase[a]	93.7	531.1	111.3
Laminarinase[a]	0.7	24.4	17.9
β-Glucosidase[b]	14.2	0.2	0.7
β-Xylosidase[b]	1.8	0.1	0.4
pNPCase[b]	17.8	106.7	0.8
Avicelase[a]	ND[d]	<0.001	<0.001

[a] Micromoles of reducing sugar min/mg protein.
[b] Micromoles of pNP min/mg protein.
[c] Sc, supernatant concentrate.
[d] ND, not done.

5.3.3 *pH and temperature optima*. The CMCase activity of C_A exhibited a pH optimum of 4 and 75% of its maximum activity was retained at pH 7 (Fig. 5.5A). The CMCase and xylanase activities of C_B exhibited pH optima of 6 and 5 respectively. The former retained 70% or more of its maximum activity between pH 4 and 7, while the latter exhibited a broad pH profile between pH 4 and 9.

The temperature optima for CMCase activity from C_A and CMCase and xylanase activities from C_B were determined at pH 4, pH 6 and pH 5 respectively (Fig. 5.5B). The CMCase from both C_A and C_B had temperature optima of 50°C. The former inactivated rapidly above this temperature with its activity at 60°C decreasing to 41% of the maximum during a 30 min incubation. The latter was more stable at higher

temperatures, retaining 98% of its maximum activity at 60°C. The xylanase activity from C_B had an optimum of 60°C, and retained 80% and 65% of maximum activity at 63°C and 70°C respectively during a 30 min incubation.

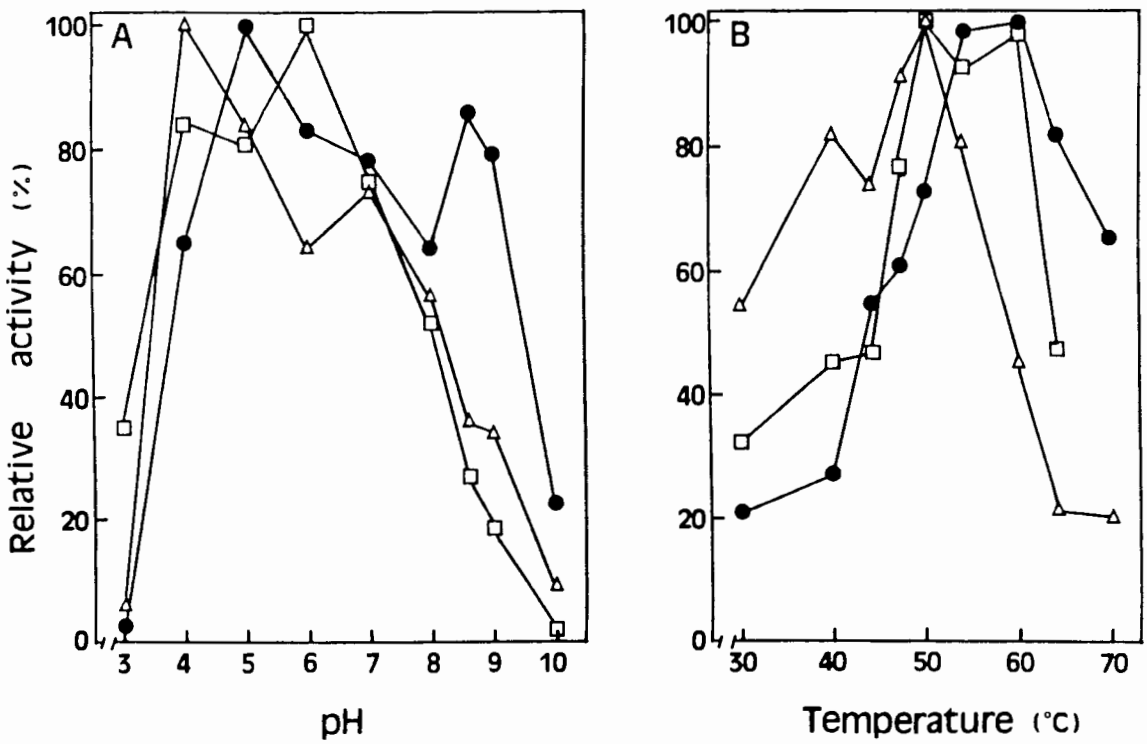


Fig.5.5 Effect of pH and temperature on CMCase or xylanase activities of pooled fractions from hydroxylapatite column. Δ, CMCase activity of C_A; □, CMCase activity of C_B; ●, xylanase activity of C_B.

5.4 Discussion

It has previously been reported that the xylobiase activities of *B. fibrisolvens* are cell associated while the xylanase activities are found predominantly in the culture fluid (Hespell *et al.*, 1987). However no analysis of the extracellular cellulase or xylanase activities has been reported for this species. Two major enzyme complexes have been identified in the culture fluid of *B. fibrisolvens* H17c. Complex A (C_A) contained predominantly CMCase and pNPCase with some laminarinase activity, while complex B (C_B) contained predominantly xylanase and CMCase with some laminarinase activity.

C_B eluted from a DE-52 column as a single peak with an apparent M_r greater than 669,000. Non-denaturing PAGE revealed 8 bands with CMCase and four with xylanase activity. Analysis by SDS-PAGE zymograms showed only 3 bands with CMCase activity. Thus some of these enzymes appear to be sensitive to denaturation. There were 11 bands with xylanase activity, ranging in size from 45 to 180 kDa, only three of which also had CMCase activity. It is therefore likely that the C_B complex exists as a multi-subunit protein aggregate. The multienzyme nature of the complex was supported by the finding that there was a range of pH and temperature optima in both xylanase and CMCase activities.

Some of the CMCases in C_A were sensitive to denaturation as the five active bands revealed by non-denaturing PAGE

zymograms were reduced to two on SDS-PAGE analysis. Unexpectedly, the latter analysis revealed a faint band with an approximate M_r of 59 kDa which exhibited xylanase activity. However, when the non-denaturing PAGE zymograms were incubated for 12 h, a faint band of xylanase activity was observed. Neither complex contained the β-glucosidase or β-xylosidase activities which were found in the concentrated supernatant. These results are therefore in agreement with those of Hespell *et al.* (1987) who showed that *B. fibrisolvens* H17c, grown on glucose or cellobiose, secreted xylanase into the culture supernatant while xylobiase activity was cell associated.

The concept of a cellulosome, a cell-associated multi-protein complex containing cellulases, some of which also have xylanase activity, and cellulose-binding factors has gained considerable acceptance for certain cellulolytic bacteria such as *C. thermocellum* (Lamed and Bayer, 1988). Recently Cavedon *et al.* (1990) have shown that a free-living, mesophilic *Clostridium* (strain C7), secretes an extracellular 700,000-M_r multiprotein complex into the culture supernatant. This complex was shown to be significantly different from the extracellular cellulase system produced by *C. thermocellum*. It consists of at least 15 proteins and is capable of hydrolysing crystalline cellulose. SDS-PAGE analysis of mildly denatured preparations showed that CMCase activity was retained in 6 of the 15 protein bands, but no report was made of xylanase activity. Another example of the secretion of extracellular multi-protein cellulase complexes is from *R. flavefaciens*

FD-1 (Doerner and White, 1990). Two such complexes were found, one containing 13 and the other 5 endoglucanase components, all of which also had xylanase activity. The complexes also contain noncatalytic polypeptides. There are, therefore, precedents for the secretion by cellulolytic and xylanolytic bacteria of multi-protein complexes into the culture supernatant. However, this is the first demonstration of the secretion of a complex composed predominantly of xylanase enzymes, a xylanosome. Perhaps this is because *B. fibrisolvens*, one of the most xylanolytic of rumen bacterium, is found predominantly in ruminants surviving under adverse conditions. It is possible that the secretion of a xylanase enzyme complex enables this bacteria to scavenge available hemicellulose in the rumen.

Chapter 6

General Discussion

This study was aimed at obtaining an understanding of the genes and enzymes involved in cellulose and hemicellulose degradation by the rumen bacterium *B. fibrisolvens* H17c. Two libraries, constructed by Dr E. Berger and Dr E. Rumbak respectively, of chromosomal DNA fragments from *B. fibrisolvens* H17c in pEcoR251 and pEB1 respectively have been screened for genes expressing cellulolytic ,xylanolytic and amylolytic activities. To date, the genes for three cellulases, one xylanase and two starch-degrading enzymes have been isolated and sequenced in this department. These data have been useful in understanding the strategy of codon usage in *B. fibrisolvens* H17c. In future this information could be used when synthesizing oligonucleotide probes for detecting further genes expressed in enzymatically inactive, but antigenically active forms. This is of particular interest as although *B. fibrisolvens* is the dominant cellulolytic and xylanolytic rumen bacterium under adverse nutritional conditions, to date only one xylanase and three cellulase genes have been cloned from H17c. Further analysis of the extracellular cellulases and xylanases could assist in understanding fibre degradation by *B. fibrisolvens* H17c. In addition to using probes, the fractionated enzymes could be used to raise antibodies to detect enzymatically inactive forms of the enzymes.

The two genes analyzed in this thesis were both expressed from their own promoters in *E. coli* as demonstrated by deletion analysis and subcloning. The nucleotide sequence of the upstream region of the *bgl*A gene contains one putative promoter consensus sequence but no such sequence was found in the upstream region of the *xyn*B gene. The promoter activity of these regions could be tested by fusing the region to a promoter-less *lac*Z gene and assaying for β-galactosidase activity. In addition, to determine what sequences are recognized by *B. fibrisolvens* as promoters, RNA could be isolated from H17c and subjected to primer extension analysis.

The *bgl*A gene codes for a β-1-4-glucosidase, and the *xyn*B gene for an endo-β-1-4-xylanase. Both enzymes were found predominantly in the cytoplasm of the *E. coli* host. Neither BglA nor XynB contain a signal sequence typical for procaryotes. Although the β-glucosidase is likely to be intracellular in *B. fibrisolvens* H17c, it is unlikely that a xylan-degrading enzyme would be located intracellularly. Therefore the XynB enzyme may be secreted via an other route. Antibodies specific to the xylanase could be used as a probe to test for the presence of the cell-bound or extracellular xylanase B.

To date, more than 70 microbial β-1-4-glycanases have been sequenced. Analysis and comparison of the sequences have revealed conserved domains which are common to both cellulases and xylanases. The conserved catalytic and non-catalytic domains are connected by linkers which allow the

domains to function independently (Gilkes *et al.*, 1991). Hydrolysis by glycosyl hydrolases is proposed as a general acid catalysis, usually promoted by an aspartate or a glutamate residue, or both. The active site residues are highly conserved in type II β-glucosidases of which BglA is a member, therefore be interesting to address the involvement of the targeted residues in the active site by site-directed mutation.

In fungi, the synthesis of cellulases and xylanases is regulated by a variety of "signal" molecules (Sternberg and Mandels, 1979; Messner *et al.*, 1988). However the expression of rumen bacterial glycanases can be either constitutive (McGavin *et al.*, 1990; Huang and Forsberg, 1990) or regulated (Howard *et al.*, 1960; Fusee and Leather, 1972; Smith *et al.*, 1973). A few reports deal with the regulation of cellulase or hemicellulase production of *B. fibrisolvens* H17c but little information is available on the transcription of individual glycanase-encoding genes. Further studies to investigate the *in vivo* regulation and initiation of transcription in *B. fibrisolvens* H17c could be carried out by using Northern blot and nuclease S1 protection analysis. This approach could address whether the synthesis of cellulase and xylanase activity is subjected to carbon source regulation.

Appendix A

Media, buffers and solutions

Appendix A

Media, buffers and solutions

All media, buffers and solutions were sterilized by autoclaving at 121°C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 µm membrane filters (Millipore).

A.1 Media

A.1.1 *B. fibrisolvens non-rumen medium (M10)*

Ingredient	Amount/l
Glucose	10.0 g
Cellobiose	0.5 g
Mineral solution 1[a]	38.0 ml
Mineral solution 2[b]	38.0 ml
Cysteine HCl (5%, w/v)	10.0 ml
Na_2CO_3 (5%, w/v)	50.0 ml
Resazurin (0.1%, w/v)	1.0 ml
Trypticase	2.0 ml
Yeast extract	0.5 g
Volatile fatty acid[c]	3.1 ml
Hemin[d]	10.0 ml
Distilled Water	850.0 ml

[a]Mineral solution 1

K_2HPO_4		6.0 g
Distilled water	to	1000 ml

[b]Mineral solution 2

KH_2PO_4		6.0 g
NaCl		12.0 g
$(NH_4)_2SO_4$		6.0 g
$CaCl_2$ $2H_2O$		1.6 g
$MgSO_4$ $7H_2O$		2.5 g
Distilled water	to	1000 ml

[c]Volatile fatty acid solution

Acetic acid	17 ml
Propionic acid	6 ml
Butyric acid	4 ml
Isobutyric acid	1 ml
n-Valeric acid	1 ml

Isovaleric acid	1 ml
2-Methylbutyric acid	1 ml

Solution is stable at 4°C

[d]Hemin solution

KOH	0.28 g
Ethanol (95%)	25.0 ml
Hemin	0.10 g
Distilled water	75 ml

Solution is stable at 4°C

All the ingredients were added and the pH adjusted to 6.8 prior to autoclaving.

A.1.2 *Luria-Bertani medium (LB)*

Bacto tryptone	16 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000ml

Solid media contained 1.5% (w/v) agar

A.1.3 *YT medium (X2)*

Bacto tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	1000ml

A.2 Media additives

Media were cooled to 50°C before addition of antibiotics, XGal, XP, or IPTG. Plates containing these additives were stored for no longer than one week at 4°C.

A.2.1 *Antibiotics*

Antibiotic stock solutions were as follows:

Ampicillin	100 mg/ml water
Kanamycin	62.5 mg/ml water

All antibiotics were filter sterilized and stored at -20°C.

A.2.2 *IPTG (Isopropyl-β-D-thio-galactopyranoside)*

IPTG (100mM)	23.4 mg
Distilled water	1 ml

The solution was stored in aliquots at -70°C

A.2.3 *XGal (5-bromo-4-chloro-3-indolyl-β-galactoside)*

Xgal	0.2 g
DMSO	10 ml

The solution was stored at -70C

A.2.4 *XP (5-bromo-4-chloro-3-indolyl phosphate)*

XP	80 mg
DMSO	1 ml

The solution was stored at -70°C

A.3 Buffers and solutions

A.3.1 *ATP (10X) (Maniatis et al., 1982)*

Adenosine triphosphate	30 mg
Distilled water	5 ml

Ajust pH to 7.0 with 0.1N NaOH before making up to 5ml. Store in 100 µl aliquots at -70°C. Discard remainder once defrosted.

A.3.2 *Azocasein*

Azocasein	0.8 g
Phosphate buffer (0.1M, pH6.8)	100 ml

Stir overnight to dissolve azocasein

A.3.3 *Bradford solution (Bradford, 1976)*

Coomassie Brilliant Blue (G-250)	100 mg
Ethanol (95%)	50 ml

Dissolve, then add 100ml phosphoric acid (85%). Dilute to final volume of 1 l. Filter through Whatman GF/C filter paper. Store in dark bottle.

A.3.4 *Denhardt's solution (10X) (Maniatis et al., 1982)*

Ficoll	1 g
Polyvinylpyrrolidone	1 g
BSA (Fraction V)	1 g
Distilled water	to 100 ml

The solution was stored in aliquots (10ml) at -20°C

A.3.5 *Dinitrosalicylic acid solution (DNS) (Miller, 1959)*

3,5 Dinitrosalicylic acid	10.6	g
NaOH	19.8	g
Rochelle salts (Na K Tartrate)	306	g
Phenol	7.6	ml
Na-meta bisulphite	8.3	g
Distilled water	1416	ml

The dinitrosalicylic acid, NaOH, and Rochelle salts were dissolved completely in the water before adding the other constituents and dissolving each in turn. The phenol was melted at 50°C. A 3ml sample was titrated to the end-point with 5-6ml HCl (0.1M) using phenolphthalein as an end-point indicator. However, if less HCl was required then solid NaOH was added to the DNS solution at the rate of 2g/ml of HCl less than 5ml, and the titration repeated. The DNS solution was stored in a dark bottle under N_2.

A.3.6 *DNA loading solution (6X)*

Bromophenol blue	0.25	g
Sucrose	40	g
Distilled water	to 100	ml

The solution was stored at 4°C

A.3.7 *EDTA (0.5M, pH8.0) (Maniatis et al., 1982)*

EDTA $2H_2O$	168.1	g
Distilled water	to 1000	ml

EDTA will only dissolve when pH has been adjusted to 8.0

A.3.8 *Ethidium bromide solution (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide)*

A solution of 10mg/ml was made in distilled water and stored in a dark bottle.

A.3.9 *Exo-nuclease III shortening solutions (Henikoff, 1987)*

A.3.9.1 Exo buffer

Tris-HCl (1M, pH8.0)	660	µl
$MgCl_2$ (0.1M)	66. 4	µl
Distilled water	9.27	ml

A.3.9.2 Klenow mixture

Tris-HCl buffer (0.1M, pH8.0)	3	µl
$MgCl_2$ (1M)	6	µl
Distilled water	20	µl

A.3.9.3 Ligase mixture

Ligase buffer	144	µl
Distilled water	1440	µl

A.3.9.4 S_1 buffer (10X)

KoAc (3M)	1.1	ml
NaCl (5M)	5	ml
Glycerol	5	ml
$ZnSO_4$	30	mg

A.3.9.5 S_1 mixture

S_1 buffer (10X)	41	µl
Distilled water	259	µl
S_1 nuclease (60U)	1.5	µl

A.3.9.6 S_1 Stop

Trisma base (no HCl)	0.3	M
EDTA (pH8.0)	0.05	M

A.3.10 *Isopropanol (salt saturated)*

Isopropanol was saturated with aqueous 5M NaCl, 10mM Tris-HCl and 1mM EDTA (pH8.0).

A.3.11 *Ligase dilution buffer*

The buffer was made according to the following table and stored at -20°C.

Stock solution	Final conc.	/10ml
Tris-HCl (1M, pH7.6)	20 mM	0.2 ml
EDTA (0.5M, pH8.0)	1 mM	2 µl
DTT (0.5M)	5 mM	10 µl
KCl (1M)	60 mM	0.6 ml
Glycerol	44 % (v/v)	4.4 ml
Distilled water		4.788 ml

A.3.12 *Ligation buffer (10X)*

The buffer was made according to the following table and stored in aliquots at -70°C.

Stock solution	Final conc.	/10ml
Tris-HCl (1M, pH7.6)	66 mM	0.66 ml
$MgCl_2$ (1M)	6 mM	66 µl
ATP (0.1M)	1 mM	0.1 ml
DTT	0.1 mM	15.4 mg
Distilled water		0.174 ml

A.3.13 *Phenol (TE saturated)*

Phenol (200g, Merck) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted three times with TE (10X) or until the pH of the aqueous phase was approximately pH7.6. The phenol was stored under TE (1X) at -20°C.

A.3.14 *Prehybrization solution*

SSC buffer (6X)	100	ml
SDS	0.5	g
Denaturated salmon sperm DNA	1	ml
Denhardt's solution (50X)	10	ml
EDTA (0.5M, pH8.0)	2	ml

A.3.15 *Restriction enzyme buffers (10X)*

Stock solution	Final conc.
Tris-HCl (1M, pH7.9)	0.1 M
$MgCl_2$ (1M)	0.1 M
DTT (0.5M)	10 mM
BSA (10mg/ml)	1 mg/ml
Glycerol	44 % (v/v)
NaCl (5M)	0, 50, 100 or 150 mM

The buffers were made using the following table and stored at -20°C

Stock solution	Salt concentration (mM)			
	0	50	100	150
Tris-HCl	1ml	1ml	1ml	1ml
$MgCl_2$	1ml	1ml	1ml	1ml
DTT	0.2ml	0.2ml	0.2ml	0.2ml
BSA	1ml	1ml	1ml	1ml
Glycerol	4.4ml	4.4ml	4.4ml	4.4ml
NaCl	-	1ml	2ml	87.7mg
H_20	2.4ml	1.4ml	0.4ml	2.4ml

A.3.16 *Restriction enzyme dilution buffer*

The buffer was made according to the following table and stored at -20°C.

Stock solution	Final Conc.	/10ml
Tris-HCl (1M, pH7.5)	10 mM	0.1 ml
NaCl (5M)	50 mM	0.1 ml
Distilled water		5.3 ml
Filter sterilize this solution and then add the following constituents:		
2-Mercaptoethanol		7 µl
Gelatin (10mg/ml)		0.1 ml
Glycerol		4.4 ml

A.3.17 *SDS-Polyacrylamide gel electrophoresis (Laemmli, 1970)*

A.3.17.1 Acrylamide-bis-acrylamide stock solution

Acrylamide	29.2	g
Bis-acrylamide	0.8	g
Distilled water	to 100	ml

The solution was filtered through Whatman's paper (No.1) and stored in a dark bottle at room temperature

A.3.17.2 Resolving gel buffer

Tris (1.5M)	18.17	g
Distilled water	to 100	ml

Adjust pH to 8.8 (approximately 5.5 ml conc. HCl)

A.3.17.3 Stacking gel buffer

Tris-HCl (0.5M)	6.06	g
Distilled water	to 100	ml

Adjust pH to 6.8 (approximately 5.5ml conc. HCl)

A.3.17.4 Reservoir buffer (10X)

Tris base (0.25M)	15.15	g
Glycine (0.192M)	72.05	g
10% SDS (0.1%, w/v)	50	ml
Distilled water	to 5000	ml

The pH should be approximately 8.5.

A.3.17.5 Coomassie Blue staining solution

Coomassie Blue R250 (0.25%,w/v)	2.5	g
Destaining solution	1000	ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No. 1)

A.3.17.6 Destain solution

Acetic acid	250	ml
Methanol	750	ml
Distilled water	1500	ml

A.3.17.7 Sample treatment buffer

Stacking gel buffer	2.5	ml
SDS (10%)	4	ml
Glycerol	2	ml
2-Mercaptoethanol	1	ml
Distilled water	0.5	ml

A.3.17.8 Polyacrylamide gel preparation table

Stock solution	Stacking gel	Resolving gel (%)	
		10.0	7.5
Acrylamide	2.5ml	10.0 ml	7.5 ml
Resolving gel buffer	-	3.75ml	3.75ml
Stacking gel buffer	5.0ml	-	-
10% SDS	0.2ml	0.3 ml	0.3 ml
Ammonium persulfate (1.5%, w/v solution)	1.0ml	1.5 ml	1.5 ml
Water	11.3ml	14.45ml	16.95ml
TEMED	15 µl	15 µl	15 µl

A.3.17.9 Solutions for CMC or xylan-PAGE

Phosphate buffer (0.2M)

$NaH_2PO_4.H_2O$	13.8	g
Distilled water	to 500	ml

Adjust pH with NaOH to pH6.8

CMC solution

CMC	0.6	g
Phosphate buffer (0.2M,pH6.8)	100	ml

The solution was sterilized by autoclaving

Xylan solution

Xylan	0.5	g
Phosphate buffer (0.2M,pH6.8)	100	ml

The solution was sterilized by autoclaving

Triton X-100 (2.5%)

Triton X-100	50	ml
Distilled water	to 2000	ml

Congo red

Congo red	1	g
Distilled Water	to 1000	ml

Adjust pH to approximately 12.0 with NaOH

NaCl (1M)

NaCl	116.88	g
Distilled water	2000	ml

Adjust pH to approximately 12.0 with NaOH

Acetic acid (5%)

Acetic Acid	100	ml
Distilled water	to 2000	ml

A.3.17.10 Preparation table of overlay with incorporated CMC or xylan

Stock solution	1 overlay	2 overlays
0.2 M phosphate pH6.8	17.4 ml	34.8 ml
CMC or xylan (0.5%,w/v)	5.0 ml	10.0 ml
Acrylamide	7.5 ml	15.0 ml
TEMED	15 µl	30 µl
10% Ammonium persulphate	150 µl	300 µl

A.3.18 *Salmon sperm DNA*

A 10mg/ml solution was made in TE buffer. The DNA solution was sonicated at full power (20 microns) for 10 min in a MSE Soniprep sonicator. The solution was aliquoted and stored at -20°C.

A.3.19 *Sodium acetate (3M,pH5.2)*

Sodium acetate $3H_20$	4.08	g
Distilled water	to 10	ml

Adjust pH with glacial acetic acid. Autoclave.

A.3.20 *SSC (20X)*

NaCl (3M)	175.3	g
Sodium citrate (0.3M)	88.2	g
Distilled water	to 1000	ml

Adjust pH to 7.0 with NaOH (10N). Autoclave.

A.3.21 *Tris-acetate buffer (50X)*

Trisbase	242	g
Acetic acid	57.1	ml
EDTA (0.5M, pH8.0)	100	ml
Distilled water	to 1000	ml

A.3.22 *TE (Tris-EDTA) buffer (100X)*

Tris-HCl (pH7.6)	121	g
EDTA (0.5M, pH8.0)	200	ml
Distilled water	to 1000	ml

Autoclave and dilute with sterile water before use

A.3.23 *TSB solution*

LB	150	ml

pH to 6.1 with 2 drops conc. HCl.

PEG 4000	15	g
$MgSO_4$ (1M)	1.5	ml
$MgCl_2$ (1M)	1.5	ml

Dispense in 20 ml aliquots and autoclave. Add DMSO (1ml) and glucose (0.5M, 400µl) immediately before use.

A.3.24 *Z-buffer (pH7.0)*

Na_2HPO_4 (60mM)	16.1	g
NaH_2PO_4 $2H_20$ (40mM)	5.5	g
KCl (10mM)	0.75	g
$MgSO_4$ $7H_20$ (1mM)	0.246	g
2-Mercaptoethanol (0.05M)	2.7	ml
Distilled water	to 1000	ml

Appendix B

General Techniques

Appendix B

General Techniques

B.1 Plasmid preparations

B.1.1 Small scale *E. coli* plasmid DNA isolation. Plasmid was isolated from a 5 ml overnight culture (LB + Ap, 100 µg/ml) as described by Ish-Horowicz and Burke (1981). Cells were harvested from 1.5 ml of the culture by centrifugation in an Eppendorf microfuge for 1 min. The pellet was drained and then resuspended in 100 µl Solution I (50mM glucose; 25mM Tris-HCl, pH 8.0; 10mM Na_2 EDTA). The sample was left at room temperature for 5 min, vortexed and then placed on ice for 1 min. 200 µl of Solution II (0.2M NaOH; 1% SDS, w/v) was added and mixed briefly on a vortex mixer. The sample was held on ice for exactly 5 min before 150 µl pre-cooled Solution III (5M KoAc, pH 4.8) was added. The tube was placed on ice for a further 5-10 min. The sample was microfuged for 5 min and the supernatant fluid was transferred to a fresh tube. Two volumes of 95% ethanol were added, the sample was allowed to stand at room temperature for 5 min and then it was spun for 15 min in a microfuge. The pellet was dried and resuspended in 50 µl TE buffer.

B.1.2 Large scale *E. coli* plasmid DNA isolation. A 200 ml LB broth culture, grown overnight at 37°C in the presence of the appropriate antibiotic, was harvested by centrifugation at 6,000 x g for 5 min. The cell pellet was resuspended in 4

ml of Solution I and incubated at room temperature for at least 5 min. Freshly made solution II (8 ml) was added, mixed thoroughly and the sample put on ice for exactly 5 min. Precooled Solution III (6 ml) was added, mixed thoroughly and the tube returned to ice for a further 5-10 min. The cellular debris was removed by centrifugation at 12,000 x g for 10 min. The supernatant was removed into clean tubes. An equal volume of isopropanol was added to the fluid and the sample left to stand at room temperature for at least 2 min. The DNA was precipitated by centrifugation at 15,000 x g for 15 min. The pellet was washed with 70% ethanol and then resuspended in 5 ml TE buffer. Cesium chloride (1g/ml, DNA solution) and Ethidium-bromide (200 µl of 10 mg/ml) were then added before a clearing spin at 27,000 x g for 15 min. The refractive index of the DNA solution was adjusted to 1.396. The DNA preparation was sealed in Quick seal tubes and spun in a Beckman Vti 65.2 rotor at 55,000 rpm at 15°C for 12h. The plasmid DNA band was visualized by long wave UV light (350nm), and removed in the smallest volume possible. EtBr from the resulting plasmid band was extracted with salt-saturated isopropanol (2-3 times) until no pink colour remained. The DNA was precipitated from the CsCl solution by the addition of four volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 200 µl TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of 10 µl (diluted in TE) between 220 and 310nm. The concentration

was determined by using the relationship A_{260} = 1 for 50 µg/ml double-stranded DNA.

B.2 Restriction endonuclease digestion. Restriction digests were carried out using one of the four restriction buffers (Appendix A) according to the salt requirements of the particular enzyme. Digestion volumes were routinely 20 µl containing 300-500 ng DNA and one unit of restriction enzyme. Digestions were done at 37°C (most enzymes) for 1h. Concentrated enzyme stocks were diluted to 1 or 2 units using universal restriction enzyme dilution buffer (Appendix A). For electrophoretic analysis, the digestions were terminated by the addition of 5 µl DNA loading solution (Appendix A) to the 20 µl digestions. If the sample was to be used for ligation the digestion was terminated by a phenol-chloroform extraction. The DNA solution was extracted with the addition of phenol (1/10 volume, TE-saturated) and an equal volume of chloroform : isoamyl alcohol (24 : 1). The mixture was vortexed briefly, and the two phases were separated by centrifugation. The aqueous phase was extracted twice with water-saturated ether. The DNA was precipitated by the addition of 5M $NaClO_4$ (1/10 volume), an equal volume of isopropanol, and 15 min centrifugation. After centrifugation the pellet was washed with 70% ethanol and resuspended in TE buffer.

B.3 Agarose gel electrophoresis. Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-acetate buffer (Appendix A) was used routinely. Sigma type II agarose was used at varying concentrations. The

amount of DNA loaded per lane also varied with the sizes and number of fragments but under normal circumstances about 330 ng of plasmid DNA was used. The gels were electrophoresed at 2 V/cm for 16h. Gels were stained in electrophoresis buffer containing EtBr (0.5 μg/ml) for 15-30 min. DNA bands were visualized using a 254nm transilluminator. A 310nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3,000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f4.7 was used.

Fragment sizes were calculated by extrapolation from a standard curve of the mobility of λ DNA fragments, digested with either *Hind* III or *Pst* I, plotted against the $\log_{10}$ of their molecular weights. Approximately 0.8-1.2 μg of a λ digest was loaded per gel lane.

B.4 Ligation reaction. The methods of Maniatis *et al.* (1982) were generally used. Recircularization reactions for isolating deletion plasmids contained DNA concentrations of 1 pmole/ml or less. A DNA concentration in the order of 5 pmole/ml was used for recombination reactions. Ligation reactions containing DNA, ligation buffer (Appendix A) and water to the required volume, were performed in sterile microfuge tubes. Sticky-end ligations were performed at

room temperature for 3 h or at 15°C overnight using 0.1-0.25 U of ligase , whereas blunt-end ligations were performed at room temperature for 2-20 h using 20-100X more ligase.

B.5 Subcloning protocol. The rapid subcloning protocol of Struhl (1985) was used. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (1%) (Seaplaque^{R}) in Tris-acetate buffer (50 mM, pH8.2). The gel was stained with EtBr after electrophoresis and the DNA bands were viewed under UV light (310nm), as briefly as possible. The desired bands were excised using sterile scalpel blades, in as small a volume as possible. The gel slices were melted at 70°C for 5 min in a microfuge tube and the required amounts (2 µl vector DNA, 8 µl insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ligase and water (10 µl). The ligation was incubated at room temperature for 3 h. Before transformation of *E. coli* competent cells, the gelled ligation reactions were melted at 70°C for 5 min, and then diluted with 4 volumes TSB solution (Appendix A).

B.6 The preparation and transformation of competent *E. coli* cells. *E. coli* cells were made competent for DNA uptake according to the method of Chung and Miller (1988). A 1/100 dilution of an overnight *E. coli* culture in LB was inoculated into 25 ml prewarmed LB and incubated at 37°C, with shaking, until the culture had reached early exponential phase (OD_{600} = 0.3 - 0.6). The cell culture was poured into a pre-cooled sterile SS-34 tube and the cells were harvested at 5,000 x g for 5 min at 4°C. The cell

pellet was resuspended in 2.5 ml (1/10 volume) ice-cold transformation and storage buffer (TSB) and held on ice for 10 min. The *E. coli* cells (100 µl) were then mixed with DNA and held on ice for a further 30 min. TSB solution (0.9 ml) containing glucose (20 mM) was added to each transformation mixture and incubated at 37°C for 60 min, to allow expression of the plasmid borne antibiotic marker.

B.7 Radioactive labelling of DNA probes. DNA probes were labelled with [α - ^{32}p]dCTP to high specific activity by nick-translation (Rigby *et al.*, 1977). The reagents were obtained in kit form (Amersham) and used according to the suppliers specifications. Contaminating nucleotides were removed from the radioactively labelled probe preparation using a Sephadex G-50 spin column as described by Maniatis *et al.*, (1982). Radioactively labelled probes were stored in lead containers at -20°C. Probes were denatured by boiling (5 min) in a fume food just before use.

B.8 DNA hybridization. DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond-N hybridization membrane (Amersham) essentially by the protocol of Reed and Mann (1985). The use of a nylon transfer membrane allows the capillary transfer of DNA restriction fragments in alkali rather than in neutral, high ionic strength solvents (used in conventional Southern transfer), and eliminates the need for post-transfer fixation (Reed and Mann, 1985). After electrophoresis the gel was rinsed in 2 volumes of HCl (0.25M) for 20 min at room temperature with gentle agitation, followed by a brief

rinse in distilled water. The gel was then placed on top of 2 sheets of Whatman 3MM filter paper (wetted with 0.4N NaoH, and placed on top of an inverted gel-casting tray in a plastic box, such that the filter paper touched the base of the box, forming a wick), and was flooded with 50-100 ml of 0.4N NaoH, and then immersed in, distilled water was placed on top of the gel, and any air bubbles were removed. Three sheets of Whatman 3MM filter paper, wetted in 0.4N NaoH, were laid onto the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer lift to continue overnight. After transfer, the membrane was removed and rinsed for 20 min in 2 X SSC (Appendix A). Hybridization and washing conditions were essentially according to Maniatis *et al.*, (1982). The membrane was gently shaken in pre-hybridization solution (Appendix A) for 4 h at 65°C, while the probe was being prepared. The radioactively-labelled probe to be used was denatured by boiling for 10 min and was added to the pre-hybridization fluid. Hybridization was carried out at 65°C for 30 min, and after checking the radioactivity by means of a Geiger-counter, the washing was terminated and the membrane sealed in a plastic bag. The membrane was exposed to autoradiographic film (XAR-5) overnight at -70°C.

B.9 Nucleotide sequencing.

B.9.1 *Primer annealing reaction.* The supercoiled DNA (6-10 µg, in TE buffer) was diluted to a final volume of 20 µl in distilled water. Alkaline denaturation in 0.2N NaoH (5 min at room temperature) was followed by the addition of 5 µl of

3M sodium acetate (pH5.2), 25 µl of distilled water and 150 µl of ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 µl of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 µl of sequencing buffer (40mM Tris-HCl, pH7.5; 20mM $MgCl_2$; 50mM NaCl) and 12 ng of primer. This mixture was annealed for 30 min at 40°C immediately prior to sequencing. The primers used were the forward sequencing primer as supplied in the sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, Ohio) and the M13 reverse sequencing primer (Amersham).

B.9.2 ***Sequencing reactions.*** DNA sequencing was done by the dideoxy chain termination method of Sanger *et al.*, (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a "sequenase" sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [α - ^{35}S] dATP (1200 Ci/mmol; Amersham).

B.9.3 ***Gel electrophoresis and autoradiography.*** The sequencing reactions were analysed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 sequencing Handbook. After electrophoresis the gels (0.3mm thick) were dried onto whatman No.3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing ^{35}S-labelled DNA were placed under XAR-5 autoradiographic

film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

B.10 SDS-Polyacrylamide gel electrophoresis of proteins. SDS-Polyacrylamide gels were prepared according to the method of Laemmli (1970) using a Hoefer gel apparatus (SE600) with 1.5 mm spacers assembled according to the manufacturer's specifications. All buffers and a preparation table for resolving and stacking gels are given in Appendix A. Propan-2-ol was layered on the gel to promote a sharp interface. After the gel had polymerized, the propan-2-ol was removed by rinsing with the stacking gel buffer, and the stacking gel was cast.

Samples were prepared in sample treatment buffer (Appendix A) and placed in a boiling waterbath for 2 min before being loaded onto the gel. Electrophoresis was continued at 35 mA (constant current) per gel until the dye front had migrated to the end of the gel (four to five hours). After electrophoresis the gels were stained for 3 h in staining solution with gentle agitation, destained and dried.

B.11 Non-denaturing (native) polyacrylamide gel electrophoresis. Proteins were separated under non-denaturing conditions using conditions identical to SDS-PAGE with the exception that SDS was omitted from all buffers. Samples were combined with an equal volume of sucrose-dye solution (50% [w/v] sucrose, 0.1% [w/v] bromophenol blue) and loaded directly on the gel.

B.12 Determination of protein concentrations. Protein concentrations in solutions were determined by the method of Bradford (1976). Assays were performed in triplicate using disposable cuvettes. The reaction contained protein solution (0.1 ml) and 2 ml Bradford solution (Appendix A). The mixture was left for 5 min at room temperature and the optical density of the reaction monitored at OD_{595}. Protein concentrations were calculated using a standard curve (BSA Fraction, 10-200 µg/ml). Protein samples were diluted such that OD_{595} did not exceed 0.8.

Appendix C

Bacterial strains, genotypes, and references

Strains	Relevant characteristics	Reference
B fibrisolvens H17c	wild type	Dohority (1966)
E. coli C600	*sup*E44 *hsd*R *thi*-1 *leu*B6 *lac*Y1 *ton*A21 *thr*-1	Appleyard (1954)
E. coli K514	*thr*-1 *leu*B6 *thi*-1 *supp*E44 *lac*Y1 *ton*A21 r_k^-, m_k^+	Wood (1966)
E. coli LK111	*lac*I^q *lac*Z ΔM15 *lac*Y^+ (K514 derivative)	Zabeau and Stanley (1982)
E. coli JM103	Δ(*lac pro*) *thi str*A *sup*E *end*A *sbc*B *hsd*R^- F'*tra*Δ36 *pro*AB *lac*I ZΔM15	Messing *et al.* (1981)
E. coli CC118	*ara*Δ139 Δ(*ara leu*) 7697 Δ*lac*X174 *pho*A Δ 20 *gal*E *gal*K *thi* *rps*E *rpo*B *ara*Eam *rec*A1	Manoil and Beckwith (1985)

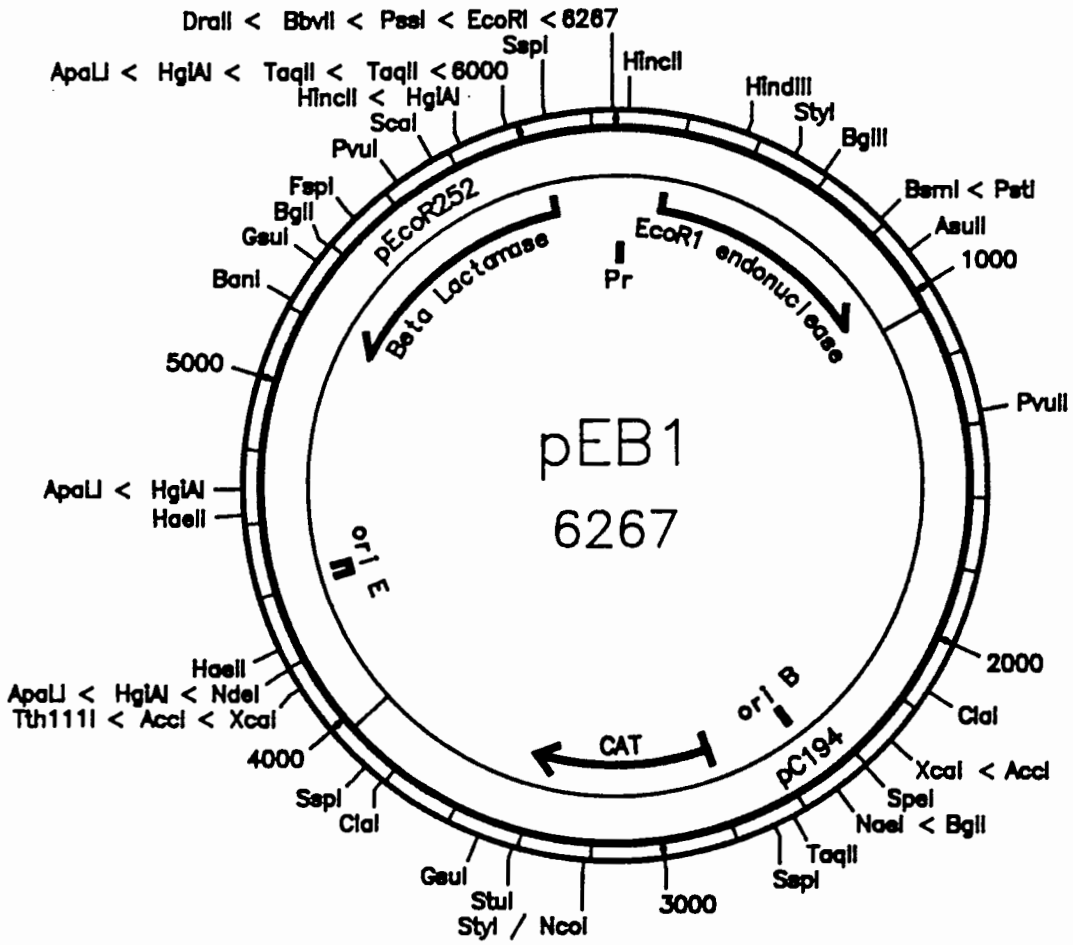
Appendix D

One- and three-letter codes used for amino acids

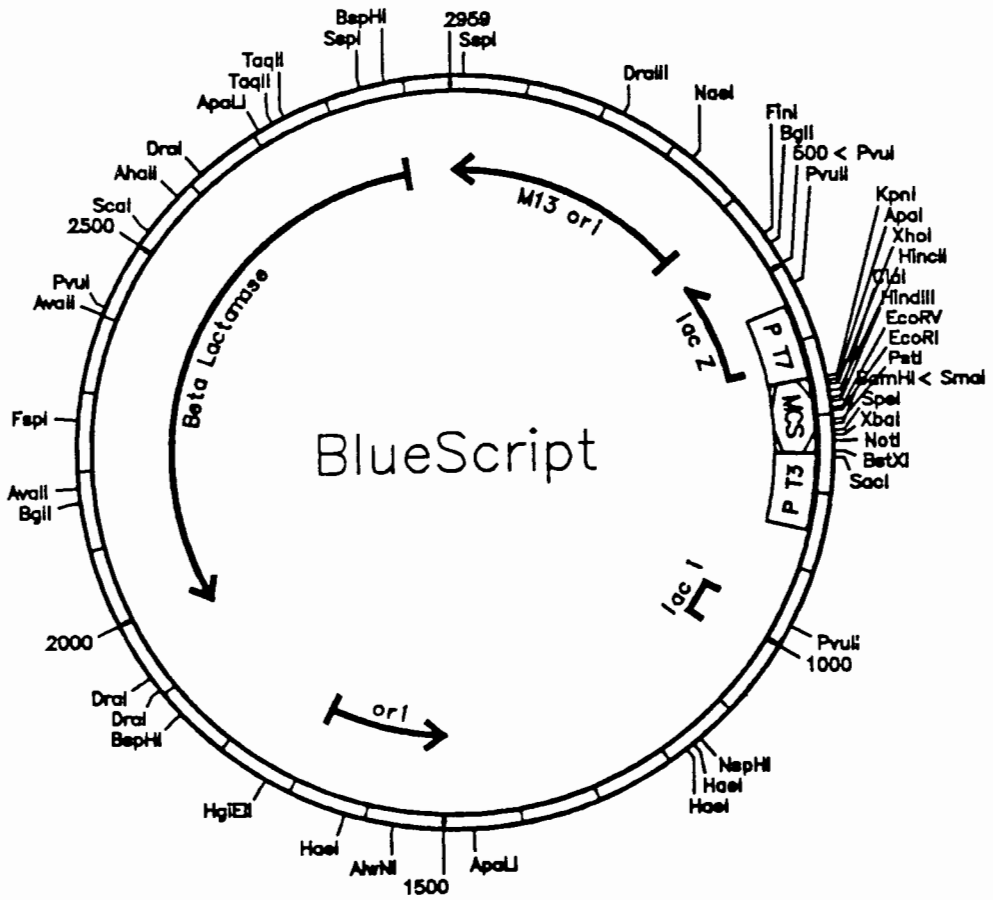
Amino acid	Code		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine

Appendix E

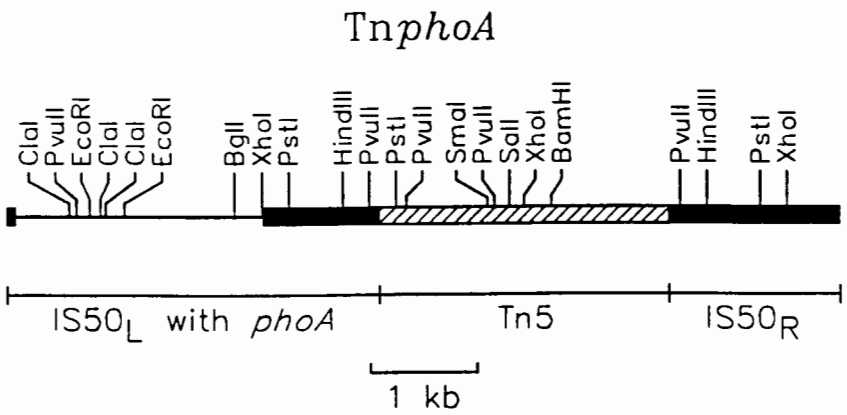
Plasmid vector and phage maps



Reference: H. Zappe (personal communication)



Reference: Stratagene, San Diego, California



Partial restriction map of Tn*pho*A showing position of Tn5 relative to the leftward and rightward insertion sequences (J. Beckwith, personal communication)

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