Examination of Cellulolytic activity in Activated sludge, Leading to Elucidation of the Role of β-1,4-endoglucanase enzyme in *Aeromonas* sp. YS3

By

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DECLARATION

The work presented in this thesis was performed while I was enrolled for the degree of Doctor of Philosophy in the department of Biotechnology and Environmental Biology at the Royal Melbourne Institute of Technology.

To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged in this report.

The report has not been submitted, in whole or in part, for any other award.

Signed......................................

Date..........................................
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ABSTRACT

The initial aim of this project was to uncover novel cellulolytic organisms or enzymes from the diverse microbial source, activated sludge. Two isolation methods were used; either directly inoculating the sludge material onto filter paper as a carbon source, or using the Evolver™ technology as an enrichment device. In both cases, as expected, cellulase activity was evident, however attributing this activity to one species was difficult in either case. This highlighted the complex interrelationships that existed between the many microorganisms present as the cellulosic carbon sources were degraded. In one instance, a *Cellvibrio* sp. was isolated. This genus of bacteria is known to possess both types of cellulase activity (exo- and endo- acting) and was therefore likely to contribute to the degradation of the cellulose. However, the isolate, once purified, did not display significant cellulolytic ability as compared to the unpurified consortium of microorganisms. Therefore, in each case, microorganisms responsible for the cellulolytic activity were not uncovered. It was suspected that the microorganisms responsible for some of the cellulolytic activity were protists.

During the isolation of microorganisms, an *Aeromonas* sp. bearing the novel phenotype (for this genus) of CMCase activity was isolated. This activity was at first suspected to contribute to the degradation of the filter paper that was seen during isolation. However, tests with the pure isolate suggested that the *Aeromonas* sp. CMCase was not used for cellulose catabolism. Ironically, the enzyme may instead function in the production of a cellulose-like exopolysaccharide by the bacterium. Part of a cellulose synthase operon was found in the genome of the *Aeromonas* sp. isolate, including a gene coding for an endoglucanase that gives a predicted molecular weight enzyme similar to the 39 kDa CMCase purified from the bacterium.

The CMCase enzyme, operating as part of of a synthetic operon is expected to be important in terms of the biofilm forming ability of this *Aeromonas* strain. Such capabilities of the bacterium were investigated here, including observing motility behaviour of the organism on agar surfaces. Studying the biofilm forming ability of this genus in general will be important in understanding how the fish and human pathogens persist in aquatic environments.
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1. Cellulase literature review

1.1 Introduction

Cellulases (E.C.3.2.1.4) are the enzymes that carry out hydrolysis of the β-1,4-glycosidic linkages in the natural polymer, cellulose. Three types of enzyme activity are needed for the complete solubilization of the cellulose substrate; endocellulase (syn. endoglucanase) activity, which cleaves cellulose chains internally; exocellulase (syn. exoglucanase) activity, which acts upon the chain ends and β-glucosidase activity, which hydrolyzes soluble cellobiooligosaccharides (mainly cellobiose) to produce glucose. Endo- and exocellulases (also known as cellobiohydrolases) were originally classified as separate types of enzymes with different modes of action, but over the past ten years it has been recognized that these distinctions are not absolute, rather, a single cellulase enzyme may have either a predominant exo- or endo- mode of action (Henrissat et al. 1998). The structure of the catalytic site of the enzyme determines the mode of action and this is discussed in detail below. Regardless of the fact that many cellulases can carry out both endo- and exo-glycanolytic modes of action, it has long been recognized that a system of enzymes, not just a sole enzyme is needed for complete solubilization of the recalcitrant substrate (Lamed et al. 1985). Evidence for this is the often observed synergy between cellulase enzymes (discussed further below). A true cellulase system is one that can completely solubilize crystalline cellulose to glucose or small oligosaccharide products (Johnson et al. 1982). Many natural organisms have been found to have endocellulase activity whilst lacking exocellulase activity, therefore these are not true cellulase systems (Robson and Chambliss 1989). Additionally, many natural cellulase systems have a shortage of β-glucosidase activity (Breuil et al. 1986).
Other enzymes have also been implicated in the cellulose degradation process. The fact that oxygen was seen to enhance the degradation of cellulose by some fungi (Eriksson et al. 1974) led to the discovery of two oxidative enzymes, cellobiose oxidase and cellobiose:quinine oxidoreductase that are involved in the cellulolytic process (Westermark and Eriksson 1975; Ayers et al. 1978). Fungal cultures grown on cellobiose did not display these activities, suggesting that they are only required for cellulolysis (Renganathan et al. 1990). In addition to fungal cultures, this enzyme activity has been found in the bacterium *Cytophaga* sp. LX-7 (Li and Gao 1997). Similarly, enhanced cellulose degradation was seen in *Cytophaga hutchinsonii* with increased oxygen supply (Walker and Warren 1938). It has also been suggested that in brown rot fungi, extracellular hydrogen peroxide and Fe$^{2+}$ are utilized in cellulose decomposition although the sources of these chemicals have not been identified (Goyal and Eveleigh 1992). Therefore, the use of isolated cellulase enzymes in industrial applications may not result in the same extent of hydrolysis as displayed in the native host, if factors other than the cellulase enzymes are needed.

Another class of enzymes implicated in cellulose degradation are the expansins. These enzymes disrupt the hydrogen bonds between cellulose, to allow sliding of the molecules during plant cell wall elongation (Whitney et al. 2000). These enzymes were originally thought to only be produced only by plants, but have since been discovered in organisms from other Kingdoms. An expansin-like enzyme, also containing a cellulose-binding domain, has been reported in *T. reesei*, and was co-expressed with the cellulase enzyme system, suggesting it was involved in the degradation of cellulose (Saloheimo et al. 2002). Such enzymes have also been found in plant parasitic nematodes and are co-expressed with cell wall degrading enzymes, suggesting that they contribute to the high rate of penetration of plant tissues by the nematodes (Qin et al. 2004). Enzyme domains bearing sequence similarity to expansins have also been found in bacteria (Laine et al. 2000).

Due to the abundance of cellulosic materials and a developing interest in using this renewable resource more efficiently, a great deal of research has been carried out on cellulase enzymes. “Cellulose is the most abundant renewable resource on Earth, accounting for about half of the organic
material in the biosphere” (Divne et al. 1994). It has been estimated that 144 million tons of cellulosic agricultural residues could be collected for bioconversion each year (Sheehan and Himmel 1999). The cellulose in forestry, agricultural and municipal wastes, once broken down into the resultant glucose, can be converted into ethanol as a fuel source or be used as a chemical feedstock (Ng et al. 1983).

1.2 Industrial applications of cellulase enzymes

Applications of cellulase enzymes are in the textiles, food, agriculture, detergent and pharmaceutical industries, in fact, cellulases are amongst the largest selling enzymes (Amritkar et al. 2004). Additionally, the cellulase market is expected to expand upon industrial scale ethanol production from cellulose (Cherry and Fidenstef 2003). Cellulases are used in the textile industry for ‘stone-washing’ of denim, improving colour brightness and depilling (Andreas et al. 1999). In the food industry cellulases can be used to increase digestibility of high-fibre foods and to enhance food flavour and texture (Hallemeersch and Vandamme 2003). Expressing cellulase genes in the shoyu koji mould may improve the use of the fungus in the production of soy sauce (Kitamoto et al. 1996). Cellulases have been found to improve the oleoresin extraction from marigold flowers, a process that yields valuable carotenoids (Navarrete-Bolaños et al. 2003). Cellulases are used in detergents to brighten colour in cotton textiles, soften fabric and help remove particulates (Niehaus et al. 1999). Cellulases may be useful in the manufacture of paper by improving the drainablity of pulp from recovered paper (Blanco et al. 1998). Paper made from pulp derived from agricultural residues such as cereal straw is of lower strength and poorer quality than that made from wood pulp, however, treatment of the agricultural pulp with endocellulase has been shown to increase the mechanical strength of paper made from crop residues (Blanco et al. 1998). Many of the applications of cellulase enzymes do not require the application of a complete cellulase system, as can be seen in the removal of barley β-glucan in beer brewing accomplished by expressing a single endoglucanase gene in yeast (Béguin 1990) and results in improved filtration and clarity of the broth (Bisaria and Mishra 1989). Another application of expressing cellulase genes in a non-native host is the modification of ruminal bacteria to improve the digestion of fibrous plant material in ruminant animals (Aylward et al. 1999). All of these separate
industrial applications of cellulases require enzymes with differing optimal conditions. For instance, psychrophilic enzymes are desired for use in cold-wash detergents and in brewing (Akila and Chandra 2003). Thermostable enzymes, on the other hand, possess an advantage in applications such as ethanol fermentation, where temperature is raised to avoid contamination by unwanted microorganisms (Bhat and Maheshwari 1987). The pH optima of the enzymes is also important, for example, biowashing of denim garments requires an operating pH below 6.0 to prevent the corrosion or loss of gloss of metal fittings (Arifoglu and Ögel 2000).

Cellulase enzymes occur in many forms in a wide variety of organisms. Fungal and bacterial cellulases are most often studied but plants, protists and even invertebrates (Lo et al. 2003) have also been found to produce cellulase enzymes. Because of its recalcitrant nature cellulose accumulates in environments, with the result that micro-organisms in virtually every niche and clime have evolved to decompose it (Ljungdahl and Eriksson 1985). Approximately 5-10% of cellulosic materials are degraded anaerobically (Wolin and Miller 1987). Psychoactive cellulases have been found in a Clostridium strain (Akila and Chandra 2003) and many thermophilic cellulases have been found in both bacteria and fungi, the most thermostable being from the aerobic Rhodothermus marinus bacteria (Hreggvidsson et al. 1996). The diverse evolutionary history of cellulase enzymes has resulted in many different forms of the enzyme with different capabilities and enzyme kinetics.

The diversity of cellulolytic organisms may be partially due to horizontal gene transfer. This is a common event in bacteria, although in fungi and other eukaryotes, DNA duplication is thought to be more often responsible for new enzyme sequences (Gilbert and Hazlewood 1993). Relatively recent transfer of an endocellulase gene was thought to occur in Cellulomonas uda, as the cellulase gene was found to have a different G/C content than the rest of the genome (Béguin 1990). An endoglucanase of Clavibacter michiganensis ssp. sepedonicus was found to be carried on a plasmid (Laine et al. 2000), making it possible to transfer to other species. Alternatively, because the structures of cellulases or their subdomains in fungi and bacteria vary considerably they are thought to have arisen from convergent evolution (Kraulis et al. 1989).
Cellulases are glycosyl hydrolases (GH) and this group of enzymes has been sorted into 87 different families according to amino acid sequence similarities (Henrissat et al. 1998; Henrissat and Davies 2000). Cellulases are present in 12 of these families (Hakamada et al. 2002). The classification scheme is useful in that it reflects structural features and helps to predict the mechanism of catalysis of members of the same family (Henrissat et al. 1998). The similarities between the sequences of members of a family can also be used to predict evolutionary relationships. For example when a phylogenetic tree was constructed with GH family 7 cellulases it was concluded that the designated exo- and endocellulases evolved independently (Hamada et al. 1999). The fact that distantly related organisms bear cellulases from one family and therefore share amino acid similarities, lends support to the theory that interspecific exchange of cellulase genes has occurred widely (Béguin 1990).

Cellulases within a glycoside hydrolase family can be further subdivided to delineate closer relationships (Gilad et al. 2003).

Table 1.1 outlines the main features of glycoside hydrolase families that contain cellulase enzymes, full information about each glycoside hydrolase family (and the enzymes they contain) is found on the Carbohydrate-Active Enzymes database (http://www.cazy.org/index.html, 2007).

Most cellulases have a multi-domain structure, where one domain carries out the catalytic function and additional domains are required for binding to solid cellulose. These binding domains were originally termed CBDs (cellulose binding domain) but now the more inclusive term CBM (carbohydrate binding module) is also used, as similar domains have been found that bind to other insoluble/soluble carbohydrates (Boraston et al. 2004). The structure and binding characteristics of cellulases is discussed further below.
Table 1.1 A brief description of glycoside hydrolase families that contain cellulase enzymes, showing the diversity of substrate specificities and origins of the enzymes in each family.

<table>
<thead>
<tr>
<th>Glycoside hydrolase family</th>
<th>Former classification (cellulase family)</th>
<th>Enzyme activities</th>
<th>Organism diversity</th>
<th>Comments</th>
</tr>
</thead>
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<tr>
<td>5</td>
<td>A</td>
<td>Most of the ‘cellulases’ are endocellulases (E.C. 3.2.1.4) rather than cellobiohydrolases (exocellulases) but other enzymes include chitinases, mannanases and xylanases.</td>
<td>Cellulase enzymes in this family arise from bacteria and fungi.</td>
<td>The largest and most diverse family containing cellulases, both in terms of substrate specificity of the enzymes and the organisms that they arise from.</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>Endocellulases and cellobiohydrolases (E.C. 3.2.1.91) are listed in this family.</td>
<td>Both bacteria and fungi.</td>
<td>The cellobiohydrolases listed in this family seem to comply with the original definition of this enzyme classification, that is, they act only from the non-reducing end of the cellulose chain.</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>Endocellulases and exocellulases.</td>
<td>Both bacteria and fungi.</td>
<td>The exocellulases in this family seem to be able to act on either end of the cellulose chain.</td>
</tr>
<tr>
<td>8</td>
<td>D</td>
<td>Endocellulases as well as chitinases, xylanases and lichenanases. No exocellulases recorded.</td>
<td>The endocellulases in this family arise from bacteria only.</td>
<td>The most diverse family in terms of organisms, with cellulases from rice, strawberries and cockroaches listed, but no fungal cellulases.</td>
</tr>
<tr>
<td>9</td>
<td>E</td>
<td>Endocellulases and exocellulases.</td>
<td>Bacterial cellulases as well as enzymes from plants, arthropods and protists.</td>
<td>A small family with few entries.</td>
</tr>
<tr>
<td>12</td>
<td>H</td>
<td>Endocellulases, xylanases and enzymes active on mixed linkage polysaccharides.</td>
<td>Bacterial, fungi and archaea enzymes.</td>
<td>The enzyme listed from Shipworm is the only eukaryotic enzyme in this small family.</td>
</tr>
<tr>
<td>44</td>
<td>J</td>
<td>Endocellulases and xylanases.</td>
<td>Bacterial enzymes and one Shipworm cellulase.</td>
<td>The sequences of these enzymes are distantly related to plant expansin enzymes.</td>
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<tr>
<td>45</td>
<td>K</td>
<td>Endocellulases.</td>
<td>Mostly fungal cellulases.</td>
<td>The exocellulases in this family do not conform to the standard definition of cellobiohydrolase.</td>
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<tr>
<td>51</td>
<td></td>
<td>Mostly arabinofuranosidase enzymes.</td>
<td>Only one bacterial cellulase listed.</td>
<td>A very small family.</td>
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<tr>
<td>61</td>
<td></td>
<td>Endocellulases only.</td>
<td>Fungal enzymes only.</td>
<td>A very small family.</td>
</tr>
<tr>
<td>74</td>
<td></td>
<td>Endocellulases and xylanases.</td>
<td>Bacterial and fungal enzymes.</td>
<td>A very small family.</td>
</tr>
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1.3 Substrate considerations

To understand the action of cellulase enzymes, consideration must be given to the physically complex substrate, cellulose. “Cellulose is a linear polymer of anhydroglucose units joined together by β-1,4-glucosidic bonds.” (Bae et al. 2004). In the cellulose chain each glucose residue is rotated 180° with respect to its neighbours (Coughlan 1985), therefore cellobiose is the repeating unit (Lucas et al. 2001). Cellulose is a chemically simple molecule but the nature of the crystalline solid is much more complex. Cellulose chains are held together in layers by van der Waals forces as well as intra- and intermolecular hydrogen bonds (Gan et al. 2003). Depending on the origin of the cellulose, the chain length and degree of interaction between the chains vary (Mandels et al. 1976). The crystallinity of a given cellulosic substrate has a large effect on the rate of hydrolysis by cellulases (Fan et al. 1980). The following types of cellulose are commonly used in cellulase assays: Valonia cellulose (from the algae Valonia ventricosa), Bacterial microcrystalline cellulose (BMCC) and Avicel (prepared from cotton) and their relative crystallinities vary considerably, being 1.0, 0.076 and 0.50 respectively (Mandels and Reese 1956). Due to the different crystallinity of these substrates, a single enzyme may have a different rate of hydrolysis on each one. For example, when Trichoderma reesei cultures were used to hydrolyse two different crystalline cellulose preparations (Solka-Floc and Avicel), a difference of 39% was seen, in terms of soluble sugars released via hydrolysis (Ferchak et al. 1980).

The regions in cellulose substrates that are not crystalline are described as ‘amorphous’ and are more amenable to attack by cellulase enzymes (Robson and Chambliss 1989). One way to increase these amorphous regions, thereby reducing the crystallinity of the cellulose, is by treatment with phosphoric acid (Mandels and Reese 1956). In many cases using phosphoric acid swollen cellulose (PASC) as a substrate in cellulase assays has led to an increase in the cellulase activity as compared to that observed when more crystalline substrates are used. For example, cellulases from the fungus Chalara paradoxa displayed a three-fold increase in reducing sugar release (a measure of solubilization of the substrate) when PASC (prepared from Avicel) was used as substrate rather than Avicel (Lucas
et al. 2001). The advantage of swollen cellulose may be that it increases accessibility of the enzymes to more of the underlying cellulose chains. A limitation of cellulose action on crystalline cellulose is the ability of the enzyme to liberate a single cellulose chain from the hydrogen bonded network, as the catalytic sites of cellulases are thought to only accommodate one cellulose chain at a time (Koivula et al. 1998). A similar effect was seen by introducing an expansin enzyme into a hydrolysis reaction. Expansins are natural plant enzymes that loosen the structure of cellulose (allowing the plant cell wall to expand at times of growth) and when added to T. reesei cellulases an increase in saccharification was seen when the more recalcitrant portion of the substrate was being attacked (Baker et al. 2000). One advantage of crystalline cellulose however, is that it may be a better inducer of cellulase enzyme activity than amorphous forms as has been seen with T. reesei (Mandels and Reese 1956) and Acetivibrio cellulolyticus (Sanchez et al. 1999), when grown on the two forms of cellulose.

Cellulosic substrates from higher plant sources are even more complex than the purified forms listed above, as they are present in a matrix of hemicellulose and lignin, the two other main structural polymers of plants. It has been noted that cellulolytic prokaryotes cannot utilize the cellulose in wood unless the lignin is removed or somehow chemically modified (Willoughby and Hayward 1988). The lignin may not have to be totally removed, as was seen when Clostridium thermocellum had similar growth rates on Avicel and a treated wood substrate containing insoluble lignin. In this case, the soluble lignin was washed off the wood substrate in the pre-treatment step, so it is not known if the soluble lignin inhibits growth of the bacteria and prevents hydrolysis of the cellulose (Lynd et al. 1989). Hydrolysis of a wood pulp with T. reesei cellulase preparation was also seen to be limited due to the presence of lignin and termination of hydrolysis was thought to be due to non-specific adsorption of the enzymes to the residual substrate (Boussaid and Saddler 1999).

Another constraint on cellulase action is the physical micro-heterogeneity of cellulose. A particular substrate may have a limited number of sites for a particular enzyme. An illustration of this was the action of a Clostridium thermocellum cellulase (CelD) on BMCC. Hydrolysis stopped after only a small proportion (2.5%) of the substrate had been solubilized, but resumed upon the addition of fresh
substrate, implying that a limited number of sites for the enzyme on the substrate was causing termination of hydrolysis (Carrard et al. 2000). Where the enzyme binds to the substrate is likely to effect hydrolysis, some CBMs (type VI) have been found to have higher affinities for amorphous rather than crystalline cellulose (Sakka et al. 1996). In addition, it has been seen that cellulose-specific CBMs have very tight specificities to target enzymes to particular cellulose microstructures (McLean et al. 2002). It has even been suggested that the diversity of cellulases in nature is a reflection of the requirement for enzymes that will perform hydrolysis of cellulose in different physical micro-environments (Gilbert and Hazlewood 1993).

The size and surface area of cellulosic substrates also have an effect on rates of hydrolysis. Krishna (1999), found that a particle size of 400µm supported maximum cellulase production when a Bacillus subtilis strain was grown on banana fruit stalk material, although this could be an effect on growth characteristics of the bacterium, rather than enzyme activity. Lamed et al. (1985), concluded that surface area of a particular substrate effects adsorption as well as enzymatic activity of Clostridium thermocellum cellulases. Crystallinity and surface area are the most important factors influencing the initial rate of hydrolysis, substrate particle size also has a strong influence (Gan et al. 2003).

Because of the differences between particular cellulosic substrates especially the different levels of heterogeneity within each substrate, it is difficult to compare the action of cellulases unless they are assayed on exactly the same substrate formulation. For example, different sugar yields were obtained from three different purified microcrystalline celluloses after the action of Cellulomonas cellulases (Bott and Kaplan 1991). Additionally, very similar experiments with T. reesei cellulases gave significantly different results and it was suggested that this could have been due to the use of different substrates (Sigmacell or bacterial cellulose ribbons, respectively) (Boisset et al. 2001).

Most cellulosic substrates have regions that are more easily attacked than the more recalcitrant, crystalline regions. For this reason, reaction rates of cellulases on solid cellulose substrates are not linear. Evidence for this was when the value of specific activity for CelD from Neocallimastix pataciarum
differed depending on whether activity was measured in the first 30 min or when 2.5% hydrolysis had been achieved (Aylward et al. 1999). Therefore, the initial rates of hydrolysis on substrates with particularly high crystallinity values (i.e. Avicel PH 102, 80%) may not reflect subsequent attack on the crystalline component of the substrate (Aylward et al. 1999). This has been displayed when T. reesei cellulases were used to hydrolyze either Avicel or a wood pulp substrate. The wood pulp gave higher initial hydrolysis rates (0.50 g sugar g\(^{-1}\) cellulose, compared to 0.35 g sugar g\(^{-1}\) cellulose for Avicel) but the sugar yield from Avicel was higher over the long term (Boussaid and Saddler 1999). Using cellulosic materials from different sources (i.e. from alfalfa or birch) results in different patterns of hydrolysis (Bae et al. 2004). The complexity of both amorphous and crystalline forms of cellulose means that the reaction kinetics of hydrolysis do not conform to typical Michaelis-Menten behaviour (Koivula et al. 1998).

Carboxymethylcellulose (CMC) is a modified form of cellulose, which retains the ß-(1→4)-linked glucopyranose units of native cellulose ([http://www.lsbu.ac.uk/water/hycmc.html](http://www.lsbu.ac.uk/water/hycmc.html), Martin Chaplin, 2007) and is therefore useful as a substrate to measure hydrolysis of the ß-(1→4) linkage. Hydrolysis of CMC can be seen with the use of Congo red dye, as the dye only binds to CMC polymers with more than 5 contiguous glucopyranosyl units (Wood, 1980). The binding of Congo red to CMC can be incorporated into an agar plate method for a sensitive measure of endocellulase activity, which may be an indication of an organism's ability to degrade cellulose (Teather and Wood, 1982). Additionally, substituted oligo- and disaccharides are often used to measure cellulase activity because they are soluble and can be measured with more sensitivity than normal sugars. Hydrolysis of methylumbelliferyl-cellobioside (MUC) is usually interpreted to be exocellulase activity, although some xylanases have been found to be active on MUC whilst having no activity on crystalline cellulose (Lüthi et al. 1990).

BMCC is thought to be a suitable substrate for cellulase assays because it has less substrate heterogeneity than cotton or pulp fibres (Stålbrand et al. 1998). Helbert et al. (2003) have stated that BMCC still contains most of the structural and morphological character of plant cellulose materials.
BMCC, as well as *Valonia* microcrystals, are more uniform in character because they are prepared by a harsh acid treatment that hydrolyzes the structural defects that are originally distributed throughout the microfibrils (Boisset *et al.* 2000). Therefore although BMCC may be a useful substrate to compare the activity of different cellulases, the results of such experiments should not be interpreted as the extent of hydrolysis that would be seen with native plant celluloses, because these substrates would have higher complexity than BMCC. Long ribbons of bacteria-produced cellulose have also been used, and the morphological changes that can be observed such as thinning or internal cutting of the ribbons, are thought to give valuable information on the mode of action of cellulases (Boisset *et al.* 2000).

**1.4 Enzyme structure**

Many cellulases have more than one sub-domain, this modular nature was first noticed in 1986 (Boraston *et al.* 2004). A typical cellulase is now known to consist of a catalytic domain connected to a binding module/domain (CBM/CBD) via a linker sequence rich in hydroxy-amino acids (usually proline and threonine) (Wang *et al.* 2001). The linker sequence is thought to be flexible, thereby enabling the enzyme to access multiple cellulose chains whilst remaining bound to the substrate.

Data that supports this theory is the fact that the linker sequence in exocellulases from *T. reesei* is 90 Å in length whereas cellulose chains in a typical microfibril are only 6-10 Å apart (Irwin *et al.* 1993).

Some cellulases, such as E4 from *Thermomonospora fusca*, have been found to have more than one CBM, in this case one of the CBMs is thought to cooperate with the catalytic domain as the active site cleft is directly in line with the putative binding site on the CBM (Sakon *et al.* 1997). In addition to their binding role, CBMs have been found to increase the stability of the enzyme overall (Kataeva *et al.* 2002). In the above example of E4, the two loop regions where the domains interact are thought to be responsible for increased thermostability (Sakon *et al.* 1997). The fact that cellulases bear multiple domains is thought to result in a domain-domain interplay that influences the affinity of the enzyme for the substrate (Linder *et al.* 1996). Cellulases have also been found that lack CBMs (Kitamoto *et al.*
1996; Li et al. 2003), however exocellulases are thought to usually possess a binding domain (Tomme et al. 1988).

Another type of domain that has been found in cellulases is the fibronectin type III-like domains. Although commonly occurring in animal proteins, in bacteria these sequences have only been found in glycosyl hydrolase enzyme sequences, and it is suggested that they may play a role in substrate utilization (Kataeva et al. 2002). The E4 cellulase from Thermomonospora fusca contains one of these domains in between the catalytic domain and a CBM (Irwin et al. 1998), as is the usual position of these domains in most glycosyl hydrolases (Kataeva et al. 2002). Kataeva et al. (2002) have shown that the fibronectin III-like domain in CbhA from Clostridium thermocellum is able to change the surface of the substrate thereby promoting hydrolysis.

Most of the fungal and a few bacterial cellulases are glycoproteins with sugars attached at asparagine (N-linked) or serine and threonine (O-linked) residues (Knowles et al. 1987). The carbohydrate moieties are thought to facilitate H-bonding with the substrate via their many hydroxyl groups as well as increasing the solubility of the proteins (Bisaria and Mishra 1989). Additionally, they may affect the conformation of the protein, making it more resistant to thermal inactivation and proteolytic digestion (Bisaria and Mishra 1989). Cellulomonas fimi cellulases, when expressed in E. coli as non-glycosylated forms were more susceptible to attack by a C. fimi protease when they were bound to the cellulose substrate, as compared to the glycosylated wild type enzymes (Langsford et al. 1987). Significant amounts of glycan were seen to be attached to cellulases (CelA and CelB) from Aspergillus oryzae (Kitamoto et al. 1996). Three distinct cellulases from Irpex lacteus were found to have an N-glycosylation site in the same position in the catalytic domain (Hamada et al. 1999). Interestingly, some hypercellulolytic mutants of T. reesei that produce cellulase of higher specific activity, were thought to have modified modes of glycosylation (Bisaria and Mishra 1989).
1.5 Catalytic domain structure

The classification of the catalytic domains of cellulases into glycoside hydrolase (GH) families based on amino acid similarities reveals insights into the structure of the enzymes (Henrissat et al. 1998). For example, enzymes within a GH family may share a similar active site topology as can be seen with glycoside hydrolase family 5 members (including CelE from Clostridium thermocellum) that all bear an cleft-shaped active site (Abdeev et al. 2001). Alignment of amino acid sequences from GH family 9 led to the discovery of a conserved amino acid that was essential for enzyme action even though it plays no direct catalytic role (Téllez-Valencia et al. 2003). Additionally, even if amino acid sequence has not been conserved, major protein folds that are similar in various glycoside hydrolases have enabled the grouping of families into clans (Henrissat et al. 1998). For example, members of clan GH-A, containing 11 families, all have the structure of (k/a)₈ barrels with the catalytic glutamate near the carboxy-terminal ends of k-strands 4 and 7 (Henrissat et al. 1998).

The structure of the active site of cellulases determines their endo- or exo- mode of action. Endocellulases have a groove-shaped active site, whereas exocellulases, which act in a processive manner, have a tunnel shaped active site (Reverbel-Leroy et al. 1997). The groove or ‘open cleft’ shaped active site has been confirmed in the following endocellulases: Cel7B, Cel5A and Cel12A of T. reesei (Karlsson et al. 2002); Cel45A and Cel7B from Humicola insolens (Boisset et al. 2001); CelE from Clostridium thermocellum (Abdeev et al. 2001). These endocellulase active sites are all similar but can vary in length and depth, thereby making the mechanism of action of each particular enzyme slightly different. For example Cel45A from Humicola insolens has a shallow cleft that allows interaction with six or seven glucose units, but Cel7B from the same fungus has a deeper active site that can only accommodate 4 glucose units (Davies et al. 1995). Similarly, the active sites of exocellulases differ in their dimensions; the 40 Å long tunnel of CBHI from T. reesei that is estimated to have seven glucosyl binding sites, is about twice as long as the one in CBHIII from the same organism (Divne et al. 1994). CelS and CelA from Clostridium thermocellum (Guimaraes et al. 2002) and CelF from C. cellulolyticum
(Parsiegla et al. 2000) have also had their structures resolved and the tunnel shaped active sites identified. The active site tunnel of CelS was additionally found to be extended by an open cleft at one end. Six glucosyl residues could bind inside the tunnel (subsites -7 to -2), whilst a further two could bind in the open cleft area (subsites +1 and +2). Stacking interactions between the substrate and aromatic amino acid residues, as well as protein-sugar hydrogen bonds were found to occur in the tunnel and conformational changes were thought to occur upon release of sugar from the open cleft area (Guimaraes et al. 2002).

The differences in the active site conformations are due to the loops of amino acid residues that surround the active site. An illustration of this is the GH family 6 cellulases, even though overall structures are conserved, the enzymes may have either exocellulase or endocellulase activity. The difference lies in the long loops around the active sites of the exocellulases that form the characteristic tunnel, whereas in the endocellulases the homologous loops are shortened, leaving the active sites open (Zhang et al. 2000). The loops forming the active site tunnel in exocellulases are thought to undergo large movements, thus opening and closing the tunnel roof (Varrot et al. 1999) as has been observed in Cel6A from T. reesei (Zou et al. 1999). The active site loops in endocellulases are also thought to undergo movements upon substrate binding (Davies et al. 1995). It has been proposed that the loops of exocellulases have been reduced over evolutionary time to create the more open structures of endocellulases (Rouvinen et al. 1990). This is supported by the finding that the sequence of EG1 from T. reesei is homologous to that of CBHI from the same organism but has several deletions, four of which map to the active site loops in CBHI (Divne et al. 1994). Mutational analyses have found that amino acids associated with the active site loops are highly important for enzyme activity. Asp_{446} in Cel9 from Myxobacter sp. AL-1 that interacts with the active site loops, played no direct catalytic role but was nonetheless essential for enzyme activity (Téllez-Valencia et al. 2003). A substantial increase in activity was seen when one of the loop residues was mutated in Cel6B from Thermobifida fusca (Zhang et al. 2000).
A very good review of carbohydrate binding modules (CBMs), their classification, structure and binding behaviours is outlined in Boraston et al. (2004). Main features of CBM/CBD structure are discussed below.

CBDs have been classified into 39 families (Boraston et al. 2004) but most of the reported CBDs from cellulases fall into families I, II or III (Carrard et al. 2000). Members of each of these three families have been found to have flat binding surfaces bearing aromatic amino acid residues that are involved in substrate binding (Carrard et al. 2000). The spacing of the three aromatic residues involved in substrate binding in family I CBDs was found to coincide with the spacing of every second glucose ring in the cellulose chain (Kraulis et al. 1989). In family I CBDs, an additional three amino acids are thought to stabilize the interaction with glucose residues by forming hydrogen bonds (Hamada et al. 1999) although, these hydrogen bonds are not thought to be very important for substrate binding (Linder et al. 1995). The planar binding sites of these CBDs are thought to be complimentary to the flat surface of crystalline cellulose (Tormo et al. 1996). The largest family is type 2 CBDs (Tomme et al. 1995). Type 2 domains are further subdivided into type IIa, which have three highly conserved Trp residues and type IIb, which only have two Trp involved in binding (Wang et al. 2001). The extra tryptophan residue in family 2a resides on an 8 residue loop (Tomme et al. 1995) and has been shown to interact with cellulose (Din et al. 1994). The type 2 CBD from T. reesei CBHI has three tyrosine residues on the hydrophilic side of the wedge-shaped molecule that are thought to participate in binding by forming stacking interactions with the pyranose rings of the substrate (Béguin and Aubert 1994). A type 2 CBD from Cellulomonas Cex displayed a higher affinity for crystalline cellulose than soluble celloooligosaccharides, so it was thought to interact with several chains of cellulose in the crystalline lattice (Bolam et al. 1998). Another type 2 CBD is that of CBHI from T. reesei and it was proposed that the wedge-like structure of this domain might help peel cellulose chains from microfibrils. One side of the wedge-shaped domain was hydrophilic and the other hydrophobic (Béguin and Aubert 1994). Ligand specificity of these type 2 domains has been found to be
determined by the orientation of the conserved tryptophan residues on the planar binding surface, which was found to be reliant on the identity of adjacent residues, as the mutation of one of these residues was shown to change the binding specificity of a *Cellulomonas fimi* CBM from xylan to crystalline cellulose (Simpson *et al.* 2000). It is thought that the flat face of family III CBDs (strands 1, 2, 4 and 7) interacts with 3 chains of cellulose, where one chain interacts with a planar strip of aromatic residues, a second interacts with 2 well conserved polar residues and a third interacts with non-conserved polar residues (Sakon *et al.* 1997). Most CBDs from family 2 have significant affinity with crystalline substrates, although the family 2 CBD from *C. fimi* CenC has poor affinity with such substrates (Coutinho *et al.* 1993).

Besides the stacking between glucosyl units and aromatic residues, other interactions such as direct hydrogen bonding and calcium mediated co-ordination play a role in ligand recognition (Boraston *et al.* 2004). The CBD of CelK from *Clostridium thermocellum* was found to tightly bind calcium (Kataeva *et al.* 2001). Calcium binding sites in other CBDs were found to be located apart from the binding surface (Johnson *et al.* 1996). Elimination of calcium from a CBD in the case of a domain from *Cellulomonas fimi* did not affect binding to cellulose (Goldstein and Doi 1994) although the presence of a calcium binding centre is common among CBDs (Kataeva *et al.* 2002). Molecular rigidity, due to disulphide bridges has been shown to be significant in the CBM-ligand interaction (Carrard and Linder 1999). The same CBD from *Clostridium thermocellum* mentioned above was found to have 2 of its five cysteine residues form a disulphide (Kataeva *et al.* 2001).

A CBD from *Cellulomonas fimi* is unusual in that it binds amorphous cellulose and soluble oligosaccharides but displays no affinity for crystalline cellulose. Similarly, members of families 17 and 4-1 bind amorphous cellulose, the binding site in these cases are pockets or grooves that accommodate single cellulose chains (Tomme *et al.* 1996). The family 6 CBM from *Cellvibrio mixtus* endoglucanase 5A has two binding sites, one can accommodate chain ends of β-1,4-glucans and xylans, whereas the other binds to internal regions of β-1,4-glucans and mixed β-(1,4)(1,3)-glucans (Boraston *et al.* 2004).
Both binding sites can therefore recognize cellulose, resulting in a multivalent interaction with insoluble cellulose (Boraston et al. 2004).

1.7 Cellulose binding

Attachment of cells to cellulose may indicate that degradation of the substrate is occurring. Colonization of plant tissues in the rumen occurs rapidly, within 5 min by bacteria and 15 min by protists, although attachment of fungi can take up to two hours (Bonhomme 1990). These attached microorganisms are thought to be responsible for most of the cellulose degradation in the rumen (Koike et al. 2003). In various bacteria, attachment of the whole cell to the cellulose fibre seems to be important for hydrolytic ability. *Bacteroides succinogenes* has a long lag time when grown on cellulose, as compared to growth on sugars, this was thought to be related to the attachment mechanisms of the cells (Hiltner and Dehority 1983). *B. succinogenes* as well as *Ruminococcus* species have been seen to firmly attach to cellulosic materials, *B. succinogenes* requires a longer time for attachment and this is thought to be due to differences in the extracellular surface coats of the two bacteria (Hiltner and Dehority 1983). In one experiment, the cellulolytic *Ruminococcus flavefaciens* was found only to occur attached to particles in the rumen and not detected in the liquid phase, but non-cellulolytic species were also found to bind to the plant fibres (Koike et al. 2003). Attachment of bacteria to cellulose fibres has also been seen with *Clostridium thermocellum, Cellvibrio fulvus, Sporocytophaga myxococcoides* and *Cellulomonas* sp. NRCC2406 (Kauri and Kushner 1985). Contact between the cells and the cellulose substrate, however, was not seen to be necessary for hydrolysis by various bacteria, including *Cellvibrio* and *Cellulomonas* (Kauri and Kushner 1985). The findings of Kauri and Kushner 1985, were confirmed with the studies of Bott and Kaplan (1991), using similar bacterial strains. Further evidence that bacterial cell contact is not necessary for cellulolysis was seen via TEM, when the lysis of plant cell walls was some distance from the responsible *Cellvibrio mixtus* cells (Willoughby and Hayward 1988). Additionally, *Acetivibrio cellulolyticus* differs from other cellulolytic bacteria in that it is not found to aggregate around cellulose particles (MacKenzie and Bilous 1982).
The cellulosome (a large protein complex containing cellulases) of *Clostridium thermocellum* enables the bacterial cells to adhere strongly and quickly to cellulose, these are present in protuberances on the cell surface that were seen to be lacking in adherence defective mutants (Bayer and Lamed 1986). One protein in the cellulosome, CipA, is responsible for binding the complex to both the cell surface and the substrate (Béguin and Lemaire 1996). The cellulosome has high affinity for various cellulosic substrates (Lamed *et al.* 1985).

The increase in cellulolytic activity in *Ruminococcus albus* cultures on addition of 3-phenylpropanoic acid (PPA) was attributed to an increase in affinity of the cells for cellulose, more cells were seen to be associated with the cellulose fibres (Morrison *et al.* 1990). Glucose has been shown to prevent attachment of cells to cellulose in *Cellulomonas* cultures (Kauri and Kushner 1985).

Whilst binding of cells to cellulose may not be essential for activity, the ability of cellulase enzymes (both fungal and bacterial) to bind to the insoluble substrate is highly correlated with the ability to degrade crystalline cellulose. It has been suggested that the extent of hydrolysis of cellulose is a function of the total concentration of bound cellulases (Jeoh *et al.* 2002). Additionally, a strong correlation between the capacity of cellulases to degrade crystalline cellulose and their affinity for this substrate has been found (Klyosov 1990). CBDs are important for this observed affect, as all cellulases that are found to be active against crystalline cellulose possess or are associated with a CBD (Béguin and Aubert 1994). The CBDs are thought to promote prolonged close contact between the enzyme and the substrate (Ferreira *et al.* 1991). The presence of a CBD does not always signify the ability of the enzyme to degrade crystalline cellulose, however (Béguin and Aubert 1994). Some endoglucanases lack CBDs (Gilad *et al.* 2003), for example Cell12A from *T. reesei* (Karlsson *et al.* 2002) therefore these domains may not be necessary for this type of cellulase activity. An endocellulase from *Bacillus subtilis* was found to bind to Avicel but nonetheless could not hydrolyze crystalline cellulose (Park *et al.* 1993). Cellulases from *Thermomonospora fusca* with their CBDs removed had significantly lower activity on filter paper (Irwin *et al.* 1993). Similarly the CBD from CelE of *Pseudomonas fluorescens* subsp. *Cellulosa*, enhanced the activity of the enzyme towards Avicel (Fontes *et al.* 1997). Different CBDs attached to a
particular catalytic module were seen to enhance the activity by different amounts (Carrard et al. 2000). Whilst removal of the CBD usually has a drastic effect on activity of the enzyme towards crystalline cellulose, removal of a CBD from a *Cellulomonas* cellulase only reduced the activity by a small amount (Shen et al. 1991). Bolam et al. (1998) found that even a CBD originating from a xylanase can enhance the activity of a cellulase, although this was in contrast to the results of Poole et al. (1991). Although normally thought to be essential only for activity on crystalline substrates, the presence of a CBD may also affect activity on amorphous cellulose (Tomme et al. 1988). This may involve type VI CBDs, which have higher affinities for amorphous rather than crystalline cellulose (Sakka et al. 1996).

When different CBDs were linked to a cellulase catalytic domain (CelD from *C. thermocellum*), an increase in soluble reducing sugars released from both crystalline and amorphous cellulose was seen (Carrard et al. 2000).

Although binding to crystalline cellulose may be a prerequisite for hydrolysis of this recalcitrant substrate, the strength of binding plays a role in enhancing or inhibiting activity. Mutagenesis studies of an exocellulase, Cel6B, from *Thermobifida fusca* concluded that the mutants with increased activity often had decreased binding affinities on cellulose, although not all mutations that decrease binding affinity resulted in increased activity (Zhang et al. 2000). One mutant had an increase in activity of 125-190% whilst having a 100-300% decrease in binding affinity. The endocellulase, Cel6A, from the same bacterium displayed a similar trend (Zhang et al. 2000). The binding abilities of different *Thermomonospora fusca* cellulases and their ability to degrade crystalline cellulose substrates did not correlate (Irwin et al. 1998). Maximal cellulase activity of the *Clostridium thermocellum* cellulosome was seen when 70-80% of the cellulosome was adsorbed to the cellulosic substrate (Lamed et al. 1985).

Binding of the enzyme to a small region of cellulose surrounded by non-cellulosic materials has been suggested as something that could terminate activity if the enzyme becomes immobilized in this way (Fontes et al. 1997). Of course, this is reliant upon the reversibility of the binding of the enzyme to cellulose. Irreversible binding of cellulases to cellulosic materials has been reported (Kraulis et al. 1989), but Ferchak et al. (1980), Linder et al. (1996) and Carrard et al. (2000) all observed very little or
no irreversible binding of cellulase enzymes to cellulose substrates. Additionally, Carrard et al. (2000), found no correlation between the reversible nature of a CBD (as quoted from other sources) and the ability of the same CBD to enhance cellulolytic activity. However all of these studies examined binding of the cellulases to purified crystalline cellulose substrates, so binding of the enzymes to other components in cellulosic materials could not have been observed. Cellulases may bind irreversibly to lignin (Ramos et al. 1993), which may result in a decrease in hydrolysis of the cellulose within native substrates containing lignin and hemicellulose.

Besides keeping the enzyme in close contact with the insoluble substrate, CBDs may also facilitate hydrolysis by first altering the substrate and making it more accessible to the enzyme. CBHI from \textit{T. reesei} was found to have ‘structure disrupting’ or ‘swelling’ activity on crystalline cellulose, which may be due to the CBD (Tomme \textit{et al.} 1988). This CBD has one flat face that is involved in binding and the opposite face is also flat but with a slight indentation in the middle (Béguin 1990). This shape was suggested to enable the domain to ‘plough’ or ‘unzip’ the crystalline structure of cellulose (Béguin 1990). The disruption of cellulose fibrils via the action of CBDs has been seen (Din \textit{et al.} 1992). Irwin \textit{et al.} (1998) proposed that the contribution of the CBD to hydrolysis by a \textit{Thermomonospora fusca} cellulase was important when the enzyme was active against the more recalcitrant regions of the substrate. This would correlate well with a structure disrupting mechanism being an advantage. Kataeva \textit{et al.} (2002) observed that a CBD from a \textit{Clostridium thermocellum} cellulase seemed to loosen the structure of cellulose fibres, whilst no sugars were released, confirming the non-catalytic role of the CBD. An initial proposal of how cellulase systems operate involved a nonhydrolytic component \textit{C\textsubscript{1}} that activates the substrate prior to hydrolysis (Reese \textit{et al.} 1950). Din \textit{et al.} (1994), suggest that the CBD accounts for this activity. Four different CBDs from families I, II and III were tested in their ability to increase the activity of a cellulase catalytic domain (CelD from \textit{C. thermocellum}) by Carrard \textit{et al.} (2000). These authors found an increase in activity was reliant upon the two domains being linked, suggesting that rather than the CBD having a structure-disrupting role, it is more important for maintaining enzyme in close proximity with the substrate. Similar results were seen when CBDIII from \textit{C. thermocellum} was mixed with a glycoside hydrolase family 9 cellulase catalytic domain and no
increase in the hydrolytic rate, as compared to the catalytic domain on its own was observed, however when the same CBD was mixed with a different enzyme, increases in activity on both amorphous and crystalline cellulose were seen (Kataeva et al. 2002). Alternatively, the presence of expansin-like enzymes in bacteria and fungi may be responsible for the non-hydrolytic component of cellulose degradation (Saloheimo et al. 2002), or these could act to increase the effect by the CBDs.

Some cellulase enzymes are said to be processive, that is, they carry out many bond cleavages without dissociating from the cellulose chain (Koivula et al. 1998). It was suggested that CBDs can contribute to this processive action if they have dynamic binding behaviour (Lehtio et al. 2003). CBM2a from Cellulomonas fimi is able to diffuse laterally when bound to crystalline cellulose, probably due to the lack of directionality of hydrophobic stacking interactions (Jervis et al. 1997). Removal of a CBD from a Clostridium thermocellum enzyme, whilst decreasing activity on crystalline cellulose, resulted in more activity on the substituted substrate, carboxymethylcellulose (Gilad et al. 2003). Substituted cellulose is thought to inhibit the processive ability of cellulases (Dalbøge 1997). The behaviour of the Clostridium thermocellum CBD above therefore may be attributed to the enzyme being able to dissociate more freely from the substrate, thus enhancing activity on CMC. An increase in catalytic efficiency was seen when different CBDs were attached to the catalytic domain of CelD (C. thermocellum), but this was not thought to be due to an increase in processivity, as the increase in soluble reducing sugars released from the substrate was similar to the increase of insoluble reducing sugars formed on the substrate (Carrard et al. 2000). Family 3c CBMs are thought to play a role in the processivity ascribed to family 9 'endo-processive' cellulases (bearing both endocellulase as well as processive exocellulase activity) (Sakon et al. 1997). The ratio of soluble/insoluble reducing sugars produced from the hydrolysis of filter paper with the processive endocellulase E4 (T. fusca) was seen as a measure of processivity. This ratio decreased to variable amounts when either of the native CBDs were removed from the enzyme and even more so when both CBDs were removed, indicating that these CBDs had distinct and dramatic influences on the processivity of the enzyme (Irwin et al. 1998).
Compared to other proteins that bind carbohydrates, such as lectins, CBMs have relatively weak binding (Boraston et al. 2004). This may be because CBMs mediate interactions with the substrate more via hydrophobic interactions than hydrogen bonding, leading to a more dynamic interaction (Flint et al. 2004). Mutation of the amino acids thought to hydrogen bond with the substrate in a Clostridium cellulovorans CBD, did not reduce affinity of the module significantly (Irwin et al. 1998). An increase in temperature has been seen to cause a decrease in binding affinity for certain CBMs (Linder et al. 1996; Carrard et al. 2000; Lehtio et al. 2003). The mutation of a tyrosine residue to a tryptophan in various CBMs increased binding affinity, although this was limited to one modified Trp residue as further changes had no further effect (Lehtio et al. 2003). When cellulases were bound to cotton fabric and then treated at high temperature the enzymes were denatured, leading to almost complete abolition of catalytic activity but increased binding of the enzymes (Andreas et al. 1999). Denaturation was thought to turn the hydrophobic amino acids from the interior to the surface, resulting in more hydrophobic interactions between the protein and the cotton fibres (Andreas et al. 1999). The method of pre-treatment and mode of storage of lignocellulosic material have been shown to affect the rate and extent of cellulose adsorption (Boussaid and Saddler 1999).

Different CBMs are thought to target cellulases to specific sites on cellulosic substrates (Carrard et al. 2000). The CBM3 from C. thermocellum CipA was found to have a slight tendency towards binding close to cellulose chain ends, additionally various CBMs binding to Valonia microcrystals had a preference for one specific face of the crystals (Lehtio et al. 2003). CBMs from families 17 and 28 were seen to recognize different regions of non-crystalline cellulose (Boraston et al. 2003). When binding to amorphous cellulose, a CBM2a and a CM3 were shown to have shared sites; shared sites were also seen for a CBM4-1 and a CBM17 but there were additional sites specific for these two CBMs, highlighting the heterogeneous nature of the substrate and the ability of the CBMs to target enzymes to fine substructures (McLean et al. 2002). This targeting ability may be important for complete substrate coverage, leading to more efficient hydrolysis of cellulose (McLean et al. 2002).
Synergy of binding was observed with two *T. fusca* cellulases, that is, binding of both enzymes was enhanced in the mixed reaction as compared to their respective binding in single component reactions (Jeoh et al. 2002). It was proposed that each enzyme creates additional binding sites for the other, causing synergy in binding as well as synergy in hydrolytic ability (Jeoh et al. 2002). The opposite was also seen where a mixture displaying anti-synergistic effects on hydrolysis also displayed anti-synergistic effects on binding (Jeoh et al. 2002). Cel5 from *Bacillus* sp. 1139 has two CBMs, each with relatively weak binding, but the affinities of the two domains together are 10-100 fold higher due to a multivalency effect (Boraston et al. 2003). Similarly, a domain-domain interplay was seen between an artificially constructed double-CBD and was thought to be similar to that seen when a CBD influences the affinity of native cellulases (Linder et al. 1996).

The sequence similarity between cellulose binding domains, suggests that they have arisen from a common ancestor (Park et al. 1993). It has also been suggested that the occurrence of mainly one type of CBD in a single organism is a result of them being added to genomes in a late stage of evolution (Béguin 1990). The sequences of CBMs are thought to be highly conserved (Bisaria and Mishra 1989). CBMs of type II are the most common in bacteria (Tomme et al. 1995). Family II CBMs are larger than family I CBMs, which are only found in fungi (Carrard et al. 2000). Family 2 CBMs can be subdivided into family 2a that all bind to crystalline cellulose and family 2b, which bind to xylan (Simpson et al. 2000). Family 3 CBMs can bind to amorphous cellulose as well as crystalline cellulose and some even bind chitin (Morag et al. 1995). Subfamily CBM3c members are unusual in that they lack many of the conserved binding residues and it has been suggested they may not even be important for cellulose binding (Gilad et al. 2003). Family 4 CBMs bind to amorphous cellulose but not crystalline cellulose (Coutinho et al. 1992).
1.7 Cellulase catalytic mechanisms

A general picture of cellulase enzyme action on insoluble substrates is that the endocellulase components cleave internal glycosidic linkages and exocellulases are active at cellulose chain ends. Synergy arises between the two types of cellulase action, as the action of endocellulases creates more chain ends for the exocellulases to act on (Irwin et al. 1993). Exocellulases can work on either the reducing or non-reducing end of the cellulose chain, this can be determined by the pattern of erosion seen on *Valonia* microcrystals (Boisset et al. 2001).

Cellulase enzymes have been found to have a smoothing effect on cellulose fibres, which is probably due to the hydrolysis of glycosidic bonds at disrupted areas of the substrate (Din et al. 1991). This is due to endocellulases cutting preferentially at loose segments of cellulose chains in the substrate (Boisset et al. 2001). Treatment of BMCC with both endocellulases and exocellulases saw a preference of action upon smaller cellulose chains as a shift in the relative abundance of higher degree of polymerization (DP) insoluble products was seen (Stålbrand et al. 1998). This may indicate that hydrolysis of broken chains on the substrate surface may occur first. The smooth, highly crystalline portion of the substrate that is left after this initial action is thought to be highly recalcitrant. This correlates well with the fact that the active sites of cellulases only accommodate one cellulose chain, making hydrolysis of chains that are hydrogen bonded to each other more difficult (Boisset et al. 2000). Therefore, cellulases require an additional action, besides the actual catalytic hydrolysis of glycosidic bonds to attack the recalcitrant portion of cellulose. This action must remove single glucan chains from the cellulose surface so that they can enter the active site and hydrolysis can occur (Koivula et al. 1998). This action can be carried out by the CBD of the enzyme, as has been seen with a *Cellulomonas fimi* CBD that was shown to disrupt cellulose fibres presumably by penetrating the fibre at surface irregularities and sloughing off fragments of the substrate to reveal underlying microfibrils (Din et al. 1991). Cellulases from the fungus *Irpex lacteus* were examined for their ability to act on the crystalline substrate, *Valonia* cellulose microfibrils, and one enzyme was found to produce pronounced morphological change by swelling the microfibrils. It was suggested that this was due to a high rate
of penetration of the enzyme into the microfibril network structure (Hoshino et al. 1994). Similarly, when the CBD and fibronectin type III domain of a *Clostridium thermocellum* cellulase (CbhA) were investigated, surface irregularities were seen to be created on the surface of cotton fibres and these domains were attributed as separating the cellulose chains to produce these effects (Kataeva et al. 2002). It has been suggested that the wedge-like structure of some CBDs may facilitate the peeling of cellulose chains from the microfibril surface (Béguin and Aubert 1994). Additionally, polar amino acid residues that are well conserved among CBDs may replace inter-chain hydrogen bonds to free single cellulose chains for feeding into the active site (Sakon et al. 1997). Not all CBDs can produce this effect, CBDs from *Pseudomonas fluorescens*, similar to the one examined by Din et al. (1991) showed no disruption of crystalline cellulose (Bolam et al. 1998). Other strategies may be employed for this function. The cellobiose oxidase enzyme found in a *Cytophaga* sp. was suggested to oxidize the cellulose substrate, introducing carboxyl groups and thus causing disturbance to the inter-chain hydrogen bonds (Eriksson et al. 1974).

Another mode of action that is an advantage to cellulases on their insoluble substrate is processivity. Processivity can be measured as the ratio of soluble to insoluble reducing sugars produced with a high ratio distinguishing the more processive exocellulolytic activity from endocellulolytic activity (Irwin et al. 1993). The degree of processivity is reliant on the structure of the active site of the enzyme, in particular the loops that form the tunnel shape characteristic of exocellulases and the movements thereof (Zhang et al. 2000). CelF from *C. cellulolyticum* has the tunnel shaped active site characteristic of exocellulases but it is thought that the tunnel roof can open to allow endoglucanase-like entry of cellulose chains, making this enzyme a processive endocellulase, carrying out both types of cellulase action. Some processive cellulases have little activity on CMC as the carboxymethyl substitutes block the active site tunnel (Reverbel-Leroy et al. 1997), suggesting that these enzymes do not display the conformational changes of the endo-processive enzymes. Cellulases with high activity on CMC are regarded as non-processive, that is, they dissociate from the substrate after each cleavage of the substrate (Stålbrand et al. 1998). The tunnel shape is needed to hold the cellulose chain in place during successive cleavages, aromatic residues in the active site are thought to act as lubricating
agents (Parsiegla et al. 2000). The length of the tunnel may also be important. CBHI from T. reesei has a longer tunnel than CBHII and this is thought to result in CBHI remaining attached to the cellulose chain after the catalytic reaction and being able to progress along the chain whereas CBHII separates after the catalytic event (Divne et al. 1994). The length of the active site may also be important for endocellulase activity. Endocellulase activity is seen to decrease as DP of the substrate decreases and certain endocellulases require a minimum amount of glucosyl residues for catalysis (Huang and Monk 2004). In a Clostridium thermocellum exocellulase, threading of the cellulose chain through the active site was thought to occur by two sugar units at a time (Guimaraes et al. 2002). This would explain the fact that the main product of most exocellulases is cellobiose.

Interaction of the CBD with the substrate is also thought to contribute to the processivity of some cellulases (Irwin et al. 1998; Gilad et al. 2003). The E4 cellulase from Thermomonospora fusca has amino acids that close over the substrate during hydrolysis but cleavage initiates a conformational change where the active site opens, making it possible for the enzyme to dissociate from the cellulose chain. Despite this, the enzyme displays processive action but this is reduced by removal of the CBD, suggesting that the CBD holds the enzyme onto the substrate to prevent dissociation (Irwin et al. 1998).

Cellulases hydrolyze β-1,4 glycosidic bonds with an acid catalysis mechanism that involves a proton donor and a nucleophile or base (Knowles et al. 1987). The catalytic residues are usually highly conserved among members of cellulase families. For example Glu-328 in CelA from a Bacillus sp. corresponds to the strictly conserved Glu residue that is the known active site nucleophile in family A cellulases (Blanco et al. 1998). Because of this, the catalytic residues of newly characterized cellulases can be predicted from the amino acid sequences, by using hydrophobic cluster analysis (Henrissat et al. 1998). The catalytic acidic residue donates a proton to the glycosidic oxygen and the catalytic base extracts a proton from a water molecule so that it can have a nucleophilic attack on the anomeric carbon atom (Koshland 1953). Besides the catalytic residues themselves, other amino acid residues are important for catalysis by forming hydrogen bonds with the substrate (Flint et al. 2004).
Cellulases cleave cellulose with either retention or inversion of the anomeric carbon. For example, CBHI from *T. reesei* cleaves the glycosidic linkage with a double displacement mechanism, with retention of configuration of the product (Divne *et al.* 1994) whereas glycoside hydrolase family 48 cellulases cleave cellulose with inversion of the anomeric carbon (Henrissat and Davies 1997). Tryptophan residues are often found to be involved in the active site of cellulases (Béguin 1990; Hakamada *et al.* 2002) and in the case of a *T. reesei* enzyme (Cel6A), one of these is thought to be the residue that makes initial contact with a single glucan chain on the cellulose surface (Koivula *et al.* 1998).

The action of cellulase enzymes often involves conformational changes of the protein molecule, such as the ‘tunnel opening’ mentioned above. A proposed conformational change in Cel9 from *Myxobacter* sp. AL-1 is thought to force the substrate closer to the nucleophilic water (Téllez-Valencia *et al.* 2003). Conformational changes in *C. thermocellum* cellulases occur upon occupation of the active site and result in a strained conformation of the sugar ring in the glucan chain, presumably forming an oxycarbenium-like reaction intermediate (Guimaraes *et al.* 2002). Twists in the substrate are forced by *T. reesei* exocellulases and have been proposed to be important for the crystal breaking capacity of exocellulases (Koivula *et al.* 1998). An induced fit also occurs with active site occupation in cellulase E4 from *T. fusca*, where the catalytic proton donor (Glu424) shifts towards the scissile oxygen and the substrate is forced towards the nucleophilic water (Irwin *et al.* 1998).
1.8 Additional catalytic activities of cellulase enzymes

The modular nature of many cellulases enables them to carry out more than one catalytic activity. Most of the cellulases described have more than one domain. One endocellulase from Bacillus sp. was reported to be a tetramer (Yoshimatsu et al. 1990). Some cellulases contain both endocellulolytic and exocellulolytic activities in the one active site, as mentioned above. However, cellulases have been found that contain these types of activities on separate domains of the one enzyme (Han et al. 1995). In addition to this, cellulases have been found with domains that carry out different hydrolysis reactions. Cellulases with additional xylanase activity have been described (Saul et al. 1990). CelD from Neocallimastix patriciae contains both types of cellulase activities as well as xylanase activity, the enzyme consists of three domains and surprisingly each domain contains all three activities (Xue et al. 1992). Similarly, an enzyme from Cellulomonas sp. was found to have both endocellulolytic and xylanolytic activity on one domain (Fülöp et al. 1996). An endocellulase from R. albus was also found to have α-xylosidase activity (Ohmiya et al. 1988).
2. Aims of the present study

As mentioned above, the industrial uses of cellulase enzymes are many and varied. These applications may either require highly active enzyme preparations or only weakly active cellulases to slightly modify the cellulosic substrate (for example, in the 'stone-washing' of denim). Additionally, the different applications require cellulase enzymes that work under different conditions, such as specific pH and temperature. With this in mind, the discovery of novel cellulase enzymes with differing capabilities will be valuable.

Cellulase enzymes are found broadly in nature and continue to be discovered in surprising situations, such as production by molluscs and other higher animals (Suzuki et al. 2003). Therefore, there seems to be scope for discovery of cellulase enzymes, as evidence that divergent evolutionary lineages of the enzymes exist (Lo et al. 2003). Cellulases arising from fungi are already extensively studied, therefore this study focuses on potential cellulases produced from bacteria. An interesting aspect of bacterial cellulases is that although some gram positive species such as Bacillus spp. and Clostridium spp. bear complete cellulase systems, many other bacteria, in particular gram negative species, seem to possess one or a few endoglucanase enzymes that cannot extensively degrade native cellulose in isolation. It is not known if these enzymes act synergistically with other types of enzymes, or cellulases from other organisms to contribute to the degradation of cellulosic materials in various habitats. Alternatively, such endoglucanases may serve another function in their native bacterial hosts. By examining the diversity of bacterial cellulases from a rich microbial source, this study hopes to gather information on bacterial cellulase enzymes to investigate the function of such enzymes and compare any enzymes found to those already studied. This may provide additional information on the evolution of bacterial cellulases.

Specific objectives of the study include discovering cellulase activity in bacteria where it has not been previously observed (to add to knowledge of the diversity of such enzymes); Purifying enzyme(s) responsible for such activity (in order to carry out preliminary characterization); and finding information on the gene(s) responsible for the enzymes (in order to elucidate the function of the enzyme and/or uncover information on the evolutionary origins of the enzyme).
3. Isolation of cellulolytic organisms

3.1 Introduction

The objective of this chapter was to isolate novel cellulose-degrading microorganisms. Cellulase enzymes occur broadly in nature. Fungal cellulase systems were first recognized for their efficiency in degrading native cellulose and even other components of lignocellulosic materials. Bacteria from diverse evolutionary origins also express cellulase enzymes. Some bacteria such as Clostridium spp. (Johnson et al. 1982) and actinomycetes (Ball et al. 1990) can significantly hydrolyze native forms of cellulose. In fact, the specific activity of cellulase from C. thermocellum can be higher than that produced by aerobic fungi such as T. reesei, and the extent of hydrolysis by A. cellulolyticus is comparable to that obtained with T. reesei (MacKenzie et al. 1985). Bacteria, including Cytophaga species and Actinomyces, are thought to be more important than fungi for the degradation of cellulose in manure (Godden and Penninckx 1984). It is more common however, for a bacterial species to produce one or a few endocellulases that are not necessarily active on crystalline forms of the substrate. The function of these limited cellulase systems remains unclear, although it may simply signal the redundancy that arises when various organisms co-operate to degrade this recalcitrant substrate. For example Prevotella strains possess endoglucanase and xylanase activities but cannot degrade native substrates on their own, and are thought to co-operate with other bacteria for degradation of plant material (Koike et al. 2003). Additionally, strains of Selenomonas have been found to posses CMCase (endoglucanase) but are known to survive in the rumen by cross-feeding of products from cellulolytic Fibrobacter bacteria (Koike et al. 2003). Alternatively, many bacteria may possess a few cellulase enzymes acquired by horizontal gene transfer (Chen et al. 1998). The hydrolysis of cellulosic fibres by bacteria has been studied since 1875 (Fåhræs 1947), since then however, the efficiency of the cellulase enzymes to break down and release nature's biggest carbon storage has been utilized by humans in industrial settings. All over the world, research into improving cellulose hydrolysis is undertaken, using modern techniques to manipulate the expression and structure of the cellulase enzymes from the well-studied native organisms and within new hosts. Cellulases have been studied intensively for over 60 years, yet because they are so widespread in
nature, there is the possibility that more efficient cellulases, or those that work under different conditions, are yet to be uncovered. Evidence for this is the fact that although members of the *Cytophaga/Flexibacter* group are reported to possess the ability to degrade crystalline cellulose (Fåhræs 1947), the enzymes responsible for this activity have not been extensively studied.

The rumen is studied as a site of efficient cellulose degradation, yet an analysis of the bacterial species present indicated that 56% of the species present are unknown, in that the 16s rDNA sequences obtained lacked similarity with known species (Tajima *et al.* 1999). Some of these unknown bacteria were related to the cellulolytic *Cytophaga* group (Tajima *et al.* 1999). A very similar study by Koike *et al.* (2003), also found evidence for unknown *Cytophaga* members in the rumen and a total of 42% unknown species. Therefore, cellulase enzymes with different capabilities to those already studied may be produced by these as yet uncultured bacteria.

The Evolver™ is a technology that is employed to help isolate microorganisms with a particular metabolic capability (Bridger *et al.* 2005). A culture is monitored by a dissolved oxygen (DO) probe, so that any changes in DO can highlight metabolic activity. A fast growing aerobic culture requires a lot of oxygen, this is translated as a high Evolver™ output, therefore when a single carbon source is supplied to the culture vessel, utilization of the particular carbon source can be seen as increased Evolver™ output, stated here as oxygen uptake rate (OUR), as microorganisms in the culture grow and multiply. If utilization of the particular carbon source is efficient, a nutrient limited environment is created, with the substrate always present at low levels in the culture vessel. This creates a situation in which microorganisms that can assimilate and metabolize the substrate faster are able to multiply more often than other species. Therefore the Evolver™ selects for microorganisms that more efficiently use a particular carbon source. Additional selective pressure can be put on the culture by increasing flow of the media through the vessel. Overflow from the vessel means that cells are routinely lost, therefore selection for microorganisms with a faster growth rate will occur if they continually replicate and maintain high numbers in comparison to slower growing strains that are gradually lost from the population. This selection facilitates the discovery of mechanisms and enzyme systems that most efficiently use a particular carbon source.
Using cellulose as a carbon source in the Evolver™ is potentially a more complicated scenario than if a soluble, small molecule is used as the carbon source. Cellulose in its native form is highly crystalline and insoluble, therefore amounts of cellulose in suspension are difficult to maintain and monitor. Additionally, binding to the substrate may be a parameter that is important in utilization of the carbon by the microorganisms. Cellulose particles that are not degraded quickly enough will be washed out of the culture vessel, any microorganisms that are bound will be lost also and diversity of the culture could be diminished as a result. However, equilibrium between attached cells and those of the same strain that are free in solution (planktonic) may arise, meaning that some members of the strain will be retained in the general population.

Cellulose is broken down into its glucose monomer or oligomers thereof, by a set of cellulase enzymes that are commonly secreted from the microorganism producing them. Therefore, the Evolver™ would potentially not only be selecting for those microorganisms with an efficient cellulase system but also those with a fast growth rate on glucose.

The highly diverse source of microorganisms, activated sludge, is used as inoculum for the Evolver™ in this case. The presence of cellulolytic microorganisms in the activated sludge is confirmed by using an alternative method of isolation. The method simply cultures the sludge on filter paper as a sole carbon source on agar plates. Compared to the Evolver™, there is very limited selection in this case. Subculturing from sites of hydrolysis attempts to isolate only cellulolytic organisms but it is known that other commensal organisms are also easily subcultured this way. Another difference is that while the Evolver™ maintains a population in liquid culture, growth on the filter paper occurs as a thick biofilm-type of growth associated with the cellulose fibres. Therefore, the two methods may isolate different cellulolytic organisms depending on which type of growth they are adapted to. A comparison of the two isolation methods is presented, in order to assess the suitability of the methods to isolate bacteria with good cellulolytic capability.
3.2 Materials and Methods

3.2.1 Media

461S minimal media was a modification from Nagel and Andreesen (www.dsmz.de/media), this is a defined medium with no carbon source. The use of such a media, when a sole carbon source is supplied, means that bacteria that grow on the media must be able to use utilize the particular carbon source for growth. A 100x salt stock solution was diluted in water (Reverse osmosis or MilliQ) then autoclaved at 121°C for 20 min before a 50x phosphates stock solution was added along with 0.7 mL/L of the following trace metals solution. The trace metals solution was prepared in 5 M HCl, the FeSO$_4$ was dissolved first and then the following metals were added (per L): 6560 mg FeSO$_4$.7H$_2$O, 140 mg ZnCl$_2$, 120 mg MnSO$_4$.H$_2$O, 10 mg H$_3$BO$_3$, 450 mg CoSO$_4$.7H$_2$O, 4 mg CuSO$_4$.5H$_2$O, 48 mg NiCl$_2$.6H$_2$O, 72 mg Na$_2$MoO$_4$.2H$_2$O. The final amounts of the salts and phosphates were as follows (per L media): 1.45 g Na$_2$HPO$_4$, 0.25 g KH$_2$PO$_4$, 0.01 g CaCl$_2$.2H$_2$O, 0.5 g MgSO$_4$.7H$_2$O, 0.01 g MnSO$_4$.H$_2$O, 0.3 g NH$_4$Cl, 0.05 g NaCl. Carbon sources were added at the desired concentration before autoclaving, although glucose/cellobiose were added without autoclaving (filter sterilised) to prevent the presence of possibly inhibiting caramelisation products of the sugars (Fåhræs 1947). For solid media in plates, bacteriological agar (Oxoid) was added to give a final concentration of 1.5% (w/v).

As an alternative minimal media, ST6 was used as it has been shown to be useful in the isolation of cellulolytic *Cytophaga* species (Reichenbach 1999). ST6 media was prepared similarly to 461S with sterile phosphates and trace metals being added after autoclaving the salts in water. When ST6 salts were made as a 100x stock solution, the iron precipitated out of solution after autoclaving but the amount of iron needed (0.02% (w/v) final concentration of FeCl$_3$.6H$_2$O) could be satisfied if the 461S trace element solution was used instead of the one prescribed by Reichenbach. The final concentrations of the salts and phosphates were therefore: 0.1% (w/v) (NH$_4$)$_2$SO$_4$, 0.1% (w/v) MgSO$_4$.7H$_2$O, 0.1% (w/v) CaCl$_2$.2H$_2$O, 0.01% (w/v) MnSO$_4$.7H$_2$O, 0.1% (w/v) K$_2$HPO$_4$, 0.002% (w/v) yeast extract. The inclusion of the small amount of yeast extract in ST6 means that it is not fully defined and therefore when it is used, it cannot be said that there is a single carbon source, unlike 461S media.
To highlight the hydrolysis of cellulose, pink cellulose was incorporated into agar plates used for the isolation of bacteria. Pink cellulose was made by dyeing crystalline cellulose with the reactive dye, Procion red MX5B (Kraftcolour). 50 g Sigmacell-20 was added to 1.5 L water, and then the dye solution of 100 mL 1% (w/v) Procion red in warm water was added. 300 mL salt solution (100 g NaCl dissolved in hot water) was added and the resultant dye bath was kept at 30°C for 40 min. A soda solution of 20 g sodium carbonate dissolved in 100 mL warm water was added and incubation at 30°C was continued for a further 40 min before the whole dye bath was left at room temperature overnight. The cellulose was collected by centrifugation at 1500 rpm for 3 min, then washed in 1 L 0.2M KHPO$_4$ pH 7.0 + 1 M NaCl to remove any unbound dye, then re-centrifuged. The cellulose was then filtered through a sintered glass filter (grade 2), washing with water, until the filtrate was very pale pink. The cellulose was then dried at 110°C overnight.

The pink cellulose was tested for dye release, with and without the presence of *T. reesei* as per Smith, 1976 to verify that strong cellulase activity was needed to liberate dye from the substrate. 10 mL of 461S media was set in 30 mL glass McCartney bottles and then a top layer of 461S + 1% (w/v) pink cellulose was overlaid on top. Un-inoculated bottles or bottles inoculated with an agar squash of *T. reesei* were tested in triplicate by incubating at 37°C and the basal media was checked for colour change, indicating diffusion of dye from the top layer. The expected results confirmed that the dye could only be released into the basal media with the presence of the cellulolytic *T. reesei*.

### 3.2.2 Direct isolation on Filter paper

The diverse source of microorganisms used was activated sludge from a mixed bed aerated tank at a waste water treatment plant in south eastern metropolitan Melbourne, Victoria, Australia. Direct isolation of cellulolytic organisms from the activated sludge microbial source was attempted by inoculating directly onto filter paper (Whatman #1) as a carbon source. Solids from the sludge were used, a 50 mL sample was taken from the bottom of the settled sludge, 1mL aliquots of this were centrifuged at 13 000 rpm (eppendorf 5415D microcentrifuge) for 5min, the supernatant was discarded and the solids remaining were used as inoculant. A spot of such solids, no more than 2 mm
in diameter was placed on the point of a triangle of FP, which had been placed on the surface of 461S media, with no other carbon source included. Plates were then incubated at 16, 30, 37°C for up to 3 months. Purification of isolates from zones of hydrolysis involved lifting growth carefully from the growing edge of hydrolysis with a sterile loop before subculturing onto further FP plates (on either 461S or ST6 minimal media) and eventually streaked onto nutrient agar (NA), TSA or 461S + 0.1% (w/v) cellobiose plates to obtain pure cultures. Visible signs of growth and cellulose hydrolysis were recorded with pictures of plates taken (sometimes at 50x magnification) with a Canon powershot A400 digital camera.

To maintain diversity of the cultures, some zones of hydrolysis were subcultured onto FP plates that incorporated a vitamin solution into the basal media, in these cases the filter paper was no longer the sole carbon source. The vitamin stock solution contained 50 mg/L riboflavin, 10 mg/L pyridoxamine.HCl, 20 mg/L folic acid, 25 mg/L nicotinamide, 50 mg/L thiamine.HCl and was filter sterilized before being incorporated into the minimal media at a concentration of 0.8% (v/v).

### 3.2.3 Substrate delivery

To deliver solid cellulose to the Evolver™ culture vessel, a segmented gas/liquid delivery system was set up as per Weimer *et al.*, 1991 with a 5 g/L suspension of Avicel. The suspension left the feed reservoir from underneath via tubing, and flow was maintained with a peristaltic pump. Air bubbles were regularly inserted into the stream of cellulose suspension by attaching another line with a Y-shaped joint, the other line was attached to an air pump. Silicone tubing of 6 mm internal diameter (i.d.) was used for delivery of the liquid and segmented air/liquid mixture and tubing of 1.3 mm i.d. was used to insert the stream of air into the Y-joint as per Weimer *et al.*, 1991. Magnetic stir bars were found not to be sufficient to keep the suspension from settling, so a mechanical drive, set at 200 rpm, powering a paddle-ended rod was used to keep the suspension from sedimenting. The suspension was pumped for a few hours to equilibrate the system first, this was needed to clear any air bubbles at the tubing junctions. To test the reproducibility of the suspension being delivered, samples were collected over 30 min periods and weighed or set volumes of suspension were collected and dried (at 100°C) in a pre-weighed beaker until constant weight was achieved.
Another strategy was tried, in which a thick slurry of cellulose in water was allowed to settle in a funnel, this was placed vertically above the peristaltic pump that controlled flow through tubing attached to the bottom of the funnel. The tubing had to be completely vertical before entering the pump head, so the pump was laid on its side.

3.2.4 Evolver run 1

The Evolver™ vessel used had the dissolved oxygen (DO) probe connected such that the probe would read the amount of oxygen in the air above the liquid level in the vessel. This design was thought to be useful in growing cultures at elevated temperatures. The standard design (Bridger et al, 2005) differed in that the DO probe was submerged in the liquid culture but this limited the temperature range that the Evolver™ could be maintained at. The vessel remained air tight to the outside surroundings, so that it could be examined if oxygen depletion in the liquid of the vessel, was reflected in a proportionate depletion of oxygen in the air in the vessel due to an equilibrium existing between air exchange at the interface.

A pH probe, inserted into the vessel contents, fed data back to the Evolver™ software so that pH could be maintained at pH 7.0, with a dead band of 0.5, if pH rose to higher than 7.5, 10% NH₃OH was pumped into the vessel to bring it back to pH 7. A heating mantle jacketed the culture vessel, this was set to maintain 30°C, a temperature probe inside the vessel, sent data to the software to control the heating mantle. DO and pH probes were calibrated prior to the start of the run. The DO probe was calibrated by bubbling air through the culture vessel, for a 100% reading. N₂ gas was then bubbled through the culture vessel to calibrate the DO probe for 0% oxygen. The culture vessel of the Evolver™ sat on a magnetic stirrer, and a magnetic stir bar inside the vessel kept the contents in suspension. The air in the vessel was recirculated through tubing and re-entered the vessel via a sparger at the bottom of the vessel which also helped to stir the culture as well as aerating the vessel contents.
461S media +2 g/L Sigmacel-20 was used as a source of nutrients and carbon, this was dripped into the vessel from above, overflow left the vessel at a side port keeping the volume of liquid constant at ~750 mL. Activated sludge from Lilydale sewerage farm, Victoria, was used as a source of microorganisms, 1 L of this material was stored at 4°C for no more than a few days before adding it to the culture vessel at the start of the run (the sample was first left to settle on the bench and the liquid was decanted off, leaving the heavier solids behind, which were used as inoculum). After the culture vessel had been filled with sludge, the pumps for the segmented air/liquid delivery system delivered the media at a flow rate of 27 mL/h. The software attached to the vessel controlled and stored DO measurements. DO readings were taken every 20 min and if DO was decreased to 50%, a fresh air pump was programmed to deliver air from the surrounding atmosphere for 60 s.

Samples were taken from the culture vessel at various points to measure OD_{600} of the culture and note observations of the diversity within the culture via light microscopy. Some samples were left to sit for exactly 5 min before aliquots were taken for OD readings, this enabled the heavy cellulose particles to sink to the bottom, with the supernatant giving a more accurate measure of growth, by measuring just planktonic cells in the liquid. After a week, a sample from the culture was serially diluted and then spread-plated on 461S minimal media + 0.2% (w/v) Sigmacel-20 as well as the complex media Sabouraud dextrose agar (SDA, Oxiod) + 0.2% (w/v) Sigmacel-20, the plates were incubated at 30°C for up to 5 days and examined for different types of growth. Single colonies of each type of growth seen, were further purified by streaking on the two types of media.

3.2.5 Evolver run 2

The second run was similar to the first, but due to persistent problems with the substrate delivery, the cellulose derivative carboxymethylcellulose (CMC) was used as carbon source, as it is soluble and can therefore be pumped into the culture vessel with a known concentration. 461S + 1 g/L CMC was used as media. The Evolver™ was set to measure DO every 15 min, with a longer period of aeration of 99 s, the pH dead band was decreased to 0.1 for tighter control of pH in the vessel. Media was pumped into the vessel at a flow rate of 43.3 mL/h. After 4 days the CMC concentration of the media was increased to 2.9 g/L. After 17 days, a final sample was taken and serially diluted before spread-
plating on tryptone soya agar (TSA). Isolates were later streaked onto 461S + 0.3% (w/v) CMC, to check for utilization of this carbon source.

### 3.2.6 Evolver™ run 3

The third Evolver™ run used the standard type of Evolver™ vessel, in which the DO probe was inserted directly into the liquid inside the vessel. Unlike the Evolver™ type used earlier, this meant that only a mesophilic temperature range could be used but reliable readings of DO in the liquid could be obtained. 461S + 1 g/L CMC was used as media, pumped at 25 mL/h into the culture vessel. Recirculated air from the headspace of the vessel was pumped back into the liquid through a sparger at 5 L/min. With pH set at 7.0, 10% ammonia was used as base control and 10% H₃PO₄ as acid control. Temperature was maintained at 30°C with a heating mantle as before.

After the culture vessel was initially filled with the sludge inoculant, the nutrient pumps were left off to enable depletion of the endogenous carbon in the sample. With the pumps off, liquid in the vessel evaporated and therefore had to be topped up with water or 461S media. The pumps were left off for the first week of the run. During this time Sigmacell crystalline cellulose was added at various points, and changes in DO that coincided with the addition of this carbon source were noted. Because the DO electrode was submerged in the culture, it occasionally became fouled with thick biofilm growth, which interfered with the reading of DO, this was indicated by the jagged appearance of the Evolver™ output curves as readings became less reliable. Therefore, occasionally the vessel had to be drained and the surface of the electrode wiped to clean it, before the vessel contents could be returned and the run resumed.

Diversity of the culture was examined and pictures of wet mounts of culture samples were taken with the lens of a Canon powershot A400 digital camera placed against the eyepiece of the phase contrast microscope (Olympus). After 15 days a sample was taken and used to inoculate 461S + 0.1% (w/v) CMC or 0.1% (w/v) cellobiose or 0.1% (w/v) Sigmacell plates (1 mL spread- plated) and filter paper (FP) plates (10 µL of inoculant placed on the point of a triangle of Whatman #1 filter paper on the surface on a 461S, no carbon plate), all plates incubated at 30°C. A final sample from the vessel was
taken after 22 days, and used to inoculate FP plates, with or without 25 µg/mL cycloheximide included in the 461S agar as well as 461S + 0.1% (w/v) CMC/cellobiose/Sigmacell, all plates incubated at 30°C.

3.2.7 Characterization and identification of isolates

Colony descriptions of suspected pure isolates were made when isolates were subcultured onto both rich, complex media (SDA, TSA or NA) and minimal media supplied with a single carbon source (461S or ST6). Therefore it was determined if further purification was necessary as well as determining if the single carbon sources could be utilized by the isolates. Very tiny colonies that appeared on single carbon source plates but didn’t increase in size after more than a few weeks of incubation were assumed to be due to growth from endogenous energy supplied from the inoculation and therefore not representative of utilization of that particular carbon source.

Hydrolysis of crystalline cellulose appeared as transparent clearance zones around bacterial colonies on 0.1% (w/v) Sigmacell plates, similar clearances on the pink cellulose plates (see section 2.2.1 above) that appeared as less pink regions also seemed to indicate utilization of the cellulose substrate.

To further investigate growth on various carbon sources, shake flask cultures were monitored by measuring OD$_{600}$ over the course of incubation. Generally, the shake flasks were incubated with aliquots (1 mL per 100 mL) from 10 mL starter cultures grown overnight on easily metabolized sugars, for example 461S + 0.2% (w/v) glucose, or + 0.5% (w/v) cellobiose. This gave a large inoculum, typically with OD of at least 1, which was thought to boost growth on the recalcitrant substrates such as crystalline cellulose.

Because growth on the crystalline cellulose could not be monitored by OD$_{600}$, aliquots of cultures were stained with Propidium iodide (PI), which highlighted the presence of dead cells, as only bacteria with damaged cell membranes will take up the fluorescent dye. The Propidium iodide, once inside cells, intercalates with DNA and can be detected with fluorescent microscopy (Arndt-Jovin and Jovin 1989). Culture samples (1 mL) were placed in aluminium foil wrapped tubes and had 1.5 µL
10 mM PI added and then incubated in the dark for 15 min. Aliquots of stained samples (5 µL) were examined with an Olympus fluorescence microscope (mercury arc light source, violet filter, images captured with Spot software). The proportion of dead cells (stained by Propidium iodide) as compared to total cell numbers seen by light microscopy was estimated visually, in order to determine survival of the bacteria in the presence of the different carbon sources.

Initial identification of suspected pure bacterial cultures involved Gram stains, a smear of cells was air dried, then fixed by passing the glass slide through the blue flame of a Bunsen burner. The smear was stained with 1% (w/v) crystal violet for 30 s, this was rinsed with tap water, then Jensen’s iodine was used to stain for 30 s, this was washed off with tap water, the smear was then decolourised with 70% (v/v) acetone, 30% (v/v) ethanol for 2 s and then washed in water. Neutral red was applied as a counterstain for 10s and the slide was finally rinsed with water and dried before it was examined microscopically. Some isolates that were coloured orange or yellow were tested for flexirubin pigment because this would be an indication that they belong to the Cytophagales group of bacteria, which is known to contain members with good cellulolytic capabilities. A loopful of bacteria from a single colony of a pure isolate is placed in a drop of distilled water on a slide, one drop on 10 % (w/v) KOH is placed on top and a colour change from yellow or orange to red, purple or dark brown indicates pos. for flexirubin (Reichenbach 1999).

3.2.8 16s rDNA PCR to identify bacterial isolates to genus level

Sequencing of the 16s ribosomal DNA gene was carried out in order to identify the bacterial isolates to at least genus level. 10 mL nutrient broth (NB) cultures were inoculated with a single colony and then incubated at 30°C with shaking overnight, which typically resulted in OD₆₀₀ of ~2. Aliquots of cultures (500 µL) were then centrifuged at 13 000 rpm (eppendorf 5415D microcentrifuge) for 1 min to pellet the cells. The cells were washed by resuspending in 1 mL lysis buffer (1% (v/v) TritonX-100, 10 mM Tris.Cl pH 8.0, 5 mM EDTA pH 8) and then re-centrifuged. The cells were then resuspended in 100 µL lysis buffer and heated to 100°C for 30 min to lyse the cells, then the sample was centrifuged at 13 000 rpm for 5 min and the supernatant containing the DNA was stored at -20°C. This method gave a fairly crude DNA sample, alternatively the Qiagen DNeasy tissue kit was used (as per the
manufacturers instructions) to extract pure DNA from the cultures. PCR reactions (50 µL total volume) contained 1 µL of DNA, 0.2 mM dNTPs, 1x PCR buffer (Invitrogen), 2.5 mM MgCl₂, 5 pmol each of forward and reverse bak primers, 1-5 U Taq (0.5 µL in 50 µL reaction) (Dasen et al. 1998). A negative control with no DNA was always run, a positive control involved DNA that was known to give a PCR product reliably. The PCR cycle consisted of an initial denaturation of 3 min at 95°C, then 40 cycles of 95°C for 15 s, 56°C for 30 s, 72°C for 2 min and a final elongation step of 7 min at 72°C.

PCR products were analysed by agarose gel electrophoresis on a 1% (w/v) gel, 5 µL of sample was run alongside Sigma wide range DNA marker for molecular weight comparison. Ethidium bromide was included in the gel to visualize the DNA, pictures of the gel were taken with the Gel-Doc system. PCR reactions that gave single bands of the expected size (~1 kbp) were purified using the Qiagen PCR clean up kit as per the manufacturers instructions except that the sample was applied to the column twice to maximise binding of DNA and the eluate was also re-applied to hopefully elute more DNA from the column. Purified PCR products were quantified by running 4 µL of each alongside 4 µL of molecular marker II (Roche) on a 1% (w/v) agarose (in TAE buffer) gel to estimate DNA concentration.

Sequencing of the PCR products (adjusted to the required concentration of 20 ng/µL) was carried out at Monash microbiology sequencing facility, primers used for sequencing were either Bac11w or dem1/II (Demarta et al. 1999). Chromatographs returned from sequencing results were examined for portions of the data that gave reliable results (clear peaks representing each nucleotide) and this data was used in a Blastn (Altschul et al. 1997) search to find similarity with other sequences in the NCBI databases. Matches with the database sequences giving at least 97% similarity indicated that the bacterial isolate from which the sequence had been obtained was of the same genus as those attributed to the sequences in the database.
Table 3.1 Primers used for 16s rDNA PCR, or sequencing of products.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Forward/Reverse</th>
<th>Target*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bak4</td>
<td>AGGAGGTGATCCARCCGCA</td>
<td>reverse</td>
<td>1522-1540</td>
<td>Dasen et al. 1998</td>
</tr>
<tr>
<td>bak11w</td>
<td>AGTTTGATCMTGGGCTCAG</td>
<td>forward</td>
<td>8-25</td>
<td>Dasen et al. 1998</td>
</tr>
<tr>
<td>demI</td>
<td>AGATTTGATCATGGCTCA</td>
<td>forward</td>
<td>8-26</td>
<td>Demarta et al. 1999</td>
</tr>
<tr>
<td>demII</td>
<td>GTGTGGACGCGCGGTGTGTA</td>
<td>reverse</td>
<td>1411-1391</td>
<td>Demarta et al. 1999</td>
</tr>
</tbody>
</table>

*Target = position in the *E.coli* 16s rDNA gene that the primer corresponds to. M= A/C nucleotides

3.2.9 Cellulase assays

Protein amount in samples tested for cellulase activity was estimated using the Bradford microassay procedure with Bio-Rad Bradford dye reagent, as per the manufacturer's instructions. A standard curve of 12.5 – 75 µg/mL BSA was used to test the reagent.

The release of reducing sugars from cellulose was measured as per Miller (Miller 1959). To make 100 mL DNS reagent, 1.6 g NaOH was dissolved in 20 mL water and 1 g 3,5-dinitrosalicylic acid (DNS) was added, 30 g sodium potassium tartrate (Rochelle salt) was dissolved in a separate 50 mL of water and 0.1 g sodium sulphite was added. The two solutions were added, made up to 100 mL and filtered to remove any undissolved particles. The DNS reagent was tested with a glucose standard curve, with 0.2, 0.4, 0.6, 0.8, 1 g/L glucose in phosphate buffer. Samples (typically culture supernatant from shake flask cultures) of 1 mL volume were added to 1 mL of substrate solution (25 mM NaHPO₄ pH 7.0 + 5 mg/mL SigmaCell-20) and incubated at 30°C for 1-2 h. After incubation, reactions were centrifuged at 4000 rpm (Eppendorf 5810R centrifuge) for 10 min to pellet the cellulose, then 0.5 mL aliquots of the supernatant were added to 1.5 mL DNS reagent in tubes, which were then boiled for 15 min, before being cooled on ice, diluted with 2 mL water and the absorbance was read in the spectrophotometer at 575 nm. *A. niger* cellulase (Sigma) 1 mg/mL in 50 mM NaHPO₄ pH 7.0 was used as a positive control for cellulase activity. A blank tube was included in the assay each time, containing phosphate buffer instead of sample. An additional control, the ‘unincubated control’ was also included for each sample. This control tube was the same as sample tubes (each sample tested in triplicate), however the control tube was not incubated at temperature, but kept on ice till all tubes were tested with DNS
reagent. The inclusion of these control tubes insured that any reducing sugars present in the sample, not due to cellulase action on the substrate, could be taken into account.

CMC overlay plates were used to detect the presence of CMCase as per Teather and Wood, (1982). Isolates were spotted onto 461S + 0.1% (w/v) cellobiose plates and incubated at 30°C until growth was observed (up to a few days), then 5 mL 461S + 0.1% (w/v) CMC + 0.8% (w/v) agar was overlaid on top and the plates were further incubated at 30°C, so that any enzymes arising from the colonies could hydrolyse the CMC in the top layer. After overnight incubation, the plates were stained with 1 mg/mL Congo red for 15 min, then destained with 1 M NaCl for 15 min. Activity was seen as pale orange clearance halos visible above the colonies, where the Congo red did not stain the CMC as intensely as the background (dark red) due to the chain length of the CMC being shortened. Growth from the bacterial colonies was prevented from occurring in the top layer by including 170 µg/mL Chloramphenicol in the CMC overlay.

To test culture supernatants for CMCase, 0.1% (w/v) CMC was dissolved in phosphate buffer (stirred slowly to prevent shearing) and then 1% (w/v) agar was added so that assay plates could be set in petri dishes, wells were punched out of such plates, which could hold 60 µL of sample. Samples, including the positive and negative controls mentioned for the reducing sugar assay above were added to the wells and then the plates were incubated at 30°C overnight. The plates were then stained with Congo red as above. Sensitivity of the assay was tested with dilutions of *A. niger* cellulase.

3.3 Results

3.3.1 Filter paper isolation

After a week of incubation at 30°C the filter paper plates had a thin layer of bacterial growth around the entire perimeter of the paper, as well as fungus and nematodes (seen at 50x magnification). A yellow spot of microbial growth had appeared on a plate incubated at 30°C after 5 days incubation, so the growing edge of this was subcultured onto more filter paper plates and named YS1. After 19 days incubation the original spot of yellow growth (461S basal media, 30°C) had increased and hydrolysis
of the filter paper was evident as a semi-transparent mass of bacterial growth existed where there had been paper before.

A similar yellow spot appeared on the secondary plates after only 2 days, some of this growth was used to inoculate 10mL NB cultures, incubated at 30°C overnight. A wet mount of the NB culture revealed predominantly short bacterial rod-shaped cells, -2 µm long with rounded ends and sometimes in microcolonies/aggregates. All cells in the sample were gram negative. The culture was used to inoculate streak plates on nutrient agar (NA), which were incubated at 30°C. Different colony types on the NA plates were named YS2, 3, 4, 5 and were further purified by streak-plating onto NA and 461S + 0.1% (w/v) cellobiose plates. YS2 did not grow on 461S plates with cellobiose as the sole carbon source, the other isolates all gave small, round, white colonies on cellobiose plates but differed slightly in their colony morphology on NA.

YS3/4/5 were all found to be gram negative rods, 1-2 µm long. These isolates were streaked onto 461S + 0.5% (w/v) pink cellulose, cellulose azure, Sigmacell, or CMC plates. None of the isolates showed significant growth on any of these single carbon sources after 26 days incubation at 30°C. A vitamin stock solution was included in the basal media (461S and ST6) when filter paper plates were incubated with either YS3 or YS5 (inoculant from cellobiose plates), but growth on the filter paper was still not seen.

YS3/4/5 were tested for endoglucanase activity with the CMC overlay assay. YS3 and YS5 had clear halos upon Congo red staining, indicating the presence of ß-1,4-endoglucanase activity (Teather and Wood 1982). 16s rDNA sequencing confirmed that YS3 & 5 were one of 9 possible Aeromonas species (16s rDNA sequences from 9 different species with similarity to the YS3/5 sequence of at least 97%, over 740 bases) (see Appendix 1.), therefore they may be the same species, although the different colony morphologies suggest they may be at least different strains.

Additionally, other sites of hydrolysis on the filter paper occurred without obvious (coloured) bacterial growth. Purification of isolates from these sites (named CH1 and CH2), was attempted by
lifting material from the advancing edge of hydrolysis and subculturating it onto more filter paper plates. After 4 days incubation at 30°C, CH1 appeared as a yellow spot, similar to that noted above, which was 1 x 1cm, the same on duplicate plates. CH2 had also grown after 4 days but appeared only as a faint discolouration of the paper. Growth from CH1 was streaked onto 461S + 0.1% (w/v) cellobiose, TSA and NA plates.

When CH1 was streaked onto cellobiose plates, three distinct types of bacterial growth were observed; small bacterial colonies were either white and shiny or pale yellow and strange forms could also be observed, seen all over the plates when observed at 50x magnification. The strange forms were bulbous growths attached at the base to fine, curly filaments and unfortunately could not be isolated in pure culture. The other bacteria, named CH3/4/5 were purified by streaking on TSA plates and used to inoculate 461S + 0.1% (w/v) cellobiose, CMC, Sigmacell, pink cellulose. None of the isolates had significant growth on CMC, Sigmacell or pink cellulose as a sole carbon source. CH4 did not show significant growth on cellobiose as a sole carbon source but CH3 actually produced larger colonies on cellobiose plates than on TSA plates.

CH3 & 5 were tested for endoglucanase production via the CMC overlay assay as well as using culture supernatant from an overnight culture (461S + 0.1% (w/v) cellobiose) in the CMC well plate method. CH3 was confirmed to have endoglucanase activity, giving halos above the colonies in the CMC overlay assay. To further investigate this activity, the well plate method was used, which was sensitive enough to detect 0.01 mg/mL A. niger cellulase (giving a halo size of 12 mm, seen on the edge of the 9 mm diameter well) but no activity was detected for CH3/5 culture supernatants. An overnight culture (461S + 0.1% (w/v) cellobiose) of CH3 was used to inoculate 100mL cultures with 461S + 0.1% (w/v) glucose, cellobiose or CMC as media. Growth on glucose and cellobiose were comparable, giving OD of 0.977 ± 0.001, 0.991 ± 0.051, respectively but no growth was seen on CMC. 16s rDNA sequencing found CH3 to be a Cellvibrio species, either mixtus, ostraviensis or fulvus (all having 16s genes with at least 97% similarity to that from CH3) (see Appendix 1).
Isolation of pure bacterial species from CH2 was unsuccessful, but it seemed to be responsible for the most extensive cellulose hydrolysis seen (large proportion of the filter paper degraded), additionally, the consortium (CH2) was found to cover a large area of the filter paper after only 5 days incubation at 30°C (see Figure 3.1).

Figure 3.1. CH2 growing on filter paper. Extensive hydrolysis of the paper is seen after two months incubation(a), and when a small amount of material is lifted to inoculate a secondary plate, growth is seen to cover ¾ of the plate after 5 days incubation(b).
3.3.2 Using the Evolver™ for isolation of cellulolytic bacteria

3.3.2.1 Substrate delivery

The segmented air delivery system initially gave reliable delivery of the cellulose suspension with
17.59 ± 0.019 g suspension delivered in 30 min. But when the system had been continuously pumping
for a few days and re-measured, only 15.15 g of suspension was delivered. Additionally, the dry
weight of cellulose delivered was twice that of the concentration in the feed reservoir. For example, a
20 mL sample of the cellulose suspension, taken before and after pumping, and dried to constant
weight was 49.54 g and 71.48 g, respectively. This was due to stratification of the suspension,
Weimer et al., 1991 warn that cellulose particles of 45 µm or less are needed to prevent this. Therefore,
Sigmacell-20 (Sigma, <1% is >38 µm) was used instead of Avicel PH101 (Sigma, average particle size of
50 µm).

The liquid pockets in between the air bubbles were also not holding the cellulose particles, this was
initially thought to be due to the Avicel particles being too heavy to hold within the surface tension of
the water but using Sigmacell-20 did not resolve this. Cellulose particles were found to be building
up on the inside walls of the tubing and especially at any tubing junctions, this caused the surface
tension of the air bubbles to break and regular flow could not be maintained. The liquid in between
the air bubbles no longer contained a standard amount of cellulose particles and the air bubbles did
not stay at regular intervals of the same size. Additionally, when cellulose particles built up at
particular points, the “dunes” formed would occasionally collapse when a critical size was reached,
causing sudden significant increase in the amount of cellulose being delivered.

The alternative strategy of delivering cellulose as a thick slurry was found to only work at high
speeds. If the slurry is left to sit, or pumped very slowly, the slurry breaks up, with pockets of water
without any cellulose in them forming. This was found to happen even if all of the tubing was kept in
a vertical position. The slurry, assumed to be a cellulose-saturated suspension was found to be -0.16
g/mL. At this concentration the slurry would have to be pumped very slowly to maintain a cellulose
limited environment in the Evolver™ vessel. Therefore this method was also not suitable to deliver a constant concentration of cellulose to the Evolver™.

3.3.2.2 Evolver run 1

This run used the segmented air/liquid delivery system with crystalline cellulose as carbon source, even though it was found that this method did not deliver a constant concentration of cellulose particles. Changes in the %DO within the culture vessel can indicate metabolic activity in response to increases of carbon input to the system. However, in this case it was not known when additional carbon was added to the culture, owing to the inconsistent delivery of cellulose particles. Therefore, instead of recording %DO, samples from the vessel were examined microscopically and measurements of OD were taken, which revealed changing diversity within the consortium surviving inside the culture vessel. Therefore, the Evolver™ was simply used as a chemostat in this initial experiment.

Samples taken within the first few days revealed a large diversity of types of microorganisms (including fungi, algae and protozoa as well as many bacterial types) before the initial consortium from the sludge was slowly washed out of the vessel. Initially, not many plantonic bacteria were seen, the cellulose particles seemed to be quickly colonised by bacteria of different types. Stalked protists, resembling Vorticella sp. were seen, with the stalks most often attached to flocs of material from the sludge. After 3 days, short, curved rods were the predominating planktonic bacterial species, long straight filaments resembling actinomycete species are seen on the surface on cellulose particles. At this stage, the OD of the liquid (after letting the heavy cellulose particles settle) was 0.081. In response to this, flow through the vessel was slowed by increasing the size of the air bubbles in the substrate delivery system, thereby also increasing the air input to the system. By the next day, OD had risen to 0.115 and diversity of the culture increased with more bacterial cocci as well as the rod-shaped cells seen before the flow rate was changed. The actinomycete-like growth had also increased in abundance, this appeared orange in colour when present as dense groups. At least 3 distinct types of large motile protists were seen. The next day OD had risen again to 0.125 and the culture now contained lots of Vorticella and other ciliated protists. Cellulose particles were seen to be colonised with sporulating fungi and there seemed to be less planktonic bacteria than earlier.
A significant input of fresh air to the system occurred when the vessel was opened to remove a blockage from the sparger inside the vessel. Once again, the day after an input of more air, an increase in OD was observed, when it had climbed to 0.24. At this point a sample from the culture was used to inoculate media containing Sigmacell, to investigate the diversity of the microorganisms within the culture and to see if any of the isolates could utilise cellulose as a sole carbon source. Figure 3.2 outlines the results of this isolation attempt and a subsequent sample taken at the end of the culture period.

After 12 days of continuous culture, there were still plenty of protists in the culture vessel, as well as a lot of bacterial filaments that in some cases was seen to bind cellulose particles together. After 19 days, diversity of the culture had not changed significantly and the final sample was taken and plated out on the complex and minimal medias outlined in section 2.2.1.
Initial sample taken after 8 days, plated out onto:

- SDA (complex media) + 0.2% Sigmacell (w/v)
- 461S (defined media) + 0.2% Sigmacell (w/v)

7 different types of growth observed, streak-plated onto:

- 461S + 0.2% Sigmacell (w/v)
- SDA + 0.2% Sigmacell (w/v)

One fungus and five bacterial isolates purified:
- Medium size, very pale, rough surface
- Large, pale pink, smooth, shiny surface
- Large, yellow, waxy surface
- Small, yellow center with white edge
- Small, pink/purple, smooth surface

No growth

Small, yellow, bacterial colonies (impure by gram stain) observed, streak-plated onto:

- 461S + 0.2% Sigmacell (w/v)
- SDA + 0.2% Sigmacell (w/v)

No growth

Second sample taken after 19 days, plated onto:

- 461S + 0.2% Sigmacell (w/v):
  - After 10 days incubation at 30°C, clearing zones are evident around the bacterial colonies where the cellulose particles (Sigmacell) have been degraded.
  - Subcultured by streak-plating onto “pink cellulose” plates:
    - Two types of growth observed, named BC01, BC03, both are seen to cause depressions in the agar and therefore suspected to secrete agarase and use the agar as a carbon source. BC03 is still an impure consortium of gram pos. and neg. species, therefore was subcultured onto more pink cellulose plates:
      - BC03 shows clearance of the pink cellulose after 30 days incubation at 30°C but these zones of degradation did not always correlate with colonies, rather a thin, spreading, translucent growth is seen to cover the plate. Repeated streak-plating on pink cellulose was required to purify the gram positive cells from the gram negative bacteria:

 Gram positive cells (BC03.2), identified as *Bacillus* sp. by 16S rDNA sequencing, could not grow on cellulose as a sole carbon source without BC03.1 present.

 Gram negative cells (BC03.1), identified as *Pigmentiphaga* sp. by 16S rDNA sequencing, seemed to be adapted to a biofilm mode of growth.

Figure 3.2 Flow diagram of isolation attempts from the Chemostat culture
Both BC01 and BC03 showed depressions in the agar underneath colonies, potentially due to an agarase enzyme. Prior to this, the possibility of the bacteria using the agar in the plates as a carbon source had not been considered. Therefore it could not be determined if BC01 was in fact utilizing the cellulose as a carbon source, this explained why no clearance of the cellulose substrates was seen with this isolate. BC03 showed clearance areas on the pink cellulose plates, similar to the degradation seen initially on the Sigmacell plates but these clearances did not always correlate with visible colonies. A thin, translucent, spreading growth was seen on plates inoculated with BC03, along with the bacterial colonies.

3.3.2.3 Further investigation of the cellulolytic capability of isolate BC03

Whilst not purified as a single species of bacteria, BC03 was investigated for cellulase activity in shake flask culture; 100mL cultures with 461S + 0.5% (w/v) Sigmacell + 0.001% (w/v) glucose as media were inoculated and incubated at 30°C with shaking. Growth in the shake flasks revealed an impure culture of the bacterial filaments and motile bacterial rods (similar to those observed in the Evolver™). To test the culture supernatant, 5 mL samples were taken at t = 24 h from triplicate flasks and centrifuged at 4000 rpm (Eppendorf 5810R) for 30 min to obtain the culture supernatant. Aliquots of these samples were then tested for release of reducing sugars from Sigmacell over a 2 h period (see section 2.2.8 above) but no activity was detected. After 5 days incubation, the culture supernatant was re-tested but again the results were all below the sensitivity of 0.1 g/L glucose equivalents. Extracellular protein levels in the culture supernatant were measured via the Bradford method and found to be between 70-120 µg/mL for the three flasks.

When BC03 was grown in 10 mL cultures of 461S + 0.2% (w/v) glucose at 30°C overnight, variability in growth was seen, with sometimes quite turbid liquid and at others not turbid at all, suggesting that the isolate may not have been adapted to planktonic growth. The less turbid the culture, the more likely there was strange, large, golden coloured, highly refractile particles formed.
Because no cellulase activity was detected in BC03 culture supernatants (grown on 461S + 0.5% (w/v) Sigmacell or cellobiose), it was investigated if there was cell-associated cellulase activity. A cell pellet from BC03 grown on cellobiose for 2 days was washed in 50 mL NaHPO$_4$ pH 7.0 twice, centrifuging at 4000 rpm for 5 min at 4°C between each step and then finally resuspending in 10 mL phosphate buffer. Aliquots of 5 mL were lysed with a handheld homogeniser for 60 s, and then centrifuged at 4000 rpm for 5 min, the pelleted material was resuspended in 5 mL phosphate buffer and the supernatants kept. Both fractions were tested alongside similarly washed cell pellet material that had not been homogenised, 1 mL of each sample was tested in triplicate in the reducing sugar release assay but none of the samples showed any cellulase activity.

In case the high concentration of cellobiose, used to grow the cultures, was inhibiting cellulase production, induction of cellulase activity was tried by first growing the cells to high density and then subjecting them to a much lower level of cellobiose. Shake flasks cultures were grown on 100 mL 461S + 0.5% (w/v) cellobiose until OD 2.65-2.75 was reached (triplicate flasks), then the cells were collected by centrifugation at 35 000x $g$ for 15 min and resuspended in 100 mL 461S + 0.02% (w/v) cellobiose before incubation was continued. After a further 45 h incubation, the cultures were harvested by centrifugation at 35 000x $g$ for 15 min and the cell pellets were resuspended in 20 mL 0.1 M NaHPO$_4$ pH 7.0. Culture supernatant put aside from the initial growth in 0.5% (w/v) cellobiose was tested alongside the two final fractions of the ‘induced’ samples but none were shown to release reducing sugars from cellulose in a 2 h period.

Another induction experiment involved adding cellulose to the cultures after most of the sugar had been depleted. BC03 was grown in 6x 100 mL 461S + 0.2% (w/v) glucose shake flask cultures for 66 h (OD 1.49 – 1.58 reached), when the glucose left in the cultures, as measured by DNS, was less than 0.04% (w/v). To sustain further growth, all but one flask was then supplemented with concentrated 461S nutrients (with a final concentration of 1x, not including any remaining from the initial media), cellulose was added to 4 flasks in the form of 5x 6 × 1 cm strips of Whatman #2 filter paper, and an additional flask had 1.9 mL 10% (w/v) glucose added instead (final concentration -0.2%). Change in OD in the flasks was measured between 120 and 216 h after inoculation (no measurements were taken
between \( t = 66 \) and \( 120 \) h), Figure 3.3 displays the changes in OD, values for the three test flasks with added filter paper have been averaged (with standard errors no bigger than 0.049) and the difference between the test flasks and the un-inoculated control is shown to illustrate how similar the filter paper flasks were to the no nutrients added control.

The OD of the filter paper test flasks dropped initially, unlike the control with no added nutrients that continued to rise until \( t = 120 \) h, the drop in OD with added filter paper may have been a result of the bacterial cells binding to the paper. The un-inoculated control was found to be important, as it monitored the slight increase in OD due to fibre release from the filter paper. When this fibre release was taken into account, the OD of the test flasks more closely resembled that of the control with no added nutrients.
Figure 3.3. BC03 Filter paper trial. Isolate BC03 was grown in 100mL shake flasks for 66h, till the originally supplied glucose was depleted to <0.04% (w/v). Then, filter paper, as a source of cellulose, was added to three flasks and rises in OD were compared to an addition of glucose to another flask, or no additions, or an uninoculated flask with filter paper added.

Figure 3.4. Propidium iodide staining of BC03 in flask cultures with filter paper added. a. A large microcolony of dead cells in the control flask with no added filter paper, b. a photo of the same microcolony taken a minute later to show how quickly the dye fades under the light source, c. a cellulose fibre (blue) with cells associated, d. a large microcolony of cells attached to a small cellulose fibre (arrowed), with many cells also in the background, but out of focus due to rapid movement.
16 days after the initial inoculation of the shake flasks in the filter paper trial, samples were taken and checked for living cells by staining with propidium iodide. As expected, flask 5, which had had no nutrients added, was found to contain large microcolonies of dead cells, stained red with the fluorescent dye (Figure 3.4). A few stained cells were seen in samples from the three test flasks but they only represented a small proportion of the total numbers of cells seen by light microscopy (Figure 3.4). Additionally, cells in a wet mount from the test flasks appeared to be motile and therefore living.

Overnight cultures of BC03 grown in 10 mL NB were used to inoculate filter paper strips placed on top of 461S media containing no other carbon source. No degradation of the filter paper was observed, but growth around the perimeter of the paper was associated with significant clearance of the agar underneath. On plates with CMC as the sole carbon source, a thin, translucent spreading growth was seen over the surface of the agar and even climbed the plastic walls of the petri dish. On these plates, in the spread area of a streak plate, where there would have been a heavier inoculum, small, orange/yellow, convex structures were seen that were reminiscent of myxococcal fruiting bodies. This thin, spreading growth was confirmed to contain the gram negative cells of BC03 (<1 µm long) and not the gram positive cells (resembling Bacillus sp.) and was also seen to occur on the pink cellulose plates, which also had raised structures that were 1 mm in diameter. When an NB culture of BC03 was used to streak pink cellulose plates, the yellow fruiting body-like structures occurred along with the spreading growth and large Bacillus colonies, after 12 days incubation, it was noted that there were fewer fruiting body-like structures on top of the Bacillus colonies than elsewhere on the plate.

The gram negative cells in BC03 were isolated by repeated streak-plating on pink cellulose plates and named BC03.1. BC03.1 did not grow well in liquid cultures, after 2 days growth in NB, OD of only ~0.28 (in duplicate) was seen, a small amount of pelleted growth was seen when glucose, cellobiose or acetate was the sole carbon source (in 461S media) but only seen after more than 4 days incubation. Therefore this isolate was not adapted to growth in liquid cultures either with minimal or complex media, and was assumed to be adapted to a biofilm mode of growth.
16s rDNA sequencing revealed that the gram negative rods in BC03.1 were *Pigmentiphaga kullae* or an undescribed closely related species (see Appendix 1). 97% similarity between the BC03 data (over 601 bases) and that for *Pigmentiphaga kullae* strain K24 was found, this was the only match in the database with this level of similarity. The gram positive cells that co-existed with the *Pigmentiphaga* sp. were found to be either *Bacillus cereus, thuringiensis* or *anthracis*, as the 16s gene purified from culture had 96-98% similarity (over 1125 bases) to the same gene from all of these species (see Appendix 1).

### 3.3.2.4 Evolver run 2

As for the first run, the DO probe was taking readings from the headspace of the Evolver™ vessel and was therefore in ambient temperature that rose and fell depending on the time of day (the room the Evolver™ was in was heated during the day but not at night). These temperature variations were found to have a significant effect on the readings that the probe was recording (Figure 3.5).

Additionally, the %DO never fell below 100% as would have been expected if any oxygen that was depleted in the liquid in the vessel, concurrently changed the oxygen levels in the headspace. Growth was present in the Evolver™ however, including some planktonic bacteria but not enough to give a significant OD reading after the sedimented particles had been left to settle. After 9 days the CMC concentration in the feed was increased from 1 g/L to 2.9 g/L in case the carbon levels were limiting growth. Because the DO probe didn’t seem to be measuring an indirect effect on DO in the liquid phase, a different air pump was fitted to the sparger to deliver fresh air into the vessel instead of recirculating the headspace gases; therefore two weeks after the start of the run, the Evolver™ was running as a simple chemostat. After three weeks, the liquid in the vessel had never become turbid (OD <0.09) so the run was ended and a sample was plated out on TSA. The TSA plates grew 4 different types of bacterial colonies.

After the TSA plates had been stored at 4°C, large, raised, shiny structures were seen to form, these were brightly coloured (pink, yellow and orange) and were bigger on the less crowded plates. Once again, these structures resembled myxococcal-like fruiting bodies and may have indicated that the bacteria were in a nutrient starved, biofilm mode of growth. When these structures were streaked
onto TSA plates in attempts to subculture and purify the organisms responsible, they only showed a little growth in the spread area of the plate, that is, they could not survive as single colonies. This once again highlighted the symbiotic relationships that occurred between the bacteria on the initial isolation plates and presumably in the Evolver™ vessel during culture.

One of the purified isolates (as well as yellow colonies from the original plates) was tested for flexirubin pigment, isolate BC06 was positive and therefore a member of the Cytophagales family. 16s rDNA sequencing confirmed BC06 to be a member of *Chryseobacterium* genus (730 bases run in a Blastn search gave 97% similarity with members of this genus) (see Appendix 1). A member of the Cytophagales family of bacteria had been expected to be found, because this group contains many cellulolytic species. However, the *Chryseobacterium* species found here displayed no cellulase activity and could not utilise cellulose as a sole carbon source.

All of the purified isolates were grown in overnight NB cultures to provide a heavy inoculum for streaking onto 461S + 0.1% (w/v) cellobiose plates but none of the organisms grew on this single carbon source, therefore they were not investigated further.
Figure 3.5. Temperature vs. %DO correlation. A. data collected for evolver run 2, showing the change in %DO and temperature relative to time. B. the same data displayed as a linear relation between %DO and temperature.
3.3.2.5 Evolver run 3

This Evolver™ run used the standard format Evolver™ with the DO probe submerged in the liquid, so changes in DO could be accurately recorded this time. The vessel was aerated regularly and then changes in DO were recorded every 10 min, giving the Evolver™ output data, given here as OUR. Figure 3.6 shows the OUR data that was collected, outlining the changes to the Evolver™ that resulted in increases or decreases to OUR.

At first the pumps were left off, so that the endogenous carbon sources in the sludge could be depleted whilst still maintaining diversity. After 2 days the OUR had dropped to 11.9 mg/h, so 1 g of Sigmacell-20 was added, resulting in the expected rise in OUR. By the next day OUR had risen to 34.7 mg/h and microscopy revealed that most of the cellulose particles appeared to be colonized by small bacterial cells (~1 µm long); there were also lots of protists and rotifers present. To confirm that the rise in OUR was due to the addition of the Sigmacell particles, OUR was allowed to drop before more cellulose was added.

A week after the run was started, the pumps were turned on to deliver 461S + 1 g/L CMC at a flow rate of 25 mL/h. To preserve diversity in the culture, more Sigmacell was added, and both types of cellulose were present for the rest of the run. After the pumps were turned on, 1 g of Sigmacell-20 only produced a small peak in OUR (rise from 8.3 – 12.7 mg/h), so 2 g Sigmacell was added from this point on. Each time 2 g Sigmacell was added there was a sudden but unsustained rise in OUR. With no added cellulose supplied to the Evolver™, OUR gradually dropped to -4.4 mg/h, but the addition of 2 g Sigmacell brought the OUR back to the previous level of -20 mg/h and this was maintained as long as Sigmacell was added daily.
Figure 3.6. Evolver run 3 data. Oxygen uptake rate (OUR) was calculated from readings taken by the Evolver %DO probe and was used as an indication of growth in the culture vessel. Additions to the culture vessel that may have induced changes in the population are noted.
The culture was sampled repeatedly to microscopically examine diversity of microorganisms within the vessel. Nine days after the run was started (~12800 min) samples were found to contain a large diversity of planktonic bacteria. More diversity was seen 11 days after the run was started, when the culture was seen to contain many protozoa of all types (flagellates, ciliates and amoebae) as well as lots of large rotifers but less planktonic bacteria. However the abundant cellulose particles were mostly covered in bacteria, predominantly small rods 1-2 µm long. Figure 3.7 illustrates this diversity (pictures taken of Evolver™ sample taken from vessel at ~15000 min). As well as the living protists, spherical, slightly golden coloured cells ~12 µm in diameter were abundant and assumed to be protist cysts. These cysts declined in abundance after two weeks of continuous culture (21 500 min), which coincided with an increase in ameboid protists, suggesting that they had emerged from the cysts at this point. At this point it was also noticed that the cellulose particles were not as heavily colonized with bacteria.

Both planktonic and attached bacteria seemed to be declining in abundance and this was thought to be due to protozoal grazing. Not only were rotifers seen to be feeding on the bacterial planktonic population, large ciliates appeared to be grazing on the bacteria bound up in aggregates containing cellulose particles. The type of large ciliate pictured in Figure 3.7 h. was especially seen to continually circle such aggregates.
Figure 3.7. Diversity within the Evolver™ culture. a. a large ciliated protist near some cellulose particles that are bound up with other microorganisms, including long bacterial filaments, b. a similar group of cellulose and bacteria containing three cysts, c. a larger aggregate, showing the abundance of protozoal cysts, d. a small flagellated protist is seen (centered), e. a rotifer (at rest) attached to a small cellulose particle, f. a rotifer actively feeding, g. a cellulose particle with many bacteria attached, very small (dark) flagellated protists are also seen, h. a large ciliated protist (top right) that appeared to be grazing at the surface of the aggregate. Mag x1000. (phase contrast).
To preserve the bacterial population within the vessel, after 16 days of culture the pumps were turned off whilst cycloheximide was added to the vessel to give a final concentration of 100 mg/mL. This concentration of the eukaryote inhibitor has been reported to kill off diverse protozoal communities within 48 h (Tremaine and Mills 1987). A rise in OUR was seen the day after cycloheximide addition and microscopy showed that although rotifers, ciliates and amoebae remained their movement had seemed to slow down. No active flagellated protists were seen and the colour of culture samples had changed from pink to white. Additionally, bacteria both on the surface of cellulose particles and in solution had seemed to increase in number. However, OUR was not maintained after the next Sigmacell addition (~24 000 min) and ciliated protists as well as some rotifers were still seen in abundance and actively feeding. Therefore, another 7.5 mL 1% (w/v) cycloheximide was added to the vessel and then the pumps were turned back on 24 h later. Regardless of this second addition, two types of ciliated protists survived and continued to be seen throughout the rest of the run. However there were also always bacterial rods and spiral shaped cells present, if only in small numbers.

Two samples from the culture were plated out in attempts to isolate cellulolytic bacteria, one taken before cycloheximide was added and the other taken at the very end of the culture period. Protozoa were found to survive on the plates (461S + 0.1% (w/v) Sigmacell/0.1% (w/v) cellobiose/filter paper) in either case (clearly observed at 50x magnification). Despite the presence of protozoa on the isolation plates, bacterial colony types were also seen on the 461S + 0.1% (w/v) cellobiose plates, but once again not all of these could be recovered on secondary plates, indicating the symbiotic relationships involved in the consortium cultured in the Evolver™.
The sample taken before cycloheximide addition, when plated onto filter paper, displayed a large area of growth on FP as a sole carbon source after 5 days incubation (Figure 3.8). Hydrolysis of the filter paper was clearly seen after 19 days incubation at 30°C. Therefore some of the species present, perhaps a few working in concert, could degrade this recalcitrant cellulosic substrate. On duplicate plates the advancing edge of hydrolysis was either bright yellow or pink. The final sample from the Evolver™, plated out onto filter paper also had pink and yellow areas of growth around the site of hydrolysis, indicating that the cycloheximide addition to the culture did not affect the species that produced these pigments. Material lifted from the bright yellow growth was seen to contain lots of protozoa as well as cysts but also many types of bacteria, rods, vibrios, coccis, spiral-shaped cells and endospores. Small, slender rods, ~0.5 x 3 µm were the predominating bacterial type.

Figure 3.8. Microbial consortium from Evolver™ vessel growing on filter paper. After 5 days incubation the discolouration of the paper due to microbial growth extends to the dashed line.
Only two bacterial types, named EV1 (no pigment), EV3 (pink colonies) grew on the secondary plates (461S + 0.1% (w/v) cellobiose) from the first isolation (before cycloheximide addition). With both EV1 and EV3 no growth occurred in 10 mL cultures of 461S + 0.1%(w/v) cellobiose even though the same inoculum grew in NB. In NB, OD no greater than 0.6 was found after two days incubation and this was found to be highly variable. This was similar to the difficulty in growing BC03 from the first Evolver™ run in liquid culture. That the bacteria may have been adapted to a biofilm mode of growth was not surprising because three weeks after the start of the run, irregular readings from the DO probe were found to be due to fouling caused by a thick, pink biofilm. When growth on the filter paper was lifted from the edge of the hydrolysed zone and spread plated on 461S + 0.1% (w/v) SigmaCell, a small orange colony was found to form (after 5 days incubation at 30°C) at the bottom of the agar, indicating that growth for this organism was preferably in a microaerophilic environment (the organism responsible for this growth could not be cultured further).

EV1 and EV3 were assayed in the CMC overlay assay, EV1 colonies produced clearance halos indicating the presence of ß-1,4-endoglucanase activity. 16s rDNA sequencing confirmed that EV1 was a member of Acidovorax genus (99% and 97% similarity to A. delafieldii, A. temperans, respectively over 854 bases) (see Appendix 1).

Bacterial isolates that could be recovered as pure cultures from the second isolation (after cycloheximide addition) were named EV7, 8, 9. These isolates were tested for endoglucanase activity with the CMC overlay method, EV7 & 8 gave faint halos but EV9 had no detectable activity. EV7 and 8 also had difficulty growing in liquid minimal media, and growth in NB media was clumped. Although EV7 & 8 initially had slightly different colony morphology, they were both gram negative coccobacilli, no bigger than 1 x 2 µm in size. 16s rDNA sequencing found both isolates to belong to either Sphingomonas or Flavobacterium genus, and the very similar sequences implied that they were likely to be the same species (see Appendix 1).
3.4 Discussion

The method of culturing directly onto filter paper has been used to isolate members of the *Cytophaga* group, where it was found to be important to subculture from the growing edge of hydrolysis in attempts to avoid non-cellulolytic organisms that may also be present in the culture (Reichenbach 1999). The impure YS1 consortium growing on filter paper was found to resemble the Evolver™ cultures when grown in liquid culture, in that the predominant bacteria were small, gram negative, rods. YS3/5, purified from this consortium, were some of these gram negative rods and were found to produce β-1,4-glucanase activity, therefore the predominant species within the consortium was thought to at least contribute to the hydrolysis of the filter paper. Once again though, no purified isolates were found to grow on crystalline cellulose or CMC as a sole carbon source. Even when a vitamin solution was supplied to the basal media, YS3/5 could not grow on filter paper, suggesting that their lack of a complete cellulase system rather than a missing growth requirement was responsible for this inability, although it is possible that a nutrient that was not examined was required. More likely though, was that the main organism responsible for the hydrolysis of the filter paper was not isolated in pure culture. The bacterial isolates that had been found to possess β-1,4-glucanase activity (YS3/5, CH3) were only seen to have low levels of endoglucanase detected by the sensitive CMC overlay assay, this alone would not be sufficient for the extensive hydrolysis of filter paper that was seen. Hydrolysis zones occurred on the filter paper without obvious signs of bacterial growth, the organisms responsible could have been protozoa, or bacteria that occurred as a thin, transparent layer, as had been seen in other isolations. Either of these would have been difficult to culture and may not have survived without the presence of other microorganisms, therefore preventing the study of these microorganisms in pure culture.

Solid cellulose could not be delivered to the Evolver™ culture vessel in a constant flow of low concentration, which meant that a limiting amount of external carbon applied to the system could not be maintained. Solid cellulose was reported to be delivered to a chemostat in a continuous method by Meyer and Humphrey (1982), unfortunately the necessary equipment to duplicate this method was not available for this experiment. The first Evolver™ run used the
segmented air/liquid delivery system, with different amounts of cellulose being added to the vessel at different instances. Microscopy revealed that growth in the Evolver™ vessel was occurring, but due to the problems with the feed delivery system, DO% was not monitored carefully. DO% during an Evolver™ run is monitored to see if there are any changes in Evolver™ output that might indicate a change in the microbial population, but in this instance changes in DO% could also be due to changing levels of carbon input to the system. Therefore, because changes in the concentration of cellulose could not be monitored, DO% was not closely monitored in this case, so it was not noticed at first that the vessel was not being aerated due to a decrease in DO%. The feed delivery system also delivered small amounts of air, which were assumed to be negligible in terms of the dissolved oxygen levels in the headspace of the vessel but may have in fact allowed the environment within the vessel to stay aerobic. An increase in OD was seen after the delivery system was altered to add more air, and most probably resulted from the increase in aerobic species.

Within the first few days of culture in the first Evolver™ run, Sigmacell particles that had been added to the vessel were seen to be colonized by bacteria. Although bacteria were often seen to bind to the ends of the particles, which may have more cellulose chain ends amenable to degradation, this phenomenon did not confirm that the bacteria were cellulolytic. Another scenario may be that the rough ends of the cellulose particles are more amenable to colonisation due to the nature of the surface, in comparison to the smooth flanking sides of the particles. Such colonization could have involved non-cellulolytic species that were surviving by using soluble sugars produced by enzymes secreted by other species. Colonization of the rough areas of cellulose particles was seen to occur first with cultures of the cellulolytic \textit{Cellulomonas flavigena} (Kenyon \textit{et al.} 2005), although it could not be determined why. Long, bacterial filaments resembling actinomycete species were seen to be attached to cellulose particles in the Evolver™ vessel. Actinomycetes as a group are known to contain some of the most efficient bacterial cellulase enzymes, including complete enzyme systems to degrade native lignocellulosic materials (Ball \textit{et al.} 1990; Quillet \textit{et al.} 1995; Tuncer and Ball 2002).
The fact that not many planktonic bacteria were seen at the start of the run may have impacted the subsequent diversity of the culture. Because the vessel contents are stirred and have a constant flow of material out of the overflow outlet, solid particles (including colonized cellulose particles) are routinely lost from the vessel. Therefore, a bacterial species that occurs mostly as part of a biofilm community would have trouble multiplying within the culture, as planktonic cells are needed to retain this strain in the vessel. However, it was possible that biofilm growth occurred on the walls of the vessel itself.

The planktonic bacterial population would also have been affected by predatory protists that were present, such as *Vorticella* and other ciliate species. Some ciliated protozoans are known to use bacteria as their main source of nitrogen (Bonhomme 1990). Such predation may not change the bacterial community composition, instead just diminishing the planktonic population in size (Massana and Jurgens 2003). This was noted after 5 days of culture, when a rise in OD coincided with observations of more ciliated protists and less planktonic bacteria. Members of the *Cytophaga-Flavobacterium* group, which include cellulolytic species, have been found to be resistant to protistan grazing (Simek et al. 2001). It is thought that protists can selectively graze on bacteria depending on the size, growth condition, species and motility of the prey (Kinner et al. 1997), although this is debated and protistan grazing on bacteria may be largely indiscriminate (Fuhrman and Noble 1999). Therefore, it was possible that the diversity of cellulolytic bacteria was decreased by protistan grazing, especially as the planktonic population was already limited due to binding of the bacteria to cellulose particles.

When Evolver™ samples were plated out on Sigmacell as a sole carbon source, single colonies resulted but these were found to be impure, indicating that a close relationship between different bacteria may have been responsible for utilization of this recalcitrant carbon source. In fact, even on secondary plates in a rich medium (SDA), two bacterial species were not growing separately but rather the pink colonies were always seen associated underneath another type of bacteria. None of the bacteria purified as single species could survive on cellulose as a sole carbon source. These bacteria may have been cellulolytic but requiring other
nutrients (such as vitamins) for growth that were not supplied by the 461S minimal media. The supply of additional growth factors in the Evolver™ could have been provided by other microorganisms. Addition of yeast extract to cultures of the cellulolytic *Cellulomonas uda* has been seen to increase growth as well as cellulase activity (Stoppok *et al.* 1982). Yeast extract was not included in the 461S media however, as this would result in the isolation of many types of bacteria rather than just cellulolytic ones. The bacterial species isolated on the rich TSA medium were such non-cellulolytic organisms, and were presumed to have survived in the Evolver™ vessel by cross-feeding of products of hydrolysis (such as glucose) from other cellulolytic organisms. Therefore, as expected, complex synergistic relationships between species were occurring in the culture. This was further evidenced when some types of bacteria grown in the culture vessel could not be isolated in pure culture, even by growing on the complex media. Multiple types of bacterial growth were often seen on the initial isolation plates (from the evolver), but refused to grow in pure culture when streak- plated from a single colony.

The fact that the colonies appeared underneath others implied that they favoured a microaerophilic environment, therefore, in the Evolver™ vessel they may have survived inside protected microcolonies or at the bottom of biofilm growth. A similar situation was when bacteria isolated from growth on filter paper (from an Evolver™ sample in the third run) grew only at the bottom of the agar medium.

Biofilm growth had been noticed in the culture vessel. This form of growth may have facilitated horizontal transfer of cellulase genes, as the close contact of different species within such communities can lead to increased rates of plasmid exchange (Hinnebusch *et al.* 2002). Evidence of cellulolytic bacteria growing in biofilms was seen when a *Thermomonospora* strain produced heavy wall growth in a chemostat, despite the culture vessel being lined with a silicone coating (Meyer and Humphrey 1982).
Evidence that cellulolytic capabilities had been isolated in the Evolver™ was obtained when clearing zones were seen on 461S + 0.1% (w/v) Sigmacell isolation plates. These colonies as well as the purified BC01 isolate also displayed agarolytic capability, as depressions in the agar surface were seen underneath colonies. Cellulases combined with agarases would be useful in degrading algal cell walls and would therefore be expected to be found in marine species but both capabilities have also been found in a member of Cytophaga that was isolated from soil (Li et al. 2003).

Cellulolytic activities were also seen when clearing zones were formed on pink cellulose plates. The dye on the cellulose surface in this case may have hindered degradation somewhat because it took longer (up to two months) to see clearance of the substrate, therefore the pink cellulose did not show any advantage over unmodified cellulose particles in terms of detecting cellulolytic capability. BC03 (impure) and BC01 were found to grow on the pink cellulose plates but the clearances did not always correlate with visible colonies, instead thin, spreading, transparent growth occurred. This growth was most likely responsible for hydrolysis of the pink cellulose, as no colonies were seen above the clearances. It could not be determined if this type of growth was due to the isolates themselves or another organism that was not forming distinct colonies itself. Evidence that the bacterium responsible for the thin, spreading growth produced cellulases was also seen when this type of growth was observed on plates with CMC as the sole carbon source.

BC03 that was responsible for clearances on the Sigmacell plates was seen to contain the bacterial filaments that had been found to bind to cellulose particles in the Evolver™ vessel. This suggested that these filaments may have possessed cellulase enzymes but when the BC03 isolate was grown in liquid culture no cellulase activity was detected in the culture supernatant with the reducing sugar assay. It is possible that a low level of activity was present but not detected with the somewhat short incubation period in the assay of 2 h. Only very low levels of protein (~6 µg/mL) were found in culture supernatants when BC03 had been grown for 24 h, so if these samples contained any cellulases, they would have had to be of very high specific activity to be detected. Although bacterial cellulases are usually secreted from the cell, the cells
themselves in BC03 cultures were also tested for cellulase activity but none was found. Additionally, no significant growth was seen when filter paper was added to BC03 cultures that had been grown to high density, as OD levels were comparable in flasks with or without filter paper. The bacterial cells seemed to be surviving in the filter paper flasks, as compared to dead cells (stained with Propidium iodide) in the control flasks but this was a somewhat subjective observation in that it was not quantified with the number of dead cells compared to living cells or compared between flasks. Also, just because the cells had not died did not mean that they were using the filter paper as a carbon source, bacteria are known to exist in survival states for long periods of time when suitable sources of nutrients are scarce (Morita 1988).

Once the gram negative *Pigmentiphaga* sp. (BC03.1) had been purified away from the *Bacillus* also occurring in BC03, it was seen to form fruiting body-like structures, which is a phenomena that occurs in *Myxobacterial* species when nutrients are limited (Shimkets *et al.* 2004). Therefore, although BC03.1 displayed some growth on CMC and pink cellulose it struggled to continue growing probably because of a lack of true cellulolytic capability (seen when the substrate can be extensively degraded). When BC03.1 was associated with *Bacillus* growth (BC03.2) on pink cellulose plates, less fruiting body structures were seen, providing more evidence that the two isolates co-operated during growth on this carbon source. BC03.2 was not able to grow on pink cellulose plates once purified away from BC03.1. BC03.1 also had difficulty growing in liquid culture, pelleted growth was seen rather than a turbid culture, which may have implied that this strain was adapted to biofilm growth rather than a planktonic mode of growth. BC03.1 may have been the organism causing the pink colonies seen underneath other colonies on the original isolation plates. 16s rDNA from BC03.1 was found to have 97% similarity with that from *Pigmentiphaga kullae*, which was a bacterial species that was first described in 2001 as a type species of a new genus (Blümel *et al.* 2001). A similarity of 97% confirms that BC03.1 is also a member of this genus but higher similarity is needed to confirm that it is the species *Pigmentiphaga kullae*, so it is possible that it is a new, undescribed species within this new genus. *Pigmentiphaga kullae* has been noted for it's ability to degrade certain dyes (Blümel *et al.* 2001),
therefore it is possible that isolate BC03.1 helped remove the Procion red dye from the Sigmacell particles that otherwise prevented the *Bacillus* (BC03.2) cellulases from attacking the cellulose.

In the second Evolver™ run, DO% was monitored closely but it was soon determined that not only did DO% change significantly depending on temperature of the probe, but the set-up did not facilitate indirect measurement of the dissolved oxygen in the liquid culture by measuring DO% in the headspace gases. Therefore, because the Evolver™ was programmed to aerate the vessel only after a significant drop in DO%, the vessel was not aerated for the first two weeks of culture. The environment within the culture may have become oxygen limited, which could explain the low level of growth seen. Large rotifers were seen in the culture, therefore predation may have once again inhibited the growth of planktonic bacteria, resulting in very low OD of the culture samples. Fresh air was added to the culture after two weeks, but diversity was likely to have been lost by this stage via flow through the vessel. In case there were still some aerobic, cellulolytic bacteria surviving on the CMC feed, the culture was continued for a further week but no increased growth could be detected. The final sample from this run was plated onto TSA media, a complex media that should support growth of most bacteria, but only 4 different types of growth were seen on the original isolation plates, indicating the low level of diversity present. No bacterial isolate from any of the experiments could utilise CMC as a sole carbon source, therefore it is not surprising in hindsight that no growth was seen in the Evolver™ with this carbon source.

The bacteria that were isolated from the second Evolver™ run, BC07 & 8, produced raised, aerial aggregates after they had been stored at 4°C. Such aggregates resembled the fruiting bodies of *Myxococcus* species, some of which have cellulase activity (Shimkets et al. 2004) but BC07/8 did not belong to this genus, neither could they grow on cellobiose as a sole carbon source. The formation of aggregates may have shown that they were adapted to a biofilm mode of growth, which would account for their being able to survive in the Evolver™, whilst not utilizing the CMC carbon source. A similar scenario most likely occurred in the other Evolver™ runs, explaining the isolation of non-cellulolytic bacteria each time.
The third Evolver™ run used the standard format Evolver™ so that changes in oxygen uptake rate (OUR) could be followed. At the very start of the run, OUR was high, indicating the rich inoculum containing a high density of microorganisms. To prove that an increase in growth could be achieved by addition of cellulose, the Evolver™ was first left to incubate without flow through the vessel to allow depletion of the endogenous carbon sources in the inoculum. It has been reported that high levels of oxygen uptake occur during the early stages of starvation of bacterial cells, due to cells fragmenting into ultramicrocells (Morita 1988). Rises in OUR coincided exactly with the additions of cellulose; however, suggesting that utilization of this carbon source was responsible for the oxygen uptake. However, even though the same amount of Sigmacell was added (in the first few instances), the increase in OUR was not as large as each previous instance. Additionally, when 461S was first added, the peak from the Sigmacell addition at the same time was seen to decline sharply, unlike the gradual declines seen with the two earlier additions. Therefore the 461S media may not have been optimum to allow for growth of the cellulolytic organisms. This may have contributed to the lack of isolation cellulolytic bacteria in pure culture on this medium.

Additions of cellulose to the Evolver™ culture gave very sharp peaks, instantaneously followed by larger and more gradual peaks in OUR. This is most likely a result of the small amount of residual sugars in the Sigmacell, as they would be easily utilized by many species in the culture but would be quickly used up, whereas the crystalline cellulose itself is a more recalcitrant substrate but contains a large amount of carbon that can sustain growth for longer if it is being degraded successfully. The Sigmacell addition at ~12 000 min was 2 g instead of 1 g that had been added at earlier instances, this gave a larger rise in OUR as should be expected if the utilization of cellulose was responsible for the increased growth of microorganisms. The rise in OUR from 2 g Sigmacell was more than 2x larger than the increase due to 1 g (an increase of 4.4 mg/h compared to an increase of 12.1 mg/h). Daily additions of 2 g Sigmacell maintained the OUR at ~20 mg/h. This meant however, that there was a lot of residual cellulose within the culture; it was possible that only the most easily accessible portion of the cellulose was being utilized.
In case protozoal grazing was affecting the numbers of cellulolytic bacteria, cycloheximide was added to kill a large proportion of the protists in the culture. Some of the protozoans may have been utilizing the cellulose itself. Some ciliates are known to produce their own cellulase enzymes (Michalowski et al. 2001) so it is possible that some of the ciliates in this culture are responsible for the degradation of the Sigmacell particles. Although, the cellulolytic ciliates are found in the rumen and it has been suggested that they do not survive well outside of this environment (Bonhomme 1990). Additionally, the cellulolytic ciliates are known to ingest cellulose particles before degrading them (Bauchop and Clarke 1976) and this type of behaviour was not seen by the ciliates in the Evolver™ vessel. It would have been highly interesting to study this aspect further, however the culture and subsequent examination of protozoa was outside the scope of this project, and bacteria within the culture remained the focus.

The addition of cycloheximide to the culture did seem to alleviate pressure on the other microorganisms present, as a rise in OUR, indicating increased growth of at least some of the microorganisms present, was seen to occur only 2 h after this addition. However, the next addition of Sigmacell (~24 000 min) did not bring the OUR back up to the previous level of 20 mg/h, this may have been due to the loss of protozoa that were responsible for cellulose degradation or bacteria that had been lost from the vessel by overflow when no Sigmacell was added. All further additions of Sigmacell after this point failed to maintain the OUR at -20 mg/h. Some protistan grazing could still have been occurring until the end of the run, as at least two species of ciliate were seen to persist in the culture even after two additions of cycloheximide, this may have been due to the cysts that were seen, giving the protists the chance to emerge and multiply once the cycloheximide had been washed out from the vessel (gradually after the pumps were turned back on).

Throughout the run, no significant rise in OUR could be attributed to changes in the population, except for the ones induced after cycloheximide addition. As with the first Evolver™ run, the cellulose particles were seen to be heavily colonised with bacteria. In fact, unless Sigmacell had just been added to the vessel, most of the particles were seen to have attached bacteria, suggesting that adherence to the particles occurred quickly. After two
weeks culture however, more of the cellulose particles were seen to be un-colonized, this observation coincided with the observation of more ameboid protists and the fact that ciliated protists seemed to concentrate their grazing around bacterial aggregates. Therefore, the microcolonies that formed around cellulose particles may not have been resistant to protozoal grazing.

Samples from the Evolver™ culture quickly grew on filter paper as the sole carbon source and showed clear hydrolysis of this substrate after a few weeks incubation, therefore the consortium growing in the Evolver™ produced efficient cellulase enzymes although a complex relationship between the different species present was evident to achieve this level of hydrolysis. Evidence for such relationships was seen when bacterial types failed to grow on the secondary isolation plates and no purified bacterial isolate could grow on cellulose (either CMC or Sigmacell) as a sole carbon source. Two types of growth were seen on filter paper, possessing either yellow or pink pigment, it is possible that these pigments arose from EV8 and EV3, respectively, as they produced similar pigments in pure culture. Unfortunately, this does not suggest a possible role for these bacteria within the consortium but it does imply that they were abundant during growth on filter paper.

Difficulty in growing the bacterial isolates in liquid media once again highlighted that they might have been adapted to biofilm growth. A biofilm resembling the same growth on filter paper (pink) outside of the Evolver™, occurred in the vessel. The pink colour of the liquid culture before cycloheximide addition may have been due to an organism that, once cycloheximide was added to the vessel, adapted to a biofilm mode of growth. A possible reason for this conversion to a biofilm mode of growth may have been the rise in the general planktonic bacterial population. If the addition of cycloheximide significantly diminished protozoal grazing, it is possible that planktonic bacteria that were surviving off the products of cellulose hydrolysis increased in number, thereby increasing the demand for such carbon sources. With an increased bacterial planktonic population, sugars and easily catabolized carbon sources may have been quickly diminished, creating a nutrient limited environment. *Pseudomonas* cells in starvation state are thought to initiate biofilm formation more readily than cells in other
metabolic states (Ghannoum and O'Toole 2004), a similar situation with the bacteria in the Evolver™ was possible. Biofilms are known to possess microhabitats with different levels of oxygen available to the cells through diffusion, so it was not surprising that growth of a particular isolate Sigmacell plates occurred below the surface of the agar, thereby allowing a microaerophilic organism to grow, as it would at the centre of a biofilm.

The fact that very similar species (by 16s rDNA sequence) were seen to form slightly different colonies also indicated that species with differing modes of growth were present in the culture. An example of this was EV7/8 that differed in the production of pigment and shiny colonies, but had very similar 16s rDNA sequences. With the isolates not adapted to growth in liquid culture, the CMC overlay assay became the easiest method to test for the production of β-1,4-glucanase activity, although a limitation with this type of assay is that it only detects those enzymes with endo-cellulase activity, whereas both endocellulases and exocellulases are known to be needed for complete hydrolysis of cellulose fibres, as was seen in the hydrolysis of filter paper.

In summary, isolation of cellulolytic bacteria was attempted 4 times by two methods, each time a few isolates were found that may have contributed to the hydrolysis of the cellulose substrates, evidenced by either growth on cellulosic material as a sole carbon source (BC03, Pigmentiphaga and Bacillus in synergy) or endoglucanase production (EV1, EV7, YS3/5, CH3). In each case however, no single isolate was found to be responsible for hydrolysis of crystalline cellulose, therefore either the responsible organisms were not amenable to the culture methods employed or complex synergies between organisms resulted in the catabolism of this recalcitrant substrate. To avoid the difficulties of culturing recalcitrant microorganisms, a direct molecular approach would have been useful to study the diversity within the culture. Such an approach was used by Grant et al. 2004 to carry out a phylogenetic study of cellulolytic bacteria from environmental samples found in soda lakes, which are extremely saline and alkaline.
Two of the isolates were found to belong to the genus *Bacillus* and *Cellvibrio*, respectively, and may have been species known to produce endo-cellulases. EV1 (*Acidovorax*) and YS3 (*Aeromonas*) were also found to possess such activities in this study, however members of these genus' have not previously been described to produce endo-cellulases. Therefore these isolates are possibly new strains of such species or they may be evidence that horizontal gene transfer occurred between other cellulolytic organisms in the Evolver™ or during the filter paper isolation. Biofilm modes of growth occurred in either case, and rates of horizontal gene transfer between members of a biofilm have been reported to be orders of magnitude higher than between planktonic cells in suspension (Ghannoum and O’Toole 2004).
4. A further investigation of some CMCase-producing bacteria

4.1 Introduction

Three bacterial isolates, Acidovorax sp EV1, Aeromonas sp. YS3, Cellvibrio sp. CH3, possessing β-1,4-endoglucanase activity as detected by clearance of the model substrate CMC (CMCase) (see section 3.3), were chosen for further study. The aim of this chapter was to determine if any of the observed CMCase activity could be ascribed to novel cellulase enzymes. To the best of our knowledge, CMCase enzyme activity has not been reported in Acidovorax or Aeromonas genera previously. The novel appearance of endoglucanase activity in such genera could be due to three possible phenomena. An unknown strain bearing the CMCase enzyme activity may have been isolated. Alternatively a strain of a characterized species could have acquired the genes responsible for such activity through horizontal transfer from known cellulolytic species. A third possibility is that a known species may have possessed the gene for the CMCase enzyme, but pure culture in the laboratory did not allow for expression of the enzymes. It can often be difficult to simulate the precise environmental conditions needed to observe wild-type phenotypes of bacteria in the laboratory, especially when the phenotypic characters are due to interactions of the isolate with other microorganisms. Therefore, in this chapter, close attention is paid to the phenotype of the Aeromonas strain investigated in order to highlight the origins of the CMCase enzyme. In particular, the biofilm mode of growth of the bacteria, which is thought to be the predominant mode of growth of bacteria as environmental isolates (rather than laboratory adapted strains) was examined, so as to mimic the specific phenotype within which the CMCase enzyme was expressed.

The Acidovorax, Aeromonas and Cellvibrio isolates had been preliminarily identified to genus level by sequencing of their 16s rDNA genes. The identification of the isolates was confirmed and refined, using standard biochemical tests, to identify them to species level. This information
was needed in order to determine whether the isolates were species already known to express CMCase enzymes, or if a novel phenotype of CMCase expression was observed.

*Aeromonas* sp. YS3 was chosen as the focus of this study, as it had the novel phenotype of CMCase expression and consistently produced CMCase as an extracellular enzyme. *Acidovorax* sp. EV1 also bore the novel phenotype but showed variable expression of the CMCase enzyme under similar growth conditions. The expression of *Aeromonas* sp. YS3 extracellular CMCase was monitored and optimised. Additionally, because this may be an as yet uncharacterized strain of *A. caviae*, various phenotypic characters, that were co-expressed with endoglucanase activity were examined. Such an investigation was thought to provide clues as to the role of the endoglucanase produced by this bacterium.

Many bacterial species have been found to produce one or a few β-1,4-endoglucanase enzymes, without degrading native cellulosic substrates in pure culture, therefore the function of these enzymes does not seem to be the utilization of cellulose as a carbon source. *Prevotella* spp. are an example of this, as they posses xylanase and CMCase enzymes, and are thought to have synergistic interactions with cellulolytic bacteria in the rumen, during hemicellulose and pectin degradation (Matsui *et al.* 2000).

β-1,4-endoglucanase activity has been implicated in cellulose production by some Gram negative bacteria, and in these cases a glycoside hydrolase family 8 endoglucanase is co-transcribed with other sub-units, in a cellulose synthase operon (Römling 2002). Therefore the presence of CMCase activity does not imply that the bacterium utilizes cellulose as an energy source. Rather, such enzymes are expressed in Gram negative bacteria with an undefined function. These CMCase enzymes may represent a separate evolutionary lineage of cellulases as compared to the complete systems of Gram positive bacteria such as *Clostridium*, which can extensively degrade native cellulosic substrates (Johnson *et al.* 1982). Therefore the investigation of CMCase in *Aeromonas* sp. YS3, presented in this chapter, provides additional information regarding the diversity of bacterial endoglucanases.
4.2 Materials and methods

4.2.1 Biochemical characterization to identify bacteria to the species level

Bacterial isolates EV1, CH3 and YS3 were grown on tryptone soya agar (TSA, Oxoid) slants prior to testing for various biochemical abilities, as they had previously been grown on minimal media and may not be expressing a wide variety of phenotypic traits. The results of the biochemical tests were used to differentiate which species the isolates were. The freshly grown TSA cultures were used to test the isolates for oxidase activity. A sterile toothpick was used to lift some of the culture, which was then placed onto filter paper impregnated with 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride. A positive result was indicated by the filter paper turning purple in colour within 10 s after the culture had been placed on it.

Growth on TSA was used to determine the production of brown soluble pigment by YS3 as well as growth at 4°C/mucoid growth at 30°C of CH3. After overnight growth (16 h) for YS3 and growth after 43 h for CH3 on the TSA slants, these isolates were inoculated onto the TSA plates with a heavy inoculum from the slant cultures. The TSA plates inoculated with CH3 and incubated at 4°C were monitored over a few weeks, however the other TSA plates only needed to be incubated at 30°C for a few days before brown pigment or mucoid growth could be observed.

To test for lysine decarboxylase, ornithine decarboxylase, starch hydrolysis and growth on arabinose, the YS3 TSA slant culture was used to inoculate an iron Kligler agar slant, starch plates (triplicate) and arabinose sodium phosphate slants (triplicate), respectively, all were incubated at 37°C. Growth on arabinose sodium phosphate slants was checked with up to 5 days incubation. After 4 days incubation on the starch plates (39 g Columbia agar (Oxoid) in 800 mL distilled water + 200 mL 5% (w/v) (w/v) soluble starch), after a good level of growth had been achieved, the plates were flooded with an iodine solution (1% (w/v) I$_2$, 2% (w/v) KI), with clear zones around the colonies indicating the action of amylase activity. Overnight growth on the Kliglers iron agar was used to inoculate decarboxylase tests (media components per L distilled water: 5 g peptone, 5 g lab. Lemco powder, 0.5 g glucose, 5 mg pyridoxal, 5 mL.
0.2% (w/v) Bromocresol purple, 2.5 mL 0.2% (w/v) Cresol red) containing 1% (w/v) L-lysine dihydrochloride, L-ornithine dihydrochloride (both in triplicate) or no amino acids (duplicate) as negative controls. All bottles were overlaid with 1mL paraffin oil to maintain anaerobic conditions and incubated at 37°C. The medium first turns yellow upon glucose fermentation and then turns violet if decarboxylation of the amino acids has resulted in alkalinization of the medium, the control tubes with no amino acid added should remain yellow. The decarboxylase tests were monitored for 4 days.

YS3 was tested for the production of neutral end products from glucose in the Voges-Proskauer test. Glucose peptone water (per L distilled water: 7 g peptone, 5 g D-glucose, 5 g K$_2$HPO$_4$) tubes, in triplicate, were incubated with YS3 from the TSA slant culture or the control organism Enterobacter aerogenes and E. coli from LB plates. All tubes, including an uninoculated control were incubated at 37°C and tested after exactly 48h incubation. 1 mL of the culture was removed and tested by adding 0.5 mL α-naphthol (5% (w/v) in ethanol) and 0.5 mL 40% (w/v) KOH containing 0.3% (w/v) creatine. The tests were shaken thoroughly and then left to stand for 5-30 min before a colour change to crimson indicated a positive result (Seeley and Vandemark 1962).

To test for gas production from glucose, YS3 was inoculated into peptone water (per L distilled water: 10 g bacteriological peptone (Oxoid), 5 g NaCl, 1 mL 2% (w/v) (in ethanol) Bromocresol purple) with 1% (w/v) glucose in Bijoux bottles with an inverted glass Durham tube to check for gas production. A colour change of the media to yellow indicated growth and acid production from glucose had occurred. Accumulation of gas in the Durham tube was monitored with up to 5 days incubation at 30°C.

Another triplicate set of peptone water bottles, without glucose, were inoculated and incubated at 30°C to test for indole production from tryptophan by YS3. After 24 h incubation these bottles were tested for indole by adding a few drops of Kovacs reagent (per 100 mL: 75 mL amyl alcohol, 25 mL 18 M HCl, 5 g p-dimethylamino-benzaldehyde), this forms a surface layer
on the culture and a colour change from pink to red in this layer confirms the presence of indole.

Esculin hydrolysis by YS3 was tested by inoculating triplicate esculin broth (per L distilled water: 25 g nutrient broth, 3 g yeast extract (Oxoid), 1 g esculin, 0.5 g ferric citrate scales) bottles and then incubated (along with an un-inoculated control) at 30°C. A colour change from yellowish brown to brownish black indicated positive for hydrolysis. YS3 was tested for utilization of L-lactate as per (Popoff and Lallier 1984) by inoculating cultures of M-70 broth + 0.25% (w/v) L-lactate, in triplicate plus un-inoculated negative controls, incubated at 30°C. EV1 was similarly tested for utilization of D-ribose. Any growth as compared to the un-inoculated controls was deemed a positive result.

CH3 was tested for nitrate reduction by inoculating 3 Bijou bottles containing 3.5 mL nitrate broth (per L distilled water: 25 g nutrient broth (Oxoid), 1 g Potassium nitrate (nitrite free)) with CH3 from the TSA slant culture, then incubating at 30°C. The nitrate broth bottles were tested for the presence of nitrite after 1, 2 and 3 days incubation with the spot test method. This involved mixing one drop of solution A (0.8 g sulphanilic acid in 100 mL 5 N acetic acid) with one drop of solution B (0.5 g α-naphthylamine in 100 mL 5 N acetic acid) and then adding a large drop of the culture to be tested. Sterile nitrate broth is tested simultaneously as a control. A red colour in the spot test confirms reduction of nitrate to nitrite has occurred, whereas the un-inoculated control should remain orange in colour.

YS3 was further identified using the API-20E test strips (bioMerieux). YS3 and an *A. hydrophila* culture were streak plated onto nutrient agar (Oxoid) plates and grown overnight at 37°C before a single colony of each was used to inoculate the API strips, as per the manufacturer’s instructions. The API tests were incubated at 35°C for 24 h before being tested as per the manufacturer’s instructions.
4.2.2 Enzyme assays

The CMC well-plate assay was used to test culture supernatants, performed as per section 2.2.8 but with varying depths of agar plates, so that up to 150 µL sample could be tested in each well. It was noticed that in shallow plates, applying a large sample volume for example 100 µL, caused the sample to overflow the well and resulted in better detection of enzyme activity. Therefore, applying the enzyme samples (both the culture supernatant samples and the commercially prepared *T. viride* positive control samples) onto the surface of the plate, instead of in wells, facilitated detection. As long as care was taken not to let drops of sample fuse on the plate before they diffused into the agar, the samples could quickly and easily be applied to the plate before incubation and subsequent staining with Congo red. To increase the sensitivity of the method, two drops of sample could be placed on the same spot on the plate, i.e. one drop of sample (30-50 µL) is pipetted onto the surface of the plate, and left to diffuse into the agar for 10 min and then another drop is placed on top. This resulted in more distinct clearances than if a single drop of larger diameter had been allowed to diffuse into the agar.

Although the CMC plate assay is not strictly quantitative, some comparison could be made between samples depending on what diameter clearance halo was achieved with the same amount of samples applied. The strength of clearance could also be estimated from the formation of either a diffuse or distinct edge to the clearance halo. *T. viride* cellulase (Sigma) was used as a positive control, this is a crude cellulase preparation and therefore contains various cellulase enzymes. The positive control was diluted to different concentrations and in different buffers to test the validity of the assay. The *T. viride* cellulase was found to give a linear response (halo diameter (mm) = 0.0386.concentration of enzyme µg/mL + 12.63; $R^2 = 0.9196$) between 25-200 µg/mL enzyme.

Methylumbelliferyl-cellobioside (MUC, Sigma) was used as a substrate to test for exoglucanase activity. The distinction between endocellulases and exocellulases is unclear, some enzymes that are designated endocellulases also have activity on this substrate, and it may indicate their processive action on native cellulose substrates. Detection of the methylumbelliferone
fluorophore (MU) is sensitive, therefore this assay is expected to quantify small amounts of exo-cellulase activity. The MUC assays were carried out in black walled, 96-well plates (Greiner), 95 µL of an appropriate buffer was added to each well, then 5 µL of 500 µg/mL MUC and 50 µL sample was added. Plates were incubated at 40-50°C before 10 µL 1 M NaOH was added to stop the reaction and bring the samples to pH 10, where fluorescence of MU is optimal. The plates were then read in the FLUORstar fluorescent plate reader with excitation, emission wavelengths of 355, 460nm respectively. Background controls were necessary for both the MUC substrate and the samples themselves, as these could both contribute to the level of fluorescence. These were carried out with water substituted for sample or MUC respectively and the values obtained from these controls were subtracted from the values obtained when the sample was tested with MUC. A dilution series of the fluorophore alone (MU) (in 25 mM NaHPO₄ pH 7.2) determined the saturation point of the assay. A linear response from the T. viride cellulase positive control was also confirmed by diluting this enzyme in the same buffer before testing it in the assay.

The DNS assay was used to measure the release of reducing sugars as in section 2.2.8 but was scaled down so that small samples could be preserved. Sample and substrate (typically 2% (w/v) CMC) solutions were combined, usually in equal volumes to give a total volume of 0.5 mL. Incubation was carried out at varying temperatures for many hours before 1 mL DNS reagent was added and the tubes were heated to 95°C for 15 min. After the tubes were cooled, aliquots of 125 µL were diluted in 125 µL distilled water in a 96-well plate and read at 550 nm.

4.2.3 SDS-PAGE, including zymogram (activity-stained gels) methods

Prior to attempts of visualising the CMC activity produced by YS3, on acrylamide gels, further evidence that this activity was due to a protein was obtained. A sample of YS3 culture supernatant was heated to 95°C for 15 min and then tested in the CMC plate assay, alongside unheated sample to see if activity was abolished this way. Additionally, various culture supernatant samples were treated with proteinase K to see what effect this would have on the CMCase activity. A stock solution of Proteinase K contained 20 mg/mL proteinase K in 21 mM
Tris.Cl pH 8.0, 0.07% (w/v) CaCl₂, 50% (v/v) glycerol. 25 µL of the proteinase K solution was added to 425 µL sample as well as 50 µL 10% (w/v) SDS, 500 µg/mL *T. viride* cellulase was used as a positive control for cellulase activity. All tubes, including the untreated samples were incubated at 37°C for 4 h and then tested in the CMC plate assay.

To analyse protein composition of culture supernatant samples, sample were run on 8/10% (w/v) polyacrylamide gels. The gels were poured in Bio-Rad spacer plates with a gap of 0.75 mm, and run in the protean3 electrophoresis tank system. The separating gel contained, in a total volume of 10 mL, 3/2.7 mL 30% (w/v) acrylamide mix, 2.5 mL 1.5 M Tris pH 8.8, 100 µL 10% (w/v) SDS, 100 µL 10% (w/v) ammonium persulphate (APS) and 10 µL TEMED. When CMC zymograms were employed, the remaining volume of the separating gel was composed of CMC solution, typically 1-2% (w/v), making the gel 0.23-0.45% with CMC. A stacking gel was poured on top of the separating gel containing (in a total volume of 5 mL): 630 µL 30% (w/v) acrylamide mix, 630 µL 1M Tris pH 6.8, 50 µL 10% (w/v) SDS, 50 µL 10% APS and 5 µL TEMED, to give a 3.8% (w/v) acrylamide stacking gel. Samples were prepared for electrophoresis by adding the correct amount of 5x SDS-PAGE sample buffer (60mM Tris pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) ß-mercaptoethanol, 0.1% (w/v) bromophenol blue) and heated to 95°C for 10 min before being applied to the gel. Electrophoresis was typically carried out at 120V for 2 h or until the dye front reached the bottom of the gel, with SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) filling the tank.

Gels were either stained with Coomassie or silver stained. For Coomassie staining, the gels were gently shaken in Coomassie stain solution (0.1% (w/v) Coomassie R250, 50% (v/v) methanol, 10% (v/v) acetic acid) and then destained In 10% (v/v) ethanol, 10% (v/v) acetic acid, changing the destain solution a few times, for up to 16 h. Silver staining was carried out with a method modified from Blum et al. (1987). The gel was fixed by staining in 50% (v/v) methanol, 10% (v/v) acetic acid, 0.05% (v/v) formaldehyde for 1 h, then washed in 3x 15 min 50% (v/v) ethanol, then pre-treated for 1min in 0.02% (w/v) Na₂S₂O₅·5H₂O, then rinsed 3x for 20 s in distilled water. Then the gel was soaked in 0.2% (w/v) AgNO₃ for 25 min and rinsed in water for 3x 20 s. 250mL developing solution contained 15 g Na₂CO₃ and 1 mg Na₂S₂O₅·5H₂O, 66 µL.
38% (w/v) formaldehyde was added to 50 mL of this prior to the gel being soaked in the developing solution, until distinct bands were seen (about 40 min). The gel was rinsed in water for 2x 2 min and then colour development was stopped with 50% (v/v) methanol, 12% (v/v) acetic acid. The gel was rinsed in 50% (v/v) methanol for 20 min before pictures were taken of the stained gel.

Polyacrylamide gels containing CMC were stained with Congo red to detect activity bands, but first SDS had to be removed from the gel and renaturation of proteins was attempted. SDS was removed by shaking the gel in 25 mM NaHPO₄ pH 7.2, 40% (v/v) isopropanol for up to 1 h, either at room temperature or at 4°C. The gel was then washed in 50 mM Tris pH 8.0 before renaturation was attempted by leaving the gel in 25 mM NaHPO₄ pH 7.2, 5 mM β-mercaptoethanol, 1 mM EDTA for up to 16 h at 4°C. To enable the enzymes to act upon the CMC substrate, the gels were incubated at temperature in the appropriate buffer for many hours. The gels were then stained as per the CMC plates, in 1 mg/mL Congo red for 15 min, then destained in 1 M NaCl for 15 min, sometimes a second destain step was employed to facilitate detection of faint activity bands.

A variant on the zymogram method involved removing SDS by shaking in 50 mM NaHPO₄ pH 7.2, 40% (v/v) isopropanol for 2x 15 min and washing in 50 mM NaHPO₄ pH 7.2 for 2x 10 min before incubating in phosphate buffer at temperature without a renaturation step.

In attempts to more clearly see activity bands or protein composition some samples were concentrated by ultrafiltration. Large volume samples were concentrated with vivaspin-20 ultrafiltration tubes (Sartorius) with centrifugation at 5000x g for 20 min at 4°C. Smaller volume samples were concentrated in vivaspin-500 tubes (Sartorius), centrifuged at 5000x g for 10 min. The presence of enzyme in the flow-through fractions was tested in the CMC plate assay and no leakage of the enzymes was seen to occur through the 10 kDa MWCO membranes. In case the polyethersulphone membranes employed in the vivaspin tubes were binding some of the proteins in the samples, concentration was also tried with centriplus tubes (Millipore, 10kDa MWCO), which were centrifuged at 3000x g for 90 min at 20°C. Retentates were
collected from these tubes by spinning upside down for 3 min. Smaller samples were similarly concentrated in centricon tubes (Millipore, 10 kDa MWCO, maximum volume 2 mL), centrifuged at 5000x g for up to 90 min at 4-15°C, then upside down for 3 min at 1000x g to collect the retentates.

To better estimate the molecular weight of activity bands, duplicate samples were run on opposite sides of a single gel. After electrophoresis the gel was cut in half so that one half could be Coomassie stained, whilst the other half with the same samples could be used in the zymogram procedure. In this way, mobilities of the activity bands could be compared to their Coomassie stained counterparts and consequently to a protein molecular marker (Mark12 ladder from Invitrogen).

4.2.4 YS3 growth experiments

YS3 was grown in liquid cultures with CY broth (0.3% (w/v) tryptone, 0.136% (w/v) CaCl\(_2\) \(\cdot\) 2H\(_2\)O, 0.1% (w/v) yeast extract, pH 7.2), LB broth (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract) or ST6 media (see section 2.2.1 above) with varying amounts of glucose, cellobiose and cellulose added to try and improve endoglucanase production. 1 mL samples were taken from such cultures at various time points to monitor the CMC activity in the culture supernatant. Culture supernatant was obtained by centrifuging the samples at 14000 rpm for 1 min, which was tested for CMCase by the CMC plate method. Therefore, the appearance of CMCase in the culture supernatants could be monitored and correlated with growth phase, as OD\(_{600}\) measurements of the culture were also taken. Culture supernatant from the closely related *Aeromonas hydrophila* was also obtained under similar growth conditions, to act as a negative control for endoglucanase production, as no CMCase activity is reported for this species.

When 1 L YS3 cultures were harvested it was found that the cells did not form a strong, compact pellet after centrifugation at 30 000x g for 30 min, so the culture supernatant had to be
filtered through 0.2 µm to ensure that cells were not present and therefore determine that any activity measured was due to secreted enzymes.

A strategy to increase yields of endoglucanase from YS3, involved growing the bacteria to high cell density in LB medium, for 24 h at 30°C until stationary phase was approached and then changing the medium to the less rich CY broth. The culture was centrifuged at 31 000x g for 30 min and the supernatant was discarded before the cells were resuspended in CY broth with some glucose or cellobiose added. Therefore, growth was continued, although in a more nutrient limited environment, to encourage endoglucanase production.

In later experiments, an even less rich environment of ST6 media was employed, mainly to eliminate the high levels of yeast extract in culture samples that interfered with the measurement of methylumbelliferone in the MUC assay. ST6 media only contains 0.002% (w/v) yeast extract compared to 0.1% or 0.5% for CY and LB medias, respectively. Additionally, the lack of tryptone in ST6 media meant that the culture supernatant samples could have protein content estimated by the Bradford method.

Motility and exopolysaccharide (EPS) production of YS3 was further investigated on ST6 + 0.1% (w/v) cellobiose or 5 mM glucose agar plates with or without added tryptone. In case these behaviours enhanced cellulase activity, Filter paper (Whatman #1) was also placed on the surface of the agar plates to see if any signs of hydrolysis would appear along with growth. On other ST6 plates, the method of inoculation, either applying a drop of liquid culture with a pipette (drop inoculation) or stab inoculation (cells from a colony applied into the agar plate with a sterile needle) was investigated, and any differences in the resultant colonies observed. EPS material was extracted from YS3 culture supernatant by adding three volumes of 100% ethanol to the culture supernatant, and incubating the resultant solution at 4°C overnight. The solution was then centrifuged at 20 000x g for 3 min to collect the precipitated polysaccharide, which was then resuspended in 20mL distilled water. The polysaccharide material was washed in 5 volumes of 100% ethanol and re-centrifuged before resuspending in 10mL distilled water and freeze-drying.
4.3 Results

4.3.1 Identification of bacterial isolates

EV1 was confirmed to be oxidase positive and produced slightly turbid cultures in M-70 broth with D-ribose, and was therefore positive for growth on this sugar. 16s rDNA sequencing had already revealed that EV1 was either *Acidovorax delafieldii* (99% similarity over 854 bases) or *Acidovorax temperans* (97% similarity over 854 bases). Willems *et al.* (1990), claim that *Acidovorax temperans* cannot utilize D-ribose, therefore EV1 is most likely a strain of *A. delafieldii*.

CH3 was also confirmed to be oxidase positive. CH3 was found to be positive for nitrate reduction, when after three days incubation all three cultures tested had nitrite present. CH3 was negative for mucoid growth on TSA and also grew on TSA at 4°C after more than a week's incubation. 16s rDNA sequencing had previously matched CH3 with *Cellvibrio mixtus*, *C. fulvus* or *C. ostraviensis*. Nitrate reduction and growth at 4°C mean that this isolate is not *C. mixtus* but may be a strain of either the other two species.

Results of the YS3 biochemical tests, including those as part of the API 20E test strip are presented in Table 4.1. In some cases YS3 was tested with both the traditional method and the API test, these always gave the same result. 16s rDNA sequencing had already found YS3 to be a member of the genus *Aeromonas* but the sequencing results returned many possible *Aeromonas* species, as this group is not well distinguished using this method. Miñana-Galbis *et al.* (2002), studied 220 *Aeromonas* strains in order to consolidate the known hybridization groups with testable phenotypic properties, in order to identify members of this diverse group. A selection of 16 tests was found to be capable of describing most of the isolates, with three or more tests usually being necessary for identification of any one strain. In this case, 8 tests were needed to confirm that *Aeromonas caviae* was the closest phenotypic match to the YS3 isolate. The API 20E test was carried out to confirm this finding, although it was later found that this test does not distinguish between *A. hydrophila* and *A. caviae*. The possibility of YS3 being *A. hydrophila*
however, is ruled out by the production of brown soluble pigment, and the lack of lysine decarboxylase (Miñana-Galbis et al. 2002).
Table 4.1. Biochemical tests used to identify YS3 to species level

<table>
<thead>
<tr>
<th>Test</th>
<th>Result from traditional biochemical test</th>
<th>Result from API 20E test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>Brown soluble pigment</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Esculin hydrolysis</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Indole production</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Utilization of L-lactate</td>
<td>pos(^1)</td>
<td></td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>β-galactosidase (ONPG)</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>H₂S production</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Tryptophan deaminase</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Gelatinase</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Fermentation of glucose</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Fermentation of D-mannitol</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Fermentation of inositol</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Fermentation of D-sorbitol</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Fermentation of L-rhamnose</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Fermentation of D-sucrose</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Fermentation of D-melibiose</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>Fermentation of amygdalin</td>
<td>pos</td>
<td></td>
</tr>
<tr>
<td>Fermentation of L-arabinose</td>
<td>pos</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) slight clumped growth was observed in triplicate cultures
4.3.2 Enzyme assays

The CMC plate assay was used widely throughout the experiments owing to its ease and sensitivity. Using a *T. viride* cellulase preparation, it was seen that a lower concentration of enzyme always gave a smaller diameter clearance halo unless the assay had reached saturation. For example, 30 µL of 200-500 µg/mL *T. viride* cellulase gave clearance halos that did not differ significantly (21-23 mm), therefore the assay is saturated with these high concentrations of enzyme. Any one sample applied to the plates could give a halo varying by up to 2 mm, so clearance halos from different samples that did not differ by more than 2 mm were assumed to contain approximately the same amount of endoglucanase enzyme. In this way, culture supernatant samples obtained from different growth conditions could be compared for endoglucanase production from the bacteria.

*T. viride* cellulase was additionally diluted in various buffers (20 mM MES, Tris, NaHPO₄, MOPS, HEPES) at pH varying from 5-9, as well as 0.1% (w/v) SDS solution, ST6 media, ST6 media with ammonium sulphate at 30% saturation. None of these solutions were found to interfere with the assay, as drops of the enzyme solutions once again gave halos differing by no more than 2 mm in diameter (30 µL aliquots gave 16-18 mm halos). Although the enzymes (both the positive control and YS3 enzyme) would be expected to have specific pH values for optimum catalysis, the fact that the *T. viride* cellulase did not give varying clearance halos when in varying pH buffers can be explained. The CMC plates are made in 50 mM NaHPO₄ pH 7.2, which provides strong buffering compared to the small volumes of sample buffers that are applied to the surface of the plate.

To verify the β-1,4-endoglucanase activity of the bacterial isolates, they were grown in the presence of crystalline cellulose and the resultant culture supernatants were assayed. CH3, EV1 and YS3 were grown on 461S + 0.1% (w/v) cellobiose + 0.02% (w/v) yeast extract plates, incubated at 30°C until single colonies were seen, these were then used to inoculate 10 mL CY starter cultures, incubated at 30°C with shaking overnight. 1 mL of the overnight cultures were used to inoculate 100 mL CY + 0.5% (w/v) Sigmatell cultures (duplicate), which were then
incubated at 30°C with shaking for 69 h before a sample was taken and culture supernatant (150 µL aliquots) was tested for CMCase in the well plate method. Both YS3 culture supernatant samples and one of the EV1 samples gave clear halos upon Congo red staining of the substrate, however CH3 samples displayed no activity. The consistent endoglucanase activity of *Aeromonas* sp. YS3 made it a candidate for further study.

Whilst the CMC plate assay clearly shows endoglucanase activity, another method had to be employed to check for any possible exoglucanase (exocellulase) activity. Both exoglucanase and endoglucanase activities are needed before extensive hydrolysis of crystalline cellulose can be achieved. Methylumbelliferyl-β-D-cellobioside (MUC) is used as a model substrate of exocellulase enzymes (Chernoglazov *et al.* 1989). To determine the range of the assay, 1mM MU was diluted in 25 mM NaHPO₄ pH 7.2 to prepare a standard curve of the fluorophore with the concentration range 2-250 µM. Concurrently, the positive control cellulase from *T. viride* was diluted in the same buffer and tested against the MUC substrate. After incubation at 42°C for 1 h, 4 µL 1 M NaOH was added to each well to increase fluorescence and then these standard solutions were read in the plate reader. Figure 4.1 shows the result of this experiment, a linear response from MU alone was seen in the concentration range 2-30 µM, or up to 30 000 fluorescent units. The fluorescence of MU reached saturation as 60 000 fluorescence units were approached. The response from the *T. viride* cellulase was somewhat linear up until 25 000 fluorescence units, concentrations of cellulase greater than 100 µg/mL seemed to reach saturation with this amount of MUC (16 µg/mL final concentration).
Figure 4.1. Saturation curves for MUC assay. a. Results of duplicate wells with different MU concentrations. b. T. viride cellulase was incubated with 16 µg/mL MUC at 42°C for 1h, average of duplicate wells, adjusted for background fluorescence, with standard error shown. All samples were read in the FLUORstar plate reader at 355 nm (excitation) and 460 nm (emission).
**Aeromonas** sp. YS3 culture supernatant tested in the MUC assay gave only low levels of activity, although this was not surprising as these samples had less than 10 µg/mL protein, estimated by the Bradford microassay. Therefore, activity was similar to that arising from 12.5 µg/mL *T. viride* cellulase, which gave 1847 ± 14 fluorescence units in the same assay (see Figure 4.1). Samples from cultures grown with more cellobiose added to ST6 media gave higher CMCase but lower MUCase (Table 4.2).

<table>
<thead>
<tr>
<th>ST6 grown cultures with Additions of:</th>
<th>Halo diameter on CMC Plate (mm)</th>
<th>Fluorescence in MUC assay</th>
<th>MU released (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% cellobiose</td>
<td>12</td>
<td>1523 ± 49</td>
<td>1.37</td>
</tr>
<tr>
<td>0.064% cellobiose</td>
<td>19</td>
<td>599 ± 25</td>
<td>0.54</td>
</tr>
<tr>
<td>0.064% cellobiose +</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01% yeast extract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.07% cellobiose</td>
<td>21</td>
<td>876 ± 18</td>
<td>0.79</td>
</tr>
</tbody>
</table>

YS3 culture supernatant grown in ST6 + 0.07% (w/v) cellobiose was also tested for the release of reducing sugars from CMC via the DNS assay. 100 µL aliquots of sample were incubated with 400 µL 0.5% (w/v) CMC at 42°C for 2 h. After the reaction tubes were tested for reducing sugars, 12.5 µg/mL *T. viride* cellulase gave a result of 0.12 g/L glucose equivalents, which is very close to the limit of sensitivity of the assay (0.1 g/L) but the YS3 sample had no detectable activity.

### 4.3.3 SDS-PAGE and Zymograms

To test that the activity on the CMC plates was due to an enzyme, it was first determined if this activity was protein based. YS3 was grown for 94 h in CY + 0.5% (w/v) Sigmacell, at which time samples were taken to obtain culture supernatant. 150 µL of the culture supernatant samples were heated to 95°C for 15 min, 75 µL aliquots of heated and non-heated samples were then assayed via the CMC well-plate method. The non-heated ‘native’ samples displayed clearance of the CMC substrate whilst the heated ‘denatured’ samples did not, therefore the
molecule responsible for the CMCase activity was heat-labile. Other YS3 culture supernatants, grown in varying media formulations, along with 500 µg/mL *T. viride* cellulase as a positive control, were tested to see if activity could be abolished by the action of Proteinase K enzyme. Once again, the samples were tested alongside the ‘native’ untreated samples in the CMC plate method. The expected result of no activity in the treated samples was obtained, Figure 4.3 shows a representative assay plate. The 4 h incubation was long enough to cleave the high concentration of the positive control cellulase enzyme, so that no activity was seen with this sample.

![Figure 4.2. Action of Proteinase K on YS3 CMCase.](image)

The action of culture supernatants, after treatment with Proteinase K is seen to be abolished. Similar results were achieved with the *T. viride* cellulase.

To further verify that the YS3 CMCase is due to a protein in the culture supernatant, samples were run on SDS-PAGE in gels containing 0.45% (w/v) CMC. *A. hydrophila* culture supernatant was obtained under the same growth conditions so it could be used as a negative control for CMCase in the zymogram. Figure 4.4 shows the gel, which was first stained with Coomassie, before it was incubated at 37°C for 5 h and subsequently stained with Congo red. Not much protein was seen with the Coomassie stain in the culture supernatant samples, but when these samples were concentrated 5-fold with ultrafiltration, at least 10 protein bands could be seen in the YS3 sample and one of these was found to have faint CMCase.
Figure 4.3. CMC Zymogram showing YS3 activity. a. Coomassie stained gel, b. Congo red stained gel, lane 1 Mark12 molecular marker, 2 100 µg/mL *T. viride* cellulase, 3 500 µg/mL *T. viride* cellulase, 4 *A. hydrophila* culture filtrate, 5 *A. hydrophila* concentrate, 6 YS3 culture filtrate, 7 YS3 concentrate. Arrows highlight the position of activity bands.

The gel shown in Figure 4.4 had been Coomassie stained before SDS was removed from gel and it was allowed to renature overnight prior to activity staining. A more distinct activity band in the YS3 sample (concentrated 5-fold) was visualised when the SDS was removed prior to the renaturation and Coomassie stain steps (Figure 4.5). The activity band appears the same colour as the stacking gel seen above it. The stacking gel did not have CMC included so this suggests that all the CMC in the area of the activity band has been cleaved into chain lengths too short to allow binding of the Congo red dye.
Figure 4.4. Concentrated YS3 culture supernatant. The same acrylamide gel is shown after each stain. 20 µL of concentrated (5-fold) *Aeromonas* sp. YS3 culture supernatant was run on the gel. a. Coomassie stained. b. Congo red stained.

Figure 4.5. Estimation of MW of YS3 CMCase. The Congo red half of the gel (left) was fixed with 1N HCl, activity band and the Coomassie band responsible (same mobility) are boxed. Mark12 protein ladder was used as a standard, seen on the right of the Coomassie stained half of the gel.

Coomassie staining the gel prior to Congo red staining meant that some of the Coomassie stain was retained in the gel, possibly obscuring any activity bands from proteins with Coomassie still bound. Therefore, molecular weight of the enzyme was estimated by running a concentrated sample on opposite sides of a single gel, so that the same proteins either side of
the gel could be stained differently. Therefore, the YS3 CMCase was found to give an activity band of size, 39 kDa (estimated using Quantity One software) (Figure 4.6).

4.3.4 Growth of YS3

Expression of the observed β-1,4-endocellulase (CMCase) activity of YS3 was examined by growing the bacterium in either LB or CY broth (100 mL cultures) with 0.01% (w/v) cellobiose or 0.5% (w/v) SigmaCell (crystalline cellulose) added in attempts to induce CMCase activity. Growth in the different media was compared by measuring optical density (OD) at 600 nm of culture samples taken at various time points. The solid cellulose substrate adds to the turbidity of the liquid media, therefore an uninoculated control had to be included to take this into account. The control flask contained the same media as the cultures grown with SigmaCell. All samples containing cellulose were shaken briefly, then allowed to settle for exactly 5 min before OD measurements were read in the spectrophotometer. This resulted in small sample errors when measuring OD of flasks containing solid cellulose particles. The difference between the un-inoculated controls and those containing YS3 growing in the presence of cellulose, should reflect any increases in OD that are due to growth of the bacteria.

Figure 4.7 shows the growth curves obtained from this experiment. Higher densities of growth were seen with the richer LB medium. The additions of 0.01% (w/v) cellobiose and 0.5% (w/v) SigmaCell did not enhance growth significantly, as measured by OD. The samples were also tested for the presence of CMCase, by the CMC well-plate method. In the first 48 h of culture, no CMCase was detected. Final samples were taken after 112 h incubation to confirm that the cultures had entered stationary phase and these samples were found to possess CMCase activity (Table 4.3) (the uninoculated control samples were confirmed to lack CMCase).
Table 4.3. CMCase arising from YS3 cultures

Culture supernatants were tested after 112h growth.

<table>
<thead>
<tr>
<th>Media</th>
<th>Halo on CMC plate (mm)</th>
<th>Halo diameter (average ± st dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY</td>
<td>12, 9, 11</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>CY + 0.01% cellobiose</td>
<td>16, 17, 16</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>CY + 0.5% Sigmacell</td>
<td>13, 13, 15</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>LB</td>
<td>21, 22, 22</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>LB + 0.01% cellobiose</td>
<td>23, 24, 24</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>LB + 0.5% Sigmacell</td>
<td>24, 24, 23</td>
<td>24 ± 1</td>
</tr>
</tbody>
</table>

Figure 4.6. Growth curves of YS3 on a. CY broth b. LB broth, error bars represent sample error resulting from triplicate cultures. Adjusted values for Sigmacell cultures took into account uninoculated controls because the crystalline substrate contributes to changing OD with time.
Due to the highest level of CMCase being detected with growth in LB medium, this was more closely investigated. YS3 was grown in 100 mL LB cultures with no additions or 0.01% (w/v) cellobiose or 0.5% (w/v) Sigmacell added, each in triplicate. OD measurements were taken at 0, 4, 8, 16, 20, 24, 28, 70 h, and confirmed that the same growth curve was obtained on each media formulation. Cell density increased exponentially up until 24 h, at which time stationary phase was reached. Culture supernatant from each time point was also tested for CMCase, with 80 µL aliquots applied to wells in a CMC plate. This activity was not observed until the cultures were in stationary growth phase, after 28 h incubation. A final sample taken after 70 h incubation confirmed that cell density had declined and each culture supernatant from these samples gave clear CMCase, with clearance halos of 18-20 mm seen on CMC plates.

![Growth curves of Aeromonas YS3 in LB media.](image)

**Figure 4.7. Growth curves of *Aeromonas* YS3 in LB media.** The higher OD values resulting from cultures with Sigmacell are assumed to be due to a contribution (in OD) from the crystalline substrate.

Growing YS3 alongside *A. hydrophila*, under the same conditions, it was seen that CMCase activity was only seen in YS3 and never in samples from *A. hydrophila*. Another difference between the two cultures was that, upon centrifugation of cultures grown to high density (30 000xg for 30 min), *A. hydrophila* easily formed a compact cell pellet, whereas YS3 did not, due to viscous material presumed to be carbohydrate produced by the cells.
In attempts to optimise CMCase production from YS3, cultures were grown until the end of
exponential growth phase (24 h incubation) and then the cells were centrifuged and
resuspended in CY broth. Therefore, nutrients that had been depleted during exponential
growth could be replenished and hopefully a concomitant increase in cell density would lead to
higher CMCase production. 100 mL flask cultures were grown at 30°C, three in LB for 72 h,
whilst another three were in LB for 24 h, then exchanged into CY broth for a further 48 h. As
expected, the cultures subjected to a change of media, produced more CMCase, evident by 17
mm clearance halos on CMC plates, compared to 10 mm halos arising from cultures with no
media change-over (30 µL aliquots of culture supernatant tested).

To eliminate the yeast extract and excess tryptone in the culture supernatant samples that was
interfering with the MUCase and Bradford assays, respectively, it was investigated whether
similar levels of activity could be achieved with the minimal ST6 media, instead of using CY
broth. To test this, 8x 100 mL LB flasks were inoculated with YS3 and incubated at 30°C for 22
h. One of the flasks was left to incubate with LB media, another had the media changed to CY
broth, 3 had the media changed to ST6 and another 3 had the media changed to ST6 + 0.01%
(w/v) cellobiose and incubation of all flasks was continued for a total of 68 h. Samples taken at
46, 51 and 68 h after the start of incubation revealed that ST6 + 0.01% (w/v) cellobiose could
give the same level of activity as CY broth, with both types of culture supernatants giving 19
mm clearance halos on CMC plates (30 µL aliquots of each culture supernatant tested). This
maximum activity was only reached after the full 68 h incubation, after which time the LB flask
(with no media change-over performed) yielded a 10 mm clearance halo on CMC. The ST6
cultures (with no added cellobiose) only had very faint activity throughout the incubation,
suggesting that the added cellobiose to the other ST6 cultures was responsible for the increased
CMCase production.
Culture supernatants from this experiment were analysed for protein content by SDS-PAGE (Figure 4.9). The media change-over to CY broth seemed to significantly increase protein levels in the culture supernatant whereas ST6 + 0.01% (w/v) cellobiose media resulted in similar levels of CMCase without this general effect.

Figure 4.8. Protein levels in different YS3 culture supernatants.

Lane 1. Mark12 molecular marker (Invitrogen), 2. LB culture supernatant, 3. CY culture supernatant, 4. ST6 culture supernatant, 5-7. ST6 + 0.1% cellobiose culture supernatant. The same volume of 20µL of each culture supernatant was applied to the gel, so that relative amounts of protein bands could be observed.
The optimum level of CMCase production was found when a media change-over with ST6 + 0.07% (w/v) cellobiose was employed (see Table 4.4). The use of ST6 + 0.1% (w/v) cellobiose seemed to inhibit enzyme production. Substituting 0.07% (w/v) cellobiose with 5 mM glucose resulted in similar levels of CMCase produced.

Table 4.4. CMCase arising from different YS3 cultures

40µL of each culture supernatant had been tested on the same CMC assay plate. Duplicate plates gave the same size halos from each sample.

<table>
<thead>
<tr>
<th>Media employed in change-over</th>
<th>Halo diameter on CMC plates (mm ± 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY</td>
<td>21</td>
</tr>
<tr>
<td>ST6 + 0.064% cellobiose</td>
<td>19</td>
</tr>
<tr>
<td>ST6 + 0.064% cellobiose + 0.1% yeast extract</td>
<td>21</td>
</tr>
<tr>
<td>ST6 + 0.07% cellobiose</td>
<td>21</td>
</tr>
<tr>
<td>ST6 + 0.1% cellobiose</td>
<td>17</td>
</tr>
</tbody>
</table>

After YS3 had been cultivated in the laboratory for more than 12 months, it was noticed that reproducible production of CMCase was no longer obtained. A significant contributing factor to this loss was thought to be that YS3 was accidentally subcultured on ST6 media containing 0.02% (w/v) yeast extract at one stage instead of the prescribed 0.002% (w/v) yeast extract. Once YS3 had been grown on the higher percentage of yeast extract, subculturing onto the properly formulated media by streak plating was not successful. To obtain growth on the minimal media plates, a heavy inoculum from liquid cultures had to be applied. Furthermore, colonies took a few days to appear instead of overnight as expected (incubation at 30-37°C). At the same time it was noticed that the procedure of growing the bacteria in LB for 24 h, then changing the media to ST6 + 5 mM glucose no longer resulted in detectable CMCase.
An error in the formulation of the ST6 media was initially thought to cause the loss of CMCase production by the bacteria. The trace metals solution being used was more than a year old by this stage, therefore some components had possibly become oxidised and either toxic or unavailable. However, replacing this with a new stock solution did not result in reappearance of the CMCase activity in cultures. To check that the media was at optimum pH for enzyme production, 100mL cultures were grown for 24 h in LB (pH 7.2) and then the media was changed to ST6 + 5 mM glucose at pH 5.2, 6.0 or 6.8. After a total incubation of 68 h, only the culture supernatant from the pH 6.8 media displayed CMCase, giving distinct 18 mm diameter clearance halos (50 µL aliquots tested). However, when this procedure was scaled up to 0.5 L shake flask cultures activity was no longer seen in the culture supernatant, therefore another factor was responsible for lack of enzyme production.

At this stage, it was noticed that when the YS3 cell pellets had previously always contained a lot of slimy polysaccharide material, making them hard to sediment by centrifugation, they now formed compact cell pellets from which the culture supernatant could easily be decanted without disturbing the cells. The disappearance of the polysaccharide material and loss of enzyme activity happened simultaneously, so they were possibly co-expressed by the bacterium. Therefore, increasing the nitrogen levels of the media was investigated as this has been seen to increase exopolysaccharide (EPS) production from bacteria within this genus (Bonet et al. 1993). Extra N in the form of KNO$_3$ (0.0002% w/v) or tryptone (0.1% w/v) was added to ST6 media, used in a media change-over of 0.5 L shake flask cultures. When 50 µL aliquots of the resultant culture supernatants were tested for CMCase, the NO$_3$ treatment resulted in no activity, whereas the extra tryptone treatment resulted in distinct 20mm diameter clearance halos. A similar experiment where either 0.1% or 0.2% tryptone was added to the ST6 media showed that no greater CMCase production was achieved with higher levels of tryptone, as 50 µL aliquots of culture supernatants gave 20 mm diameter clearance halos on CMC in either case.
Because excess tryptone had been shown to increase CMCase production in YS3, this media formulation was further investigated to see if any hydrolysis of filter paper could be observed by YS3 under these conditions. YS3 had been isolated from hydrolysis zones on filter paper but it was not known what role YS3 held within the consortium of organisms that survived in such hydrolysis zones. Filter paper (Whatman #1) was placed onto the surface of agar plates, of ST6 + 5 mM glucose media, either supplemented with 0.2% (w/v) tryptone (ST6 rich) or without such an addition (ST6 poor). YS3 was used to inoculate one corner of the filter paper and the plates were incubated at 37°C and monitored for any changes over two weeks. The YS3 inoculum was either from ST6 rich or poor plates (grown at 37°C for a few days), so that the effect due to previous growth conditions of the inoculum could be assessed. Growth on the initial ST6 rich plates was heavier, as was expected, so the inoculum taken from these plates (lifted with a sterile metal loop) was also heavier when inoculating the filter paper plates. Despite this, there was more growth observed on both the ST6 rich and poor filter paper plates when the inoculum was from the ST6 poor plates.

Two types of growth were seen on any of the filter paper plates, either a thick, mucoid growth, present on the aerial surface of both the filter paper and surrounding agar, or a tendril-like growth reminiscent of twitching motility, that was seen to extend across the agar surface (Figure 4.10) and appeared to be underneath the other type of growth. More of the twitching-motility type growth was seen when the inoculum was from ST6 poor media, which may explain the ability of this growth to colonise a greater area of the plates faster.
Figure 4.9. YS3 motility on solid surfaces. Dense growth (arrowed) was seen to occur all around the edge of the filter paper, whereas tendrils of thinner, translucent growth grew towards the outer edge of the petri dish, 40x mag. (main image). These outgrowths were seen to be composed of very thin trails of bacterial cells (inset).
Very dense, tendril-like growth structures were seen after 5 days incubation of the ST6 poor filter paper plates. These were always seen to be attached to the filter paper and most commonly on the corners of the filter paper. By lifting some of this strange growth onto a microscope slide, it was found to be composed of microcolonies of cells attached to one another somehow (see Figure 4.11).

Figure 4.10. Microcolonies of YS3 formed on filter paper. a. at 40x mag., b. 400x mag., c. 1000x mag.
After 5 days incubation of the ST6 poor filter paper plates, there were less thin tendrils of growth seen at 40x mag. and instead, refractive, golden, convex structures had appeared (see Figure 4.12). A group of such structures formed a distinct shape, oval with somewhat pointed ends, which was a structure that had commonly been seen within the initial filter paper isolation plates that YS3 had originated from. These convex outgrowths and the conditions under which they were formed are reminiscent of Myxobacterial fruiting body structures, in that, under starvation conditions the cells had retreated back into aggregates (Silakowski et al. 1998), although the presence of any spore-like cells within these aggregates was not investigated so they cannot be referred to as true fruiting bodies. After 12 days incubation, there were many of these convex structures, covering the surface of the ST6 poor plates.

Figure 4.11. Fruiting-body-like structures of YS3. Seen after 5 days growth in nutrient limited conditions. 40x mag.
On any of these plates, no hydrolysis of the filter paper was observed during the two week incubation. The motility and formation of dense microcolonies seen on the filter paper plates were also observed on similar ST6 plates without filter paper present.

The percentage of agar has been seen to modulate the type of motility seen by *Aeromonas* spp. (Bechet and Blondeau 2003). Therefore growth of YS3 was observed on ST6 plates with either 0.5, 0.7, 0.8% (w/v) agar (normal formulation included 0.8/1% agar). The dense threadlike growth seen in Figure 4.1L was more predominant on the 0.5% (w/v) agar plates, which also gave rise to faster motility in the sense that the colonies increased in diameter faster on these plates (70 mm average diameter achieved after a weeks incubation compared with 52, 36 mm for 0.7, 0.8% agar respectively). Most of the bacterial growth exhibited typical swarming motility, with the formation of concentric rings on the aerial surface of the colonies (Bechet and Blondeau 2003), although with further incubation these became obscured with extensive slime production. After 48 h incubation, on the 0.5% agar plates, a zone of transparent material was observed around the perimeter of the swarm colony, stretching a few mm from the edge of the swarm.

The ability of YS3 to form a biofilm at the air/liquid interface was investigated by inoculating 40 mL flask cultures (100 µL inoculant from overnight culture grown on CY + 0.005% (w/v) cellobiose at 37°C with shaking), which were incubated at 28, 37°C without shaking. ST6 + 0.2% (w/v) tryptone + 5 mM glucose and CY + 5 mM glucose were trialled as media to support biofilm formation. Fragile biofilm growth (easily disturbed by shaking) was seen to occur in the CY flasks at both incubation temperatures, whilst the ST6 media supported little growth, which occurred as pellets at the bottom of the flask.
4.4 Discussion

Three bacterial isolates possessing CMCase activity, belonging to the genus *Acidovorax*, *Aeromonas* or *Cellvibrio*, had been uncovered in the search for novel cellulase enzymes. *Cellvibrio* is a bacterial genus known to possess cellulases and members of this genus can degrade native cellulosic substrates (Lednická et al. 2000). CH3 was identified as a possible *C. fulvus* or *C. ostraviensis* strain. The cellulase activity from *C. fulvus* has previously been examined (Berg et al. 1971) and during the course of this study, a putative *C. ostraviensis* strain possessing cellulolytic abilities was also isolated (Nakajima-Kambe et al. 2005). Therefore, due to the cellulase activity from this genus being studied already, CH3 was not investigated further. *Aeromonas* on the other hand, is a bacterial genus that is known to possess carbohydrate hydrolytic enzymes such as chitinase (Lin et al. 1997) and xylanase (Lui et al. 2003) but β-1,4-endoglucanase activity has not been reported in this genus previously. Strains of *A. caviae*, the closest species match to YS3, contain xylanase enzymes, including one that has no homology with the commonly found xylanases in glycoside hydrolase family 10 or 11 (Suzuki et al. 1997). This may indicate a different evolutionary lineage of hydrolytic enzymes in this genus. A similar situation to YS3 has been reported in *Prevotella* species that efficiently degrade xylan and also co-express a CMCase (Matsui et al. 2000). The genus *Acidovorax* has also not been reported to possess this enzyme activity but preliminary tests, growing EV1 (*Acidovorax* sp.) in the presence of crystalline cellulose yielded variable CMCase activity (see section 3.2.2). For these reasons, YS3 (*Aeromonas* sp., most likely a strain of *A. caviae*) was chosen as the focus of this study, investigating any cellulase activity as well as growth behaviours of this particular strain.
YS3 possessed CMCase activity as well as some ability to cleave the model substrate, MUC, which is used as a measure of exocellulase activity. The culture supernatant samples tested for MUCase had to be first concentrated, implying that only a low level of this activity was present, compared to the easily detected CMCase (endoglucanase). This may imply a small amount of an exocellulase enzyme is present, separate from the enzymes responsible for the CMCase. However, although many endocellulases lack activity on MUC, a residual amount of activity can sometimes be detected with concentrated samples due to the enzymes sometimes cleaving at points in the cellulose chain other than the preferred bonds (Bhat et al. 1990). Therefore, both the CMCase and MUCase activities may be properties of a single enzyme.

However, activity against MUC present in the *Aeromonas* YS3 culture supernatants displayed a different trend in regards to growth conditions, as compared to CMCase. Culture supernatants from cellobiose grown cultures displayed less MUCase, although this could have been due to residual cellobiose in the samples interfering in the assay. If cellobiose was bound in the active site of the enzymes, there may have been fewer enzyme molecules free to act upon the MUC substrate. However, cultures supplied with extra yeast extract (0.01% (w/v) compared to the usual level of 0.002% (w/v) in ST6 media) also displayed lower MUCase, therefore conditions of excess carbon may lead to decreased production of a component of this enzyme system in the YS3 strain. The strain was isolated in nutrient limited conditions, so it is not unexpected that it would be adapted to such conditions.

Even though CMCase was easily detected by the CMC plate assays, no reducing sugars were detected with culture supernatant samples incubated with CMC for 2 h, implying the mostly endo-character of the enzymes in such preparations. The generation of more reducing sugars from cellulolytic substrates is seen as indicating a processive action on the substrate, whereas strictly endo-cellulolytic enzymes cleave the cellulose chain at internal points randomly, decreasing the chain length more rapidly than producing reducing sugars (Irwin et al. 1993). The CMC plate assay is ideal for detecting such endocellulolytic activities as the clearance
zones are produced when the CMC substrate is cleaved to oligomers of 5 or so glucosyl residues (Wood 1980).

The CMCase activity of YS3 was clearly demonstrated to be due to a 39 kDa protein, as demonstrated by Congo red stained polyacrylamide gels. The fact that such activity was not registered in the closely related *A. hydrophila* under the same growth conditions further suggests that this is a novel phenotype not usually observed in this genus of bacteria.

CMCase activity in YS3 culture supernatant was not seen until the cultures had entered stationary phase, suggesting that production of this enzyme was a response to nutrient limitation. A similar situation has been observed with the bacterium *Prevotella ruminicola*, which gradually increased CMCase production at the start of stationary growth phase (Gardner et al. 1995). It is possible that the CMCase enzyme from *Aeromonas* sp.YS3 is cell-bound during exponential growth phase and released to the culture medium after the onset of stationary phase, as has been seen in other gram negative bacteria (Matsui et al. 2000).

The higher levels of CMCase resulting from LB grown cultures were most likely a result of higher cell densities obtained on this medium rather than YS3 grown in CY broth. The addition of Sigmacell to cultures grown on either CY or LB media seemed to confer a slight increase in CMCase production by the semi-quantitative CMC plate assay (see Table 4.3). However, this may also be accounted for by the supply of more nutrients to the bacteria, as the crystalline cellulose may contain small amounts of easily utilisable sugars. Additionally, *Aeromonas* sp.YS3 did not appear to utilize the crystalline cellulose for growth, because no increase in optical density of the cultures was seen (see Figure 4.7).

In fact, cultures with Sigmacell added to CY and LB media gave lower OD values at all stages, compared to cultures grown in the CY or LB medium alone. This was most likely due to binding of the bacterial cells to the insoluble cellulose particles. The long lag time of the cellulolytic *B. succinogenes*, when grown on cellulose as compared to growth on sugars, has been...
attributed to the attachment of the cells to the cellulose particles (Hiltner and Dehority 1983) and many other cellulolytic bacteria have been seen to attach to cellulose fibres. Despite this, no conclusions can be made as to why the YS3 cells may have bound to the cellulose particles. The un-inoculated control flasks containing Sigmacell displayed a significant decrease in OD in the first 24h. This was due to aggregation of the cellulose particles, so that heavier ones were formed and therefore removed from the measurements by leaving the solution to settle. Continued agitation of the flasks was seen to slowly increase OD in these control flasks as smaller particles of cellulose were released as a fine suspension. When these changes are taken into account, the course of YS3 growth in flasks containing cellulose is seen to follow the same general pattern as growth in the media alone, although with a longer lag time. OD of YS3 cultures supplied with cellulose was always lower than the other flasks, presumably because an equilibrium between bound and planktonic bacterial cells was in place. The fact that the initial adjusted measurements for these flasks gave a negative value may imply that when the bacterial cells attached to the cellulose, even more aggregation occurred initially, as compared to the control flasks. Even though 0.5% (w/v) Sigmacell represents a large supply of carbon in such cultures, OD of the YS3 cultures with Sigmacell added declined at the same rate as those without cellulose, suggesting that YS3 was not utilizing this carbon source for growth, otherwise maintenance of a high OD would be expected for at least some time. It is possible that something else in the media was limiting growth, although this phenomenon was seen in the very rich LB medium as well as the less rich CY broth.

When media change-overs were performed with ST6 + cellobiose instead of CY broth, similar levels of CMCase were detected, despite the fact that ST6 + cellobiose did not increase overall protein production/secretion to the extent that CY broth did. Therefore, this media formulation may have resulted in either more specific expression or secretion of the CMCase enzyme. This highlights the fact that YS3 produces an extracellular CMCase in nutrient deprived conditions. Additionally, supplying the cultures with higher levels of cellobiose and therefore decreasing the extent of nutrient limitation, resulted in a decrease in CMCase production (see Table 4.4). A similar situation has been observed in *Aeromonas* previously, when another plant cell wall degrading enzyme, pectinase, was seen to be upregulated in
*Aeromonas hydrophila* (previously *liquefaciens*) when carbon was limited in the growth media (Hsu and Vaughn 1969).

In fact, this adaptation to nutrient limited conditions was crucial to extracellular CMCase expression, as the adaptation was lost at one stage by cultivation in the laboratory on richer media, resulting in total loss of CMCase production. Similar examples of loss of phenotype have been seen in laboratory strains of *E. coli*, which lack cellulose production (involving a endoglucanase) that is present in environmental strains (Zogaj *et al.* 2001). The loss of such capabilities has been attributed to repeated passaging of strains in the laboratory. Similar phenotypic differences between environmental and laboratory strains of cellulolytic rumen bacteria are thought to be due to loss of phenotypic attributes by pure culture in the laboratory (Ghannoum and O'Toole 2004). More specifically, the loss of phenotypic characters in laboratory strains has been attributed to selection of fast growing liquid cultures rather than maintaining the wild-type forms in structured communities, which involve complex multicellular processes (Branda *et al.* 2001).

The fact that CMCase activity could be recovered in 100 mL shake flask cultures but not in 0.5 L shake flasks, all other variables remaining the same, suggests that CMCase production is sensitive to slight environmental variations such as oxygen pressure, which would be lower in the larger volume cultures.

The simultaneous loss of CMCase production and EPS production may be significant in determining the role of this endoglucanase in the bacterium. Nutrient limited conditions imposed on the closely related *V. cholerae* also resulted in strains over-expressing EPS material (D’Argenio and Miller 2004). EPS production in Gram negative bacteria is part of an active adaptation program that is employed in energy/nutrient deprived conditions that enables them to attach to surfaces and confers some protection from environmental stresses (Wai *et al.* 1998). Specifically in *Aeromonas* spp., EPS production promotes biofilm formation (Bechet and Blondeau 2003), a mode of growth that is known to protect bacteria from various stresses. YS3
was seen to form an air/liquid interface biofilm when grown in stationary cultures of CY broth. The finding that no biofilm formed in the more nutrient limited ST6 media suggests that a large cell density is required before biofilm formation can occur. When EPS is incorporated in glycocalyx surface layers, it is known to play a role in adherence of bacterial cells to each other and surfaces (Kenyon et al. 2005). This may be the case in YS3, as there was evidence of adherence to microcrystalline cellulose particles (see Figure 4.7) and to filter paper in the form of dense microcolonies, with the bacterial cells bound to each other (see Figure 4.11).

EPS production in YS3 was stimulated upon addition of extra N to cultures, in the form of tryptone, as was similarly seen with A. salmonicida cultures (Bonet et al. 1993). By including extra tryptone in agar plates, additional behaviours of twitching motility and the formation of multicellular, convex-shaped aggregates were seen by YS3 (see Figures 4.10, 4.12). Under nutrient limited conditions, particular amino acids induce the formation of fruiting bodies in Myxococcus xanthus (Ghannoum and O’Toole 2004), so perhaps amino acids arising from the tryptone promoted the somewhat similar aggregation of YS3. The fact that inorganic nitrogen did not result in the same phenotypic changes as tryptone supports this hypothesis. Amino acids within the tryptone may have also induced endoglucanase production, as the CMCase activity returned to cultures of Aeromonas YS3 when supplied with extra tryptone, although this could have been an indirect effect due to other phenotypic changes. Nonetheless, both EPS and endoglucanase production were revived in the isolate with the supply of extra tryptone, suggesting that these traits may be linked genetically. The fact that YS3 exhibited unusual motility, consistent with twitching motility, which also suggested that this strain was adapted to a biofilm mode of growth. Twitching motility is implicated in the ability of Gram negative bacteria to form biofilms (Solano et al. 2002). When A. caviae strains were selected for biofilm formation, swarming motility was abolished, although reversion of the biofilm strain to the parental strain was seen at low frequency (Bechet and Blondeau 2003). The loss and subsequent retrieval of the CMCase-producing phenotypic state in Aeromonas sp.YS3 may similarly reflect that epigenetic mutations occur frequently to affect the expression of such traits. Additionally, YS3 exhibited both swarming and twitching motilities concurrently (from
the same inoculum), suggesting that expression of the different modes of growth is very finely tuned in the individual cells.

The simultaneous production of the CMCase activity and EPS material in YS3 may imply that the function of the CMCase enzyme is to modify the polysaccharide. EPS processing enzymes in *Rhizobium leguminosarum* cleave EPS produced by the bacterium but also CMC (Zorreguita et al. 2000). Additionally many bacteria produce cellulose (Römling 2002) or acetylated cellulose polymers, along with an endoglucanase that is thought to be involved in the synthesis of such polysaccharides (Spiers et al. 2003). Therefore the YS3 CMCase, rather than being a catabolic enzyme needed to utilize cellulose as a carbon source, may in fact be an enzyme involved in the synthesis of a polysaccharide implicated in biofilm formation. Inoculating YS3 directly onto filter paper, in the same conditions optimised for CMCase production, resulted in a display of phenotypic characters that are implicated in biofilm formation but no hydrolysis of the filter paper was seen. This does not rule out the possibility that the secreted YS3 CMCase contributes to filter paper hydrolysis when other bacteria are also present. It has been suggested that, in natural ecosystems, bacteria are dependent on such synergies with each other, in order for them to use what little energy sources are available (Morita 1988).

The ability of YS3 to spread across a solid agar surface, by twitching motility, was enhanced when the isolate was first grown on nutrient limited media. Movement across the agar surface occurred via two modes of growth, either very thin projections of cells, or a highly mucoid swarm from which the thinner projections originated. The ability to actively increase colony size due to such swarming was once thought to be restricted to a few bacterial groups such as *Proteus* or the Myxobacteria (Stanier 1937). This type of behaviour however, has since been found in many unrelated species (Rodriguez and Spormann 1999). The extension of colonies by this method may be driven by the search for new energy sources. This may be the case in *Pseudomonas* strains, where chemotaxis was only seen after the cells had been starved for 48h first (Morita 1988). When YS3 was adapted to nutrient limited conditions (inoculum grown on ST6 poor plates with less N), this phenotype appeared up-regulated (as seen by faster
colonisation of subsequent agar plates), supporting this theory. Notably, this behaviour occurred even on plates supplied with filter paper, further suggesting that *Aeromonas* sp. YS3 did not recognize the recalcitrant cellulose substrate as a carbon source.

In other bacteria however, such as *B. subtilis*, swarming motility (also observed in YS3 concurrently with the other type of motility) occurs even on complex media and is thought to be triggered by changes in population density (Julkowska *et al.* 2004). Swarming behaviour may be facilitated by biosurfactants produced by the organism, seen as transparent material proceeding the bacterial cells (Julkowska *et al.* 2004). A similar zone of transparent material had been observed around YS3 colonies, therefore the use of such substances may be widely employed as these are also seen in *Streptomyces* and fungi (Branda *et al.* 2001). The biosurfactant material may more easily diffuse throughout the media in lower concentrations of agar, thus explaining the ability of the bacteria to more quickly colonise 0.5% agar plates as compared to 0.8% agar plates.

After 12 days incubation, the thin projections of YS3 seen on nutrient limited agar plates, had appeared to retract, instead forming dense, convex structures, as if the cells were congregating in order to protect themselves from adverse conditions. This type of behaviour was once thought to occur only in the Myxobacteria (Shimkets *et al.* 2004) but this behaviour exhibited by YS3 provides additional evidence that such strategies may be widespread in natural ecosystems. *Bacillus* spp. are known to produce endospores to withstand harsh conditions, but whereas this was once thought of as a unicellular process, it has been shown that in environmental isolates spore formation is restricted to aerial fruiting body structures, therefore requiring multicellular behaviour to co-ordinate sporulation (Branda *et al.* 2001). The convex-shaped aggregations of YS3 cannot be referred to as fruiting bodies as no evidence of spores has been seen, although this is possibly due to not finding the right environmental cues. Mutants of *Stigmatella aurantiaca* were made with a single gene inactivated, which instead of forming typical fruiting bodies, simply formed undifferentiated aggregates (Silakowski *et al.* 1998). Therefore, formation of the aggregates in YS3 may either be an evolutionary vestige of a strain that used to
form fruiting bodies, or expression of hidden genes may still be possible. The congregation of such aggregates into a particular ovoid shape may possibly be explained by the transmission of a chemical signal communicating cell density.

The *Aeromonas* sp. YS3 isolate described here may not differ genetically from other *Aeromonas* strains already described. However, it has been found that many unexplored phenotypic behaviours (including CMCase activity) can be observed if the natural biofilm mode of growth is simulated.
5. Purification and assay of a CMCase produced by

*Aeromonas* sp.YS3

5.1 Introduction

Cellulase enzymes are commonly produced as part of a cellulase enzyme system containing both endocellulases and exocellulases, which act together in synergy to degrade cellulosic substrates. Therefore, with the possibility of more than one cellulase enzyme being present, it is always necessary to purify the enzyme of interest to a single protein before the action of the single enzyme can be determined. Hence, to further study the CMCase from *Aeromonas* sp.YS3 it was purified as a single protein molecule. This ensures that any activity is attributable to the 39kDa CMCase and is not affected by the presence of similar enzymes during assays. Furthermore, CMCase activity is sometimes present as an additional capability of other enzymes such as xylanases (Fülöp et al. 1996). Work in the previous chapter found that *Aeromonas* sp.YS3 is most likely a strain of *A. caviae*. The xylanases and chitinases of *Aeromonas caviae* have already been extensively studied (Sitrit *et al.* 1995; Suzuki *et al.* 1997). Therefore, to confirm that the CMCase activity is due to an enzyme that has not been previously studied, similar activities of xylanase and chitinase from the 39kDa enzyme were investigated.

During the purification of the *Aeromonas* sp. YS3 CMCase, CMCase activity was followed using the simple and sensitive CMC plate assay (see section 4.2.2). Endocellulolytic activity can be easily detected with the CMC plate assay. The endo-acting enzymes decrease the chain length of the CMC, leaving the Congo red dye to bind only to substrate that has not been acted upon, thereby leaving clearance halos as signs of hydrolysis of the substrate (Wood 1980). The DNS assay to test for production of reducing sugars was also used because hydrolysis of the CMC creates new chain ends bearing reducing groups due to the glucose moities still bound to the CMC chain. Therefore, the action of the purified *Aeromonas* sp. YS3 CMCase could be quantified using the DNS assay, although long incubation times were necessary to allow enough reducing groups to be produced by the small amounts of enzyme used.
5.2 Materials and methods

5.2.1 Sample preparation

Culture supernatant was used as a source of crude enzyme samples. Strain YS3 was grown for 24 h in LB liquid media, incubated with shaking at 37°C, the cells were then collected by centrifugation (27 500x g for 10 min) before being resuspended in ST6 + 0.2% (w/v) tryptone + 5 mM glucose. Cultures were then further incubated for a total of 68 h, before harvesting by centrifugation (27 500x g for 15 min) to obtain the culture supernatant.

Ammonium sulphate precipitation was used either to concentrate proteins by precipitating them out of solution, or slowly increasing the conductivity of sample, whilst keeping the proteins in solution, in order to use the sample in hydrophobic-interaction chromatography (HIC). In either case, the sample was kept stirring in a glass beaker, in an ice-bath, whilst solid ammonium sulphate was slowly added to the desired saturation level. The ammonium sulphate saturated solution was then typically incubated at 4°C overnight. Precipitated material was centrifuged at 10 000x g for 15 min, and the pelleted material was resuspended in a small volume of the appropriate buffer. Proteins concentrated by ammonium sulphate precipitation were desalted either using PD-10 columns (Amersham), as per the manufacturers instructions or by ultrafiltration.

Vivaspin-500 ultrafiltration tubes (polyethersulphone membrane, Sartorius) were used to de-salt small volumes (600 µL) of chromatography fractions prior to analysis by SDS-PAGE. The tubes were centrifuged at 10 000x g for 10 min, topped up with 20 mM NaHPO₄ pH 7.2, and repeated three times to remove all salt. It was noted that the stated maximum speed of 12 000x g resulted in non-selective binding of proteins to the polyethersulphone membranes (as seen by SDS-PAGE analysis), therefore the lower speed of 10 000x g was used to avoid this. Dialysis was used to de-salt various samples, as well as performing buffer exchanges. Regenerated cellulose tubing (Biolab) was prepared by boiling lengths in 0.1 mM EDTA for 15 min, then
rinsing well in distilled water. Samples were dialyzed against at least 5 volumes of buffer, at 4°C, with buffer changes every half hour for a few hours before leaving to stir at 4°C overnight.

Conductivity of samples was measured with a Conductivity/Salinity meter (TPS model MC-84), which had been calibrated with a 2.76 mS/cm standard solution (TPS).

An alternate method of concentrating samples was stirred-cell ultrafiltration. Membrane filters (polyethersulphone, Millipore), 63.5 mm diameter, 10kDa NMWL, were used in the Amicon 8200 stirred cell apparatus. The filters were washed by passing distilled water through with 50 psi nitrogen gas pressure for a few minutes. The apparatus was placed in an ice-bath, on top of a magnetic stirrer, when samples were concentrated under 60 psi N. Flow through from the stirred cell was collected so that it could be assayed for CMCase along with the retentate. Filters were washed in 0.1 N NaOH and rinsed well in distilled water prior to re-use.

5.2.2 Hydrophobic interaction chromatography (HIC)

Phenyl sepharose (fast flow resin, Amersham) columns of 1.2 mL were packed to a height of 1.5 cm in 1 cm diameter Bio-Rad econo-column holders, and equilibrated with at least 30 volumes of equilibration buffer (with pH and conductivity adjusted to be similar to that of a particular sample). The columns were run gravity-fed, with sample and wash/elution buffers applied manually using a pasteur pipette. Samples were prepared by adding ammonium sulphate to culture supernatant, but using the un-precipitated proteins in the high conductivity background as sample, to enable binding of the proteins to the resin. Precipitated proteins were removed by centrifugation. After sample application, the columns were washed with 10-20 mL equilibration buffer. Elution of proteins from the columns was carried out with 20 mM MES pH 5.5 with or without lithium dodecyl sulphate (LDS) added. Fractions were tested for CMCase by applying 30 µL aliquots to 0.1% (w/v) CMC plates, which were incubated at 37°C for at least 16 h before being stained with Congo red. The smallest diameter halo that can be seen with this amount of sample applied, is 9 mm. Fractions were analysed by SDS-PAGE as per section 3.2.3.
**5.2.3 Ion-exchange chromatography**

Initially, gravity fed columns, 1.2 mL in 1 cm diameter econo-column holders (Bio-Rad) were run using either Q-sepharose or SP-sepharose (fast flow resins, Amersham) to find optimum binding conditions of the CMCase enzyme. Samples were dialyzed into different pH buffers (MES, HEPES, MOPS) and then applied manually to the columns using a pasteur pipette. Elution was carried out with a step gradient, manually applying NaCl solutions to the column. Fractions were collected and tested for CMCase on 0.1% (w/v) CMC plates and stained for activity with Congo red after incubation. For more precise elution, gradient elution was carried out using a gradient mixer (Bio-Rad gradient former model 395), comprised of two chambers connected underneath that could be filled with the same volume of different concentration salt solutions. This was placed above the column, on top of a magnetic stirrer, so that tubing at the bottom of the second chamber could deliver a linear gradient of salt concentration to the column below.

After the right sample conditions for binding and correct gradient for elution was determined, the purification was scaled up to apply larger sample volumes to 8 mL columns. 8 mL SP-sepharose columns were packed in 1 cm diameter glass column holders (Pharmacia). These were attached to the Gradifrac fraction collector (Amersham). The columns were equilibrated by applying buffer with pump p-1 (Amersham) at a rate of 6 mL/min, then sample was applied at 0.3-0.6 mL/min, typically at 4°C. The Bio-Rad gradient mixer was attached to pump p-1 so that gradient elution (150 mL 0-0.4 M NaCl) could be carried out at a flow rate of 0.5-1 mL/min. Fractions were assayed for CMCase as per the previous section. Fractions were analysed by SDS-PAGE as per section 3.2.3 and protein amount was estimated using the Bradford microassay as per section 2.2.8 except that a BSA standard curve of 0 - 20 µg/mL was used.

**5.2.4 Enzyme characterization**

Substrate specificity of the purified enzyme was determined by using the following as substrates in the DNS reducing sugar assay (see section 3.2.2): CMC, laminarin, chitin, Birchwood xylan, Beechwood xylan, oat spelt xylan, Avicel (all supplied from Sigma), and YS3
purified EPS (see section 3.2.4). To detect any possible activity the reaction tubes were incubated for at least 16h at 37°C. To make sure only soluble reducing sugars were measured, tubes containing xylan, chitin and Avicel were centrifuged after reaction with dinitrosalicylic acid, at 14 000 rpm for 5 min, and the supernatant was diluted 1:2 with sterile water before the samples were analysed spectrophotometrically at 550 nm. The DNS assay was also employed to determine the temperature and pH optima of the purified enzyme. For pH optima determination, a sample of purified enzyme was exchanged into different pH buffers (sodium acetate pH 4.0, 4.5, 5.0; MES pH 5.5, 6.0, 6.5; MOPS pH 7.0, 7.5; Tris.Cl pH 8.0, 8.5, 9.0) using ultrafiltration. 2 mL of sample was centrifuged in centricon 10 kDa MWCO tubes (Millipore) at 5000xg for 70 min at 15°C, concentrating the sample to 100 µL, which was then brought up to 1 mL with each buffer. These samples were assayed against 4% (w/v) CMC, incubated at 40°C for 16 h before being tested for reducing sugars.

Purified E1 cellulase from Acidothermus cellulolyticus was kindly provided by Professor David Stalker.
5.3 Results

5.3.1 Sample preparation

To prepare samples for hydrophobic interaction chromatography (HIC), culture supernatant had ammonium sulphate slowly added so that some proteins would precipitate out, leaving the CMCase enzyme of interest in a high salt solution. It was found that all CMCase from culture supernatant could be precipitated out at 60% ammonium sulphate saturation. No CMCase could be detected in the supernatant after proteins precipitated at this level were pelleted by centrifugation. Even with as low as 20% ammonium sulphate saturation, some CMCase could be seen in the precipitated proteins. For example when 200 mL YS3 culture supernatant was brought up to 20% saturation and the precipitated proteins were resuspended in 3 mL 20 mM NaHPO$_4$ pH 7.2, faint CMCase was detected (30 µL aliquot applied to a 0.1% (w/v) CMC plate, resulting in a faint 10 mm clearance halo). Despite this, 30% saturation with ammonium sulphate was found to be necessary to bind the CMCase efficiently to the phenyl sepharose (high sub) resin, this equated to a conductivity of at least 147 mS/cm.

Sample preparation for ion-exchange chromatography (IEX), initially involved the precipitation of proteins from the crude culture supernatant sample, with 60% saturation with ammonium sulphate. In this way, the sample could be concentrated, so as to facilitate detection of the CMCase by the sensitive CMC plate assay during subsequent purification. Precipitated proteins were resuspended in an appropriate buffer before being de-salted, using either PD-10 columns (Amersham) or dialysis in regenerated cellulose tubing. It was found that the samples had to be less than 1500 µS/cm in order to bind to the SP-sepharose resin. It is notable that the enzyme could be dialyzed using regenerated cellulose tubing, even leaving samples in the dialysis bag overnight did not cause any loss in activity. This suggests that the enzyme did not bind to this cellulosic material.

Growing YS3 under the same culture conditions, the phenotype of the bacterium changed, yielding different amounts of polysaccharide in the culture supernatant (exopolysaccharide, EPS). Excess production of the EPS affected sample preparation for IEX, in that, after
ammonium sulphate precipitation, not all proteins could be re-solubilised. Initially, pelleted material after ammonium sulphate precipitation was resuspended in 2-4 mL 10 mM MES pH 5.5 (because the sample needed to be pH 5.5 for binding to the SP-sepharose resin). When too much EPS was present, it would be seen as slimy material in the pellet that would settle out of solution. This material could be separated from the liquid phase by centrifugation, at 8700xg for 5 min. CMCase (as detected by the CMC plate method with Congo red staining) could be detected in the liquid phase but equally as much in the insoluble fraction. It was found that washing the insoluble material with a few mL of 20 mM NaHPO₄ pH 7.2 released some but not all of the CMCase into solution. Therefore, the procedure of ammonium sulphate precipitation to concentrate the sample resulted in unacceptable losses of enzyme in this insoluble fraction.

Table 5.1 outlines the CMCase detected by the CMC plate assay during a typical sample preparation step that involved 910 mL of culture supernatant being brought up to 65% saturation with ammonium sulphate over a period of 5 h before being left stirring at 4°C overnight. Not all of the precipitated material stuck fast in the pellet after centrifugation, this accounts for the activity seen in the ammonium sulphate supernatant. The pellet was resuspended in 4 mL NaHPO₄ pH 7.4 but only 2 mL of this could be recovered as soluble material after centrifugation. The soluble material had to be run on two PD-10 columns in order to remove enough salt, this resulted in a 3.5 fold dilution and activity could no longer be detected, therefore the sample ended up with a lower concentration of CMCase enzyme than the starting material.
Table 5.1. IEX sample preparation by ammonium sulphate precipitation

30 µL aliquots of each fraction were applied to 0.1% CMC plates.

Note: because the CMC plate assay is only a semi-quantitative measure of activity, percentage recovery could not be calculated.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Halo diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>910</td>
<td>17</td>
</tr>
<tr>
<td>Ammonium sulphate supernatant</td>
<td>910</td>
<td>18</td>
</tr>
<tr>
<td>Ammonium sulphate pellet</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>Pellet – soluble fraction</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Pellet – insoluble fraction</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>De-salted soluble fraction</td>
<td>7</td>
<td>no activity</td>
</tr>
</tbody>
</table>

An alternative method of concentrating the sample prior to IEX was stirred cell ultrafiltration. This method also concentrated the EPS material in the sample, as it was retained inside the chamber of the stirred cell, as could be seen by the increase in viscosity of the solution. This increase in viscosity slowed down the ultrafiltration, at least 25 h was needed to concentrate 950 mL culture supernatant to less than 80 mL. The stirred cell was kept inside an ice bath during ultrafiltration, to minimise degradation of proteins, this would also have affected the speed of filtration.

Ultrafiltration was carried out under 60 psi N gas pressure. The maximum pressure for the filter membranes is stated at 70 psi and the 10 kDa MWCO membranes used would normally be expected to retain proteins larger than 30 kDa. Despite this, slight CMCase activity was detected in filtrate from the stirred cell, even though the CMCase of interest was estimated to be 39 kDa (see section 3.3.3). Therefore, the stirred cell was operated under 60 psi N to minimise loss of CMCase from the concentrated sample, although slight activity was still seen in the resultant filtrate.

After ultrafiltration, the sample was exchanged into MES buffer pH 5.5 by dialysis. Once the sample was below pH 6, the concentrated EPS material was seen to precipitate out of solution, this was then removed by centrifugation at 12000xg for 10 min (4°C) and the supernatant was
filtered through a double layer of Whatman #1 filter paper (under vacuum pressure) to yield a sample free from polysaccharide, yet still displaying the same amount of CMCase activity.

5.3.2 Hydrophobic interaction chromatography of YS3 CMCase

Ammonium sulphate had to be added to culture supernatant to a level of 30% saturation to enable the CMCase enzyme to bind to phenyl-sepharose resin, making the sample at least 147 mS/cm. This was tested by manually applying culture supernatant to 1.2 mL phenyl sepharose columns (under the force of gravity alone) and monitoring the flow-through with the CMC plate assay. With samples of >147 mS/cm, up to 100 mL of culture supernatant could be applied to the 1.2 mL columns without CMCase activity being detected in the flow-through or wash fractions. Elution of the enzyme from such columns was achieved with 20 mM MES pH 5.5, although this resulted in a slow leaching of the enzyme from the resin, CMCase was detected in up to 20 mL of eluate. The activity could be removed from the resin faster by including LDS in the elution buffer, 0.5% (w/v) LDS resulted in recovery of activity in 5 mL of eluate.

Octyl-sepharose was also tried as a resin for purification but was found to bind the CMCase enzyme too tightly. This was seen when 60 mL of the same sample (166 mS/cm, pH 7.64) was applied to either a phenyl- or octyl-sepharose column. CMCase activity was seen in the first 5 mL of eluate in either case, but for octyl-sepharose the halo on CMC (<10 mm) was smaller than the halo given by the load material (12 mm), whilst the eluate off the phenyl column contained more activity (14 mm).

When HIC was used as an initial purification step, with culture supernatant as sample, and CMCase was eluted with low salt elution buffer (10 mM MES), no great increase in purity was seen (as analysed by SDS-PAGE) as all the proteins in the load were seen to bind to the column and elute together as well. When HIC was used as a second stage purification step, a different strategy of letting most of the proteins bind to the column and the CMCase flow through was trialled. Active SP fractions were pooled and brought up to 130 or 139 mS/cm (first exchanged into 10 mM Tris pH 6.8) with ammonium sulphate and then applied to a phenyl-sepharose column. 30 mL of sample was applied to the columns, which were washed with 20 mL
equilibration buffer and then elution was carried out with 20 mL 10 mM MES pH 5.5. As expected, the enzyme bound more strongly when in the higher conductivity background (139 mS/cm), as seen by more activity in the eluate (17 mm halo as compared to 15 mm halo for the 130 mS/cm sample), as well as a more intense 39 kDa band in this fraction (see Figure 5.1).

CMCase activity is attributed to the 39 kDa band, although it is possible that the enzyme only co-migrates with this band. Letting the CMCase flow through the column, with the 130 mS/cm sample, resulted in some purification, although the 39 kDa band of interest still co-purified with at least 4 other proteins (see Figure 5.1).

Figure 5.1. Second step purification of YS3 CMCase by hydrophobic interaction chromatography

Columns were equilibrated to sample of 130 mS/cm (a.) or 139 mS/cm (b.) Lane 1. Mark12 ladder 2. load 3. 1st 10 mL of flow-through 4. 2nd 10 mL of flow-through 5. 3rd 10 mL of flow through 6. column wash 7. 1st 5 mL of eluate 8. 2nd 5 mL of eluate. 39 kDa CMCase protein band is boxed.

In attempts at gaining better purification with the HIC step, an affinity elution was attempted. Cellobiose is a product of many β-1,4-glucanases and therefore should be able to bind in the active site of such enzymes. If the YS3 CMCase is bound to the phenyl-sepharose resin in the region of the active site, it was thought that an elution buffer containing cellobiose could release the enzyme from the resin in a selective manner, leaving proteins of similar hydrophobicity bound to the resin. Alternatively, cellobiose could also release the enzyme from the resin regardless of how the enzyme is bound, if binding of cellobiose effects a conformational change in the protein molecules that cause them to no longer bind to the resin.
Pooled SP fractions (15 mL) were used as sample and brought up to 182 mS/cm with ammonium sulphate. Maintaining the same conductivity background, 20 mL elution buffer comprising of equilibration buffer (10 mM Tris pH 8.1 + ammonium sulphate, 184.6 mS/cm) + 1% (w/v) cellobiose was passed through the column. Further elution was carried out with the usual low salt buffer (10 mL 10 mM MES pH 5.5). The affinity elution was successful in that enzyme was seen to be present in these fractions, although at very low levels (only faint 10 mm halos seen as compared to a strong 21 mm halo for the load material). In fact, the levels of enzyme in these fractions were so low that they could not be detected by SDS-PAGE, even after 15-fold concentration by ultrafiltration (in Vivaspin tubes) (see Figure 5.2).
5.3.3 Ion-exchange chromatography of YS3 CMCase

To investigate binding to either cationic or anionic resins, YS3 culture supernatant was dialyzed into different pH buffers (MES pH 6, MOPS pH 7, HEPES pH 8) until conductivity below 1500 µS/cm was reached. The dialyzed samples were applied to 1.2 mL Q- or SP-sepharose columns, which were washed with 10 mL equilibration buffer and then a step-gradient elution was carried out with NaCl to remove any bound enzyme. Enzyme activity in the fractions was confirmed with the CMC plate assay. It was seen that more binding of the CMCase was achieved with a pH 6.0 sample applied to a SP-sepharose column. This was seen by a stronger clearance halo on CMC with the 0.1 M NaCl elution fraction, compared to the halos in elution fractions from the pH 7.0, 8.0 samples. As was expected, the opposite behaviour was seen on Q-sepharose, with no binding of the CMCase at pH 6.0, with activity only being seen in the flow-through and wash fractions. Therefore SP-sepharose was chosen as the resin for the IEX purification step and it was seen that binding was improved with samples.
exchanged into MES pH 5.5, although there was always a slight amount of unbound CMCCase, as faint CMCCase was detected in flow-through fractions off the columns. Total binding of the CMCCase enzyme was not seen even with variant conductivity samples of 340, 644, 817, 1008, 1206, 1650 µS/cm (columns ran under similar conditions, samples pH 5.47-5.66).

An optimized procedure to purify the CMCCase was determined, with samples of 200-250 mL volume (culture supernatant concentrated 15-fold to 15 µg/mL protein), -1150 µS/cm, pH 5.4 applied to 7.5 mL SP-sepharose columns at a rate of 0.3 mL/min at 4°C. Elution from the columns was found to result in high purity CMCCase fractions when a 150 mL 0-0.4 M NaCl gradient was applied (see Figure 5.3). The CMCCase enzyme eluted gradually over this salt range, and activity could be detected in most fractions, however the gradual elution allowed separation of the 39 kDa CMCCase from most other proteins in the range of -0.2-0.3 M NaCl.
Figure 5.3. Purification of *Aeromonas* sp. YS3 CMCase on SP-sepharose with a gradient salt elution.

A. IEX fractions from a SP-sepharose column; lane 1. Mark12 Protein ladder, 2. load (fraction 1), 3. fraction 31, 4. fraction 32, 5. fraction 33, 6. fraction 34, 7. fraction 35, 8. fraction 36, 9. fraction 37, 10. flow-through (fraction 2). B. fractions from a similar IEX purification, with activity once again seen to elute over the range of 0.2-0.3 M NaCl. C. Analysis of protein content and CMCase from the same fractions as seen in A. (note: the limit of detection with the CMC assay was a 9 mm halo). Protein estimation was with the Bradford micro-assay.
The SP purification step was found to have two main protein peaks in the elution (see Figure 5.3). Owing to the low concentrations of protein in all fractions, large standard errors were obtained in these measurements (duplicate samples used to estimate protein amount) and some fractions containing CMCase enzyme were below the sensitivity of the Bradford microassay. The fractions with the highest protein amount were also those with the most CMCase, as detected by comparing clearance halos on CMC. The purest CMCase fraction (no. 32) was not the fraction with the most CMCase (no. 33) (see Figure 5.3).

5.3.4 Characterization of YS3 CMCase

Pooled SP-sepharose fractions were used as a source of purified CMCase (39kDa) (see Figure 5.4), which was tested against various substrates from which the production of reducing sugars was analysed with the DNS assay. Only a very small amount of purified material was available, even with fractions pooled from 5 similar columns, a sample of only 11 µg/mL (estimated by Bradford microassay) of total volume ~50 mL was obtained. Therefore, the enzyme reactions were incubated for at least 16 h before the production of reducing sugars was assessed. No production of reducing sugars occurred when the purified CMCase was incubated with 1% (w/v) xylan (from different sources), 0.6% (w/v) chitin, 1% (w/v) laminarin, 2% (w/v) Avicel (incubated for 24 h) or 1% (w/v) YS3 EPS. The EPS material, purified according to standard procedures (see section 3.2.4), was further analysed by methanolysis, carried out at the Plant Cell Biology Research centre, Melbourne, Australia. This revealed that the material was actually only 10% polysaccharide, so it was presumed that salt or other media components had co-precipitated with any polysaccharide present.
Activity on chitin of crude YS3 culture supernatant samples (~15 µg/mL) had been determined earlier with 0.45 g/L glucose equivalents being released from 0.6% (w/v) chitin after 9 h incubation at 37°C. Similar crude samples had been tested against three types of xylan (from Beechwood, Birchwood and oat spelts) but no reducing sugars were found after 16 h incubation at 37°C.

A highly concentrated YS3 crude sample (culture supernatant concentrated by ultrafiltration using a stirred cell), ~370 µg/mL protein estimated by the Bradford assay, yielded no reducing sugars when incubated with 2% (w/v) Avicel for 24 h at 40°C. Lack of activity on crystalline cellulose was most likely due to the absence of exocellulase enzyme in the YS3 samples. Regardless of this, it was thought that the endocellulase present could contribute to hydrolysis of crystalline cellulose if it was combined with a mixture of other cellulase enzymes. Specific binding sites for particular cellulases, within the complex physical substrate are thought to account for the synergy of hydrolysis often seen when mixtures of cellulases act on insoluble cellulose (Jeoh et al. 2002). To test for such a synergistic effect, YS3 culture supernatant was dialyzed into 20 mM acetate buffer pH 4.5 + 10 mM CaCl₂ (dialysis buffer) before adding to T. viride cellulase (Sigma) in enzyme reactions with Avicel as substrate. The reactions included...
250 µL 2% (w/v) Avicel (in dialysis buffer), 125 µL 100 µg/mL *T. viride* cellulase, 125 µL dialyzed YS3 culture supernatant, and were incubated at 37°C for 40 h. The release of reducing sugars from Avicel was assayed with the DNS method. Un-incubated controls, where culture supernatant was added after incubation, were needed as the YS3 sample contained some reducing compounds that interfered with the DNS assay. A negative control included dialysis buffer instead of enzyme solutions and the action of *T. viride* cellulase alone was similarly tested with dialysis buffer in the place of YS3 sample. *T. viride* cellulase was found to release 0.13 ± 0.01 g/L glucose equivalents from the substrate after 40 h, but the addition of YS3 sample did not increase this amount (0.13 ± 0.04 g/L glucose equivalents).

The purified CMCase sample was assayed with 4% (w/v) CMC (for maximum sensitivity in the DNS assay) as substrate under different temperature or pH conditions for 16 h, to find the temperature and pH optima of the enzyme (see Figure 5.5). A broad temperature optima between 34 - 40°C was seen and pH optimum was pH 4.5. These figures must remain as estimates of the optimal conditions for catalysis, because a comparison of initial velocities of enzyme action would normally be employed to determine optimal conditions. In this case, the very low activity and small amount of enzyme available meant that long incubation times were necessary before reactions products could be measured.
Figure 5.5. Temperature and pH optimum of YS3 CMCase. Purified enzyme was incubated with CMC substrate for 16h under different conditions before activity was measured as reducing sugar equivalents produced.
The purified CMCase behaved similarly to other cellulases in the CMC plate assay. When the purified enzyme (~11 µg/mL) was assayed alongside cellulase from *T. viride* (crude preparation 12.5 µg/mL) and purified El cellulase from *Acidothermus cellulolyticus* (12 µg/mL), similar sized halos were seen from each enzyme preparation (see Figure 5.6). The halo arising from El cellulase had a more distinct edge than halos from the other preparations. An additional result of enzyme action was seen when the Congo red stained CMC plate was left at room temperature (~22°C) overnight. The centre of some of the halos turned blue instead of the orange colour it had first appeared. This phenomenon was commonly seen with the *T. viride* cellulase, especially when higher concentrations were assayed, but the effect was never observed with YS3 CMCase, regardless of concentration.

Figure 5.6. Appearance of clearance halos on CMC from YS3 CMCase
a. halos seen immediately after staining with Congo red, b. halos seen the day after staining, (El enzymes at top, *T. viride* enzymes in middle, YS3 enzyme at bottom).

5.4 Discussion

Hydrophobic interaction chromatography was initially chosen as a first purification step, to purify the 39 kDa CMCase from YS3 culture supernatant. This was because sample preparation simply involved adding salt to the culture supernatant, to increase the conductivity and allow it to bind to the hydrophobic resin. However, when samples were prepared that allowed binding of the CMCase to the columns, nearly all the other proteins in the sample also bound, so a good degree of purity was not achieved with this procedure. It’s possible that a
gradient salt elution off the phenyl-sepharose columns may have increased the purity of CMCase removed.

Hydrophobic interaction chromatography was also tried as a second step in the purification, using pooled active fractions from IEX as sample. A strategy of letting the CMCase flow through the column whilst removing other proteins bound to resin, or an affinity elution with cellobiose as eluent were tried. Neither of these procedures resulted in a significant amount of pure CMCase. The cellobiose affinity elution may have resulted in highly pure CMCase because no other proteins were seen in these fractions, however only very small amounts of CMCase were removed from the column in this way (not detectable by silver staining on SDS-PAGE, see Figure 5.2). Therefore this procedure was not applicable to achieving enough pure CMCase to enable characterization of the enzyme.

Ion-exchange chromatography (IEX) was more closely investigated as a method to purify the YS3 CMCase. This involved lengthy sample preparation but achieved a good degree of purity by eluting the CMCase with a salt gradient. The presence of exopolysaccharide (EPS) material in the culture supernatant samples complicated the sample preparation for IEX. Ammonium sulphate precipitation could no longer be used to concentrate the sample if a large amount of EPS was present, as the EPS remained associated with the precipitated proteins. Washing the resultant slimy precipitate with phosphate buffer removed some of the CMCase enzyme, suggesting that it was not strongly associated with the EPS. It was later found that the EPS material precipitated out of solution below pH 5, perhaps washing the ammonium sulphate pellets at a high pH would have allowed the EPS to be completely solubilized and result in release of most of the CMCase. However, the stability of the CMCase at high pH was not known and this may have damaged some of the enzyme molecules.

Concentration of the sample by stirred cell ultrafiltration also retained the EPS material in the sample, however when the EPS was precipitated out of the concentrated sample, the CMCase was left behind in solution. Perhaps the constant stirring of the concentrate over many hours shortened the chain length of the EPS, making it less likely for the proteins to become trapped
within it when it was precipitated out of solution. The stirred cell ultrafiltration was not ideal however, as increase in viscosity of the solution led to longer times needed for the filtration. Another method of filtration may have been more efficient for this process.

Elution off the SP-sepharose columns with a 0-0.4 M NaCl gradient resulted in a gradual elution of the 39 kDa CMCase but a few fractions in the range of 0.2-0.3 M NaCl contained only this band (determined by SDS-PAGE, see Figure 5.3). However the fraction containing the highest concentration of the 39 kDa CMCase also contained a slightly larger molecular weight protein band, therefore co-purification of this other band reduced the yield of purified 39 kDa CMCase from the columns. Fractions after the 0.3 M NaCl fractions also displayed CMCase activity but did not contain the 39 kDa protein band, therefore one of the higher molecular weight bands seen in these fractions may be another CMCase produced by YS3.

A purification method known as affinity digestion involves the binding of cellulase enzymes to amorphous cellulose and can successfully purify cellulases from other cellular proteins (Morag (Morgenstern) et al. 1992). This method relies on strong binding of the enzymes to the substrate, as well as catalytic efficiency to remove the substrate and therefore result in the release of pure cellulase enzyme (Morag (Morgenstern) et al. 1992). This method was attempted using Aeromonas sp.YS3 culture supernatant. None of the activity in this material was found to bind to the substrate and therefore the more traditional purification method of ion-exchange chromatography was relied upon in this case. From the relatively small size of the Aeromonas sp.YS3 CMCase, it was suspected that no carbohydrate binding domain was present, therefore explaining why affinity digestion could not purify this enzyme.

Fractions from 5 similar columns were pooled before enough pure enzyme was available for preliminary characterization. The DNS (dinitrosalicylic acid) reducing sugar assay was used to characterize the purified 39 kDa CMCase. This assay had been used to test crude YS3 samples previously but no activity had been found with enzyme reactions incubated for 2 h. An exo-acting glycoside hydrolase would be expected to cleave sugars from the ends of polysaccharide
chains, resulting in a rapid increase of soluble reducing sugars that could be detected with DNS. Because YS3 culture supernatant did not generate reducing sugars in a 2h incubation period with CMC as substrate, it was assumed that only endo-acting glycoside hydrolases were present. The presence of endocellulases, and lack of exocellulases in bacterial species is not uncommon (Robson and Chambliss 1989). The purified enzyme displayed no activity on the highly crystalline cellulose substrate, Avicel, as expected due to the lack of exo-cellulolytic ability.

Additionally, when the crude enzyme (culture supernatant) was included with the cellulase system from *T. viride* in reactions on the crystalline cellulose substrate no further release of reducing sugars was seen. The *T. viride* cellulase preparation is a crude preparation containing mostly endocellulases but also some exocellulase and is therefore able to degrade the crystalline cellulose substrate. Adding more endocellulase to such a mixture of enzymes could possibly result in more chain ends for the exocellulase component to act upon and therefore lead to an increase in reducing sugar release. The YS3 endocellulase was therefore thought to not act on crystalline cellulose at all. Whilst the active sites of cellulase enzymes can carry out hydrolysis of glycosidic linkages in the cellulose chain, another mechanism is thought to be involved in releasing single cellulose chains from highly ordered crystalline substrates so that they can enter the active site of the enzyme (Koivula *et al*. 1998). Therefore, the YS3 endoglucanase can carry out hydrolysis of β-1,4-glycosidic linkages but lacks the ability to disrupt the crystalline structure of native cellulose. This suggests that the role of the endoglucanase in the bacterium is not to access cellulose as a carbon source, but may serve another purpose.

Optimum activity on CMC with the YS3 purified enzyme was found at pH 4.5 and between 34-40°C. Similar results were obtained with crude YS3 culture supernatant, therefore the CMCase activity may be attributed to the 39 kDa sample (which may in fact be more than one single protein). The action of the purified enzyme was seen to be slightly different to that of other cellulase preparations, in that clearance halos on CMC never had a blue centre. Congo red dye turns blue under acidic conditions, therefore the blue centres of clearance halos were thought
to be the result of glucose that was released from the CMC substrate. The *T. viride* cellulase preparation from Sigma is a crude cellulase, containing both endo- and exo-acting cellulases. The E1 cellulase from *Acidothermus cellulolyticus* has been described as mildly endo-processive in action (personal communication, Professor David Stalker) and therefore contains both types of cellulase action also. Therefore, the YS3 CMCase was unable to release glucose and produce blue halos from CMC due to its complete lack of exo-glycanolytic ability.

The most important aspect of the enzyme that had to be determined was substrate specificity. *Aeromonas* sp. YS3 was found to be most closely related to *Aeromonas caviae* (see Section 3.3.1) and therefore may be a strain of this species. Other strains of *Aeromonas caviae* have been found to produce glycoside hydrolases such as chitinases (Lin *et al.* 1997) and xylanases (Suzuki *et al.* 1997). Enzymes have been found previously that have both endocellulolytic as well as endoxylanolytic activities (Fülöp *et al.* 1996). Therefore, the possibility of the 39 kDa CMCase also bearing xylanase activity had to be investigated. The purified enzyme did not have any activity, in terms of reducing sugars released, on the three types of xylan substrates tested, which was not surprising as crude YS3 samples also did not display this activity. Xylanases from *A. caviae* can release significant amounts of reducing sugars with a 1h incubation with 1% (w/v) Birchwood xylan, incubated at 40°C (Suzuki *et al.* 1997) but no such activity was found with *Aeromonas* sp. YS3, suggesting that the YS3 isolate is a previously uncharacterized strain of *A. caviae* or possibly even a new species of *Aeromonas*.

The crude YS3 samples displayed significant activity on chitin. This activity must have been due to an enzyme other than the 39 kDa CMCase however, as no such activity was seen with the purified enzyme. This was also an unsurprising result as chitinases from *A. caviae* are larger enzymes, at 100kDa (Lin *et al.* 1997).

No activity was recorded on laminarin (composed of mixed β-1,3-1,4-glycosidic linkages), therefore the enzyme specifically cleaved β-1,4-glycosidic linkages. Because the enzyme was often found to be associated with and co-expressed with the EPS material from YS3, the
CMCase was tested against this polysaccharide also, to investigate whether the purpose of the enzyme is to process the EPS. EPS processing enzymes in *Rhizobium leguminosarum* have been found to also be active on CMC, which is not surprising because the EPS from this bacterium consists mainly of β-1,4-glycosidic linkages (Zorreguita *et al.* 2000). No activity on the prepared EPS material was found, in terms of reducing sugars released. However, this was not surprising considering that the prepared EPS material was later found to be only 10% polysaccharide and the coprecipitation of other material may have modified the carbohydrate so that it was no longer amenable to hydrolysis.

In addition to the substrate specificity analysis, a full kinetic analysis (determination of $K_m$, $K_{cat}$) would have been useful. This data could have been compared to the characteristics of similar endoglucanase enzymes. β-1,4-Endoglucanase activity has not been reported in the *Aeromonas* genus previously, so it is possible that the enzyme purified here has some characteristics that are divergent from other β-1,4-endoglucanases, depending on how this activity arose in the current host. Unfortunately, the purification scheme used here only yielded small amounts of enzyme and much more would be needed to uncover the kinetic parameters on the CMC substrate. This problem is compounded by the need to use the DNS reducing sugar assay to measure cleavage of the linear substrate which is a relatively insensitive method, i.e. the enzyme under study had to be incubated for up to 16h to produce a reliable result, whereas kinetic analysis requires initial velocities to be recorded.
6. Obtaining the *Aeromonas* sp. YS3 CMCase gene sequence

6.1 Introduction

By discovering the gene for CMCase activity from *Aeromonas* sp.YS3, a role for the enzyme may be elucidated. β-1,4-glucanases are predominantly thought of as existing as part of cellulase systems, used by cellulolytic organisms to degrade native cellulose as a carbon source. However, this type of enzyme is also seen in many Gram negative bacteria, even though the bacteria cannot degrade native cellulose and therefore do not use it as a carbon source (Robson and Chambliss 1989). Rather, the endoglucanases have been implicated in cellulose production by the bacteria themselves, as in the case of *Clavibacter michiganensis* ssp. *sepedonicus* (Laine et al. 2000). Additionally, endoglucanases are produced by various phytopathogenic bacteria such as *Pectobacterium chrysanthemi* (Lim et al. 2005) and *Pseudomonas solanacearum*, and are thought to be involved in breach of the plant cell walls by the bacteria (Huang and Schell 1992). Bacteria beneficial to plants also express endoglucanases during nodule formation in roots, as seen with *Azorhizobium caulinodans*, although endoglucanase deficient mutants of this bacterium could still carry out nodulation of the plant roots (Geelen et al. 1995). Therefore, although endoglucanases are often seen to be produced by bacteria that are found associated with plant materials, the endoglucanase enzymes might not be needed for such symbiosis. Alternatively, the bacteria could have acquired the genes for the endoglucanase enzymes by horizontal transfer from the plants (Ueda et al. 2003). The retention of such genes, even though they are not necessarily beneficial to the bacteria, is likely if they are linked to other genes that have been acquired in a similar way that are beneficial, such as genes needed for pathogenicity.

*Aeromonads* are often found associated with plant material, although they may be hard to isolate from such sources as they can exist in a non-culturable form, during their close association with the plants and other materials (Shukla et al. 1995).
6.2 Materials and methods

6.2.1 Creation and activity screening of a genomic library from *Aeromonas* sp. YS3

Genomic DNA was obtained from *Aeromonas* sp. YS3 using the Qiagen genomic DNA kit, as per the manufacturers instructions. A bacterial cell pellet obtained from 30mL overnight culture was lysed by adding 500 µL 10 mg/mL lysozyme, 400 µL 20 mg/mL proteinase K and incubating the resultant suspension at 37°C for 1 h.

Genomic DNA was partially digested with *Sau*3AI restriction enzyme (ng DNA cut with 1 U enzyme in a total volume of 50µL by incubating at 37°C for 25min). The resulting DNA fragments were size fractionated by electrophoresis on a 0.7% (w/v) agarose gel (as per section 3.2.8). Without exposing the DNA fragments in the gel to UV light, the fragments ranging in size from 2 -10 kb (estimated by comparison to 1 kb ladder from Invitrogen), were excised from the gel and extracted using the Qiagen gel extraction kit.

To clone the fragments of *Aeromonas* sp.YS3 DNA, pUC19 plasmid (Invitrogen) was used as the vector. The plasmid was linearised by cutting with 6 U BamHI at 37°C for 16 h. The plasmid DNA was cleaned by ethanol precipitation by incubating in 0.3 M sodium acetate pH 5.2, plus 1 volume of isopropanol at -20°C for 30 min. The solution was then centrifuged at 13 000 rpm for 5 min to collect the DNA, then 250µL 70% (v/v) ethanol was added, before the solution was recentrifuged. After the ethanol was decanted, the pellet was air dried and finally resuspended in water. The linearised plasmid was treated with shrimp alkaline phosphatase (Invitrogen) as per the manufacturers instructions to prevent self-ligation of the vector.

To ligate the genomic fragments (inserts) to the pUC19 backbone, the vector and insert were first ethanol precipitated so that the ligation could be performed in 10 µL, including 1 U T4 DNA ligase and 1 µL 10x T4 ligase buffer. The ligation was incubated at 4°C overnight before being used to transform *E. coli* electrocompetent SURE cells. Transformation involved adding 2 µL of the ligation reaction to 50 µL of carefully thawed cells and depositing in a chilled electroporation cuvette (0.1mm gap). The electroporation machine was set to deliver current at
200 Ω resistance, 25 µF capacitance and 1.6 V. After the cells were ‘zapped’ they were added to 1mL LB broth and incubated at 37°C for 90 min. To check for transformants, aliquots of the cells were spread-plated onto LB agar + 100 µg/mL Ampicillin. The plates also had 50 µL 0.1 M IPTG, 50 µL 2% (w/v) X-gal spread onto the surface to facilitate blue-white screening.

The genomic library was screened for CMCase by growing colonies of LB agar containing 0.2% (w/v) CMC and staining with Congo red. Additionally, some clones were grown on LB agar and then screened with the CMC overlay technique (as per section 3.2.9) to avoid any false positive reactions.

To check for inserts in the pUC19 vector in the transformants, minipreps were carried out using the Qiagen miniprep kit. Plasmid DNA was linearized to check for size of the insert by using BamHI or HindIII restriction enzymes (Invitrogen). Miniprep DNA was incubated with 2-6 units of enzyme and incubated at 37°C overnight before the products of the enzyme restriction were checked by agarose gel electrophoresis. Insert sequence was determined using M13 primers provided by Micromon sequencing.

6.2.2 Degenerate PCR to obtain the Aeromonas sp.YS3 bcsZ gene

To investigate the possibility of a cellulose synthase operon in Aeromonas sp. YS3, degenerate primers were designed to amplify the endoglucanase gene bcsZ. Protein sequences of various glycoside hydrolase family 8 endoglucanases arising from closely related Gram negative bacteria were aligned using CLUSTAL W. The resulting alignment was used to design CODEhop primers by first converting to blocks using BLOCK MAKER.

Degenerate PCR involved GoTaq master mix (Promega), 1 µL DNA (from Aeromonas sp.YS3 or E. coli for a positive control), and 0.5 µM of each of the forward and reverse primers in a total volume of 25 µL. Touchdown PCR involved the following cycle parameters: an initial denaturation at 94°C for 5 min, 12 cycles of: 94°C for 30s, annealing at 60 – 48°C (with a
decrease in temperature every cycle) for 30 s, extension at 72°C for 1 - 2 min; then a further 28 similar cycles with annealing at 48°C before a final extension at 72°C for 7 min.

After the initial set of degenerate primers returned the expected product (as determined by sequencing, carried out at Micromon sequencing facility), further sets of degenerate primers were designed in order to amplify larger sections of the operon. Eventually, using a combination of degenerate and perfect match primers, the whole \textit{bcsZ} gene from \textit{Aeromonas} sp. YS3 was amplified.

6.3 Results and discussion

6.3.1 Screening of the genomic library from \textit{Aeromonas} sp. YS3

A library was created in pUC19 plasmid with various sized inserts of genomic DNA from \textit{Aeromonas} sp. YS3. The library consisted of a total of 620 clones. The average size of the inserts was estimated by selecting 10 random clones and linearizing the pUC19 plasmids bearing the genomic inserts. The average size of the inserts was found to be 6-8 kb, therefore 620 clones was not a large enough library to ensure that all of the ~4.5 Mb genome of the bacterium was represented in the library (according to the below formula, with probability set to 95%).

\[
N = \frac{\ln(1-P)}{\ln(1-1/n)}, \text{ where } N \text{ - number of clones needed, } P \text{ - probability of finding a particular sequence, } n \text{ - ratio of genome size to the average cloned fragment size (Sambrook and Russell, 2001).}
\]

Regardless of the fact that not all of the genome was represented in the constructed library, an endoglucanase gene appeared to have been cloned, as one clone displayed clear endoglucanase activity on the CMC overlay assay (Figure 6.1). Blue colonies arising from the transformation and containing the pUC19 plasmid with no insert were also tested with the assay and at no point displayed CMCase activity on the overlay plates.
Figure 6.1. Clone no.532 (top) displaying weak endoglucanase activity on CMC. The ‘squiggles’ are growth resulting from strong inoculation with single clones, including no.346, with no genomic insert to act as a negative control (right).

However, clone no. 532, displaying CMCase activity was found not to contain a genomic insert and therefore the CMCase activity could not have been due to a cloned gene. The absence of an insert in clone no.532 was confirmed by restriction mapping (linearized plasmid from minipreps was the same size as original pUC19), PCR with M13 primers directed towards the pUC19 multiple cloning site (no insert was amplified) and sequencing (M13 primers revealed only pUC19 sequence).

The *E. coli* genome bears an endoglucanase gene that yields an enzyme similar in size to the one produced by *Aeromonas* sp.YS3 (40kDa, 39kDa respectively), although it is not normally expressed in laboratory strains (Park, 1999). Therefore clone no.532 was assumed to be a reversion to wild-type *E.coli*.

Another clone expressing slight CMCase activity was investigated. Sequencing of the insert sequence (using M13 primers) found that part of the insert coded for a putative diguanylate cyclase. A Blastx search of the insert sequence found homology with other putative diguanylate cyclase sequences from various Gram negative bacteria; 40-44% homology with sequences from *Acidothermus cellulolyticus, Acidovorax* sp., *Alkililinmicola ehrlichei, Chloroflexus aurantiacus, Frankia* sp.,
*Halorhodospira halophila*, *Magnetococcus* sp., *Magnetospirillum magneticum*, *Marinobacter aquaeolei*, *Myxococcus xanthus*, *Pseudomonas* spp., *Rhodopseudomonas palustris*, *Shewanella* spp., *Sphingopyxis alaskensis*, *Thiobacillus denitrificans*, *Wolinella succinogenes* (see Appendix 3). Diguanylate cyclases, or proteins bearing the GGDEF domain, are thought to regulate levels of cyclic-di-GMP, which in turn may regulate many important aspects of bacterial physiology such as exopolysaccharides and cell surface appendages (D’Argenio and Miller 2004).

The bacterial cellulose synthesis (*bcs*) operon, originally found in *Gluconoacetobacter xylinus*, and now known to occur in other bacteria such as *Salmonella typhimurium* and *E. coli*, is thought to be regulated by c-di-GMP (Zogaj et al. 2001). The *bcs* operon includes the endoglucanase gene, *bcsZ* (formerly known as *yhjM* in *E. coli*) (Zogaj et al. 2001), therefore diguanylate cyclases indirectly regulate (to some extent) the endoglucanase production in bacteria bearing the *bcs* operon.

The cloned putatative diguanylate cyclase from *Aeromonas* sp. YS3 may have directed slight CMCase production from the *E. coli* host cells. Bacterial cellulose production was once thought to be limited to only a few species but it now seems that c-di-GMP might regulate this phenotype in various Gram negative bacteria (Zogaj et al. 2001). Therefore the possibility of a bacterial cellulose synthesis operon in *Aeromonas* sp. YS3 was investigated. Many Gram negative bacterial species have now had their entire genomes sequenced, and sequences bearing homology to the *E. coli bcsZ* gene are easy to find. With the assumption that the *bcs* operon arose in the bacteria a long time ago (evolutionarily), sequences of the *bcsZ* gene from gamma proteobacteria were collected, as these were suspected to be most homologous to the putatative *Aeromonas* sp. YS3 sequence.
Translated bcsZ sequences from *E. coli*, *Pseudomonas fluorescens*, *P. syringae*, *Salmonella typhimurium* and *Xanthomonas campestris* were aligned using CLUSTAL W (Thompson et al. 1994) to find conserved regions.

![Alignment of predicted protein sequences of GH family 8 endoglucanases from selected gamma proteobacteria.](alignment_image)

**Figure 6.2.** Alignment of predicted protein sequences of GH family 8 endoglucanases from selected gamma proteobacteria. This portion of the alignment (performed with CLUSTAL W) contains the two regions that were completely conserved (boxed), from which degenerate PCR primers were designed.
The alignment of predicted protein sequences from *bcsZ* genes (Figure 6.2) was converted to blocks of sequence using BLOCK MAKER software (Henikoff *et al.* 1995), then was used as input for CODEHOP software (Rose *et al.* 1998), which enabled the design of degenerate PCR primers. The primers designed to bind to the conserved regions of the endoglucanase (*bcsZ*) genes were as follows:

\[
\text{bcsZ-F 5'} - \text{CGAGCACCCTGCCGCNTGGYTNTG} \text{G} - 3' \\
\text{bcsZ-R 5'} - \text{CCCACATGTAGACCGGATNGCRTCA} - 3'
\]

(where N = A/C/T/G, Y = C/T, R = A/G)

PCR reactions with this primer set yielded the expected size product of ~500 bp, whether the template was *Aeromonas* sp.YS3 genomic DNA or *E. coli* DNA (included as a positive control). Good quality sequence data was obtained for 430 bases of the *Aeromonas* sp.YS3 PCR product (see Appendix 4). A Blastx (translated) search of this sequence revealed homology with various GH family 8 endoglucanases from various Gram negative bacteria, as expected. High homology (50-64 %) was found with sequences from *Yersinia* spp., *Pectobacterium carotovorum*, *Salmonella* spp., *Shigella* spp., *E. coli*, *Vibrio fischeri*, *Xanthomonas* spp., *Burkholderia* spp., *Ralstonia eutropha*, *Chromobacterium violaceum* and *Pseudomonas putida*. The wealth of similar sequences coding for a glycoside hydrolase family 8 endoglucanase in so many Gram negative species of bacteria suggested that the *bcsZ* gene has been retained over a long evolutionary timescale.

Fortunately many Gram negative bacterial genomes have been fully sequenced. This meant that not only the *bcsZ* gene sequences but also other *bcs* operon genes were available in the NCBI databases. The *Aeromonas* sp.YS3 partial *bcsZ* gene was most similar to sequences from *Yersinia* spp. and *Pectobacterium* (formerly *Erwinia*) *carotovorum*. Genome sequencing of these species has revealed *bcs* genes in the same operon structure as found in *E. coli*, *Salmonella typhimurium* and *Pseudomonas putida* (Zogaj *et al.* 2001) (Figure 6.3).
Yersinia bcs operon

Figure 6.3 VNTi diagram (constructed using Vector NTi software) of *Yersina bcs* operon, annotated to show primer binding sites and expected PCR products from *Aeromonas* sp.YS3 template.

With the assumption that the rest of the *bcs* operon was also conserved in *Aeromonas* sp.YS3, degenerate primers were designed for *bcsB* and *bcsC* genes so that the full sequence for the *Aeromonas* sp.YS3 *bcsZ* gene could be obtained. An alignment of *bcsB* genes from *Yersinia intermedia*, *Y. frederiksenii*, *Salmonella enterica*, *S. typhimurium*, *E. coli*, *Shigella boydii*, *Sh. sonnei*, *Sh. flexneri*, *Sh. dysenteriae* and *Pectobacterium carotovora* was carried out using CLUSTAL W (figure 6.4).

![Figure 6.4](image_url)

**Figure 6.4.** Part of the Alignment of *bcsB* genes showing the conserved region to which degenerate primers were designed.
As previously, the alignment was used to generate CODEHOP primers. The chosen primer was then paired with a perfect match primer designed from the compliment of the sequence data already obtained for *Aeromonas* sp.YS3 bcsZ, in order to amplify the unknown 5' region of *Aeromonas* sp.YS3:

- **bcsB-Forward primer**  
  5' – TGGTGCCGCCTAAACCNTAYGAYGC - 3'

- **Perfect match egl reverse primer**  
  5’ – GATCCAGAGATCCGAATCGGAC - 3'

Similarly, an alignment of *bcsC* genes was carried out and a conserved region to which primers could be designed was found (figure 6.5). The degenerate primer was once again paired with a perfect match primer for the known *bcsZ* sequence to amplify the unknown 3' sequence:

- **bcsC reverse primer**  
  5' – GCCACCGTGCGCCARTAYTCNAC - 3'

- **Perfect match egl forward primer**  
  5’ – GCCCTCTTGCCCAAATTGTTG C - 3'

Using the *Yersinia* bcs operon as a guide, the expected size products of the PCR using bcsB, bcsC primers were ~1.6/1.1 kb, respectively. Multiple products were seen in either case, but the expected size products were always the most abundant from the reaction. When the 1.6 kb product of the bcsB PCR was sequenced using the perfect match primer, 900 bases of sequence were obtained (see Appendix 4) and used in a Blastx search. As expected, the sequence had high homology to the products of *bcsB* and *bcsZ* genes from *Pectobacterium carotovorum* and *Yersinia* spp (58-61% homology between the endoglucanase sequences).
When PCR products from the bcsC primer set was sequenced (one of ~900 bp and another at ~1.1 kb), only very poor quality sequence data, not sufficient to use in a Blast search, was obtained. Therefore, another perfect match primer was designed from the now known Aeromonas sp.YS3 bcsB data:

Perfect match BcsB-F 5′ – AACTGTTTGCCCGTTATCC - 3′

When this primer was paired with the degenerate primer designed from bcsC sequences, it was hoped that the entire Aeromonas sp.YS3 bcsZ gene would be amplified. However, although expected size products of between 1.5-2 kb resulted from such PCRs, when sequenced they once again produced only very low quality sequence data, meaning that the perfect match primers (PMB-F as well as another nested primer designed from the bcsZ sequence data) were only binding transiently or not at all to these PCR products. Therefore the products of the reactions were thought to not contain fragments of either bcsC or bcsZ. The reason for this may be either that bcsC in Aeromonas spp. may be sufficiently different from the other sequences examined (so that the degenerate primer did not bind) or that Aeromonas spp. have a differently structured bcs operon that is lacking bcsC or has the gene at another locus. The gene product of bcsC is a putative oxidoreductase of unknown function and it is therefore not known what role it plays in cellulose biosynthesis. The operon structure of bcs genes has been seen to be conserved between various gamma proteobacteria (such as E. coli and Salmonella spp.), whilst a different order of genes is displayed in the genomes of alpha proteobacteria (Zogaj et al. 2001). Evidence that the bcs operon of Aeromonas sp.YS3 (also gamma proteobacteria) is missing the bcsC gene downstream from bcsZ is therefore surprising.

The existence of a partial or reorganized bcs operon in Aeromonas spp. may have implications for the mode of growth of the bacteria in various environments. In Agrobacterium spp. and Rhizobia cellulose produced as a product of the bcs operon is thought to facilitate the bacterial attachment to plant roots in either pathogenic or symbiotic relationships (Ross et al. 1991). Aeromonads are often found associated with plant roots (Shukla et al. 1995) and they may be beneficial to the plants by reducing Na^+ uptake (Ashraf et al. 2004) and improving nodulation.
of plants roots indirectly, by improving plant growth generally (Zhang et al. 1997). Perhaps more importantly, the exopolysaccharide (cellulose, cellulose derivatives or other polymers) produced by pathogenic bacteria, such as the close relative to Aeromonas, V. cholerae, allow them to persist in aquatic environments, where they may be a danger to human health (Tischler and Camilli 2004). Therefore an understanding of the bcs genes and the functioning of their products is of medical as well as ecological importance.
6. Conclusions and future directions

The first aim of this project was to isolate cellulolytic organisms from the diverse microbial source, activated sludge. Two selective enrichment methods were employed, using either the Evolver™ Technology or isolation on filter paper. The selection of the desired cellulolytic microorganisms was limited in either case, as microorganisms unable to use the supplied carbon source persisted, presumably surviving off the products of cellulose hydrolysis. Nonetheless, it was evident that cellulolytic microorganisms were also selected for. Degradation of filter paper (highly crystalline cellulose) could be monitored visually whether the inoculum was sludge or culture from the Evolver™.

The Evolver™ is purported to provide better selection of microorganisms able to utilise a particular nutrient. The advantages of the technology are that it provides real-time monitoring of the metabolism occurring within the culture (in this case followed as OUR) as well as assisting selection by providing a flow rate of liquid media through the culture (Bridger et al. 2005). In this particular case, cellulose is an unusual energy source in that, unlike smaller carbon based molecules it is almost chemically inert and therefore unlikely to be toxic to any form of life. Therefore, the carbon source itself provides no negative selection, in comparison to other situations where the carbon source or the concentration thereof may provide some initial selection. Additionally, cellulose is commonly broken down to either cellobiose or glucose, which can serve as carbon sources for a large variety of microorganisms. Therefore, the rise in OUR associated with addition of cellulose to the Evolver™ culture reflects not only cellulose degradation but also the microorganisms surviving off the products of hydrolysis as well as any predation or other cross feeding that can also occur. Such complex and competitive interrelationships were thought to account for the fact that high OUR values could not be maintained when cellulose was continually added to the Evolver™ culture.
The Evolver™ was expected to have an advantage over the filter paper method because selection can be enhanced by increasing the flow rate throughout the culture vessel. A consistently high level of OUR attributable to cellulose hydrolysis would have prompted an increase in flow rate, to hopefully wash out those microorganisms that weren’t using the cellulose carbon source as quickly as others. However, only low levels of OUR reflected that the cellulose was not being extensively degraded and increasing the flow rate may have had the opposite effect. That is, if degradation of cellulose by the microorganisms was slow, increasing the flow rate may have washed out the cellulolytic organisms whilst leaving those with a fast growth rate on glucose remaining. Furthermore, there was evidence of biofilm growth in the Evolver™ culture vessel, which may have hampered selection by allowing non-cellulolytic organisms to persist even when little carbon was available to them.

Maintenance energy levels of bacteria in starvation states has been found to be very low (Morita 1988) so it is possible that non-cellulolytic bacteria could persist for some time after the cellulolytic bacteria are removed, especially if lysis of some cells releases nutrients to maintain others. An advantage of the filter paper method of isolation is that hydrolysis of the filter paper itself is clearly visible, so it is always known when the cellulolytic organisms are present.

An important factor in the selection process, using either the Evolver™ or the filter paper method, is the choice of media for isolation of the microorganisms. The 461S media employed, although it was completely defined and therefore able to determine use of a sole carbon source, may not have been ideal. The addition of 461S media to the Evolver™ caused a drop in OUR, which may have reflected intolerance of some of the microorganisms to the levels of various nutrients in the media. Additionally, although cellulose hydrolysis was seen to occur in the consortiums of microorganisms cultured, no single pure culture was seen to utilise cellulose as a sole carbon source when grown on 461S. Therefore, it was suspected that the microorganisms responsible for the hydrolysis of the cellulose required nutrients that were supplied to them whilst growing in the diverse consortium but were
lacking in pure culture on 461S. The use of a semi-defined media such as ST6 (containing a small amount of yeast extract) may have been advantageous in this case.

Another possibility accounting for the fact that truly cellulolytic microorganisms were not isolated in culture may have been that the microorganisms responsible were protozoa and not the bacteria investigated. Soldo and Brickson (1980) outline a method for isolating protozoa in pure culture, from environmental samples, which may be useful in the future isolation of cellulolytic microorganisms.

Whilst truly cellulolytic microorganisms (able to degrade crystalline cellulose) were not isolated, a novel phenotype of β-1,4-endoglucanase activity was found in *Aeromonas* sp.YS3. This activity was attributed to a 39kDa enzyme that was produced in the culture supernatant at very low levels and seemed to be co-produced with an exopolysaccharide material. Therefore, although the bacterium was isolated in a situation where hydrolysis of cellulose was occurring, it was likely that the enzyme was not produced for this function. Indeed, if the enzyme is required for the synthesis of exopolysaccharide, as has been seen in other Gram negative bacteria bearing glycoside hydrolase family 8 cellulases (Römling 2002), then the maintenance of this polysaccharide material may have enabled the bacterium to survive in a biofilm at the sites of cellulose hydrolysis on the filter paper plates. In that way the bacteria could remain in close contact with the surfaces (making it hard to eliminate in subsequent subculture), whilst taking advantage of any liberated sugars from the cellulose hydrolysis to construct the polysaccharide itself.

Evidence to support the hypothesis that the endoglucanase enzyme was implicated in exopolysaccharide synthesis was the discovery of a gene homologous to *bcsZ* from the bacterial cellulose synthesis operon. Further work is needed before it can be concluded that the 39kDa β-1,4-endoglucanase from *Aeromonas* sp.YS3 is a product of this gene and if it is required for exopolysaccharide synthesis/processing. The fact that many Gram negative
bacteria with slight endoglucanase activity have also been found to bear bcs operon genes (Mølhøj et al. 2002) goes some way to understanding the function of these enzymes as they are clearly not sufficient to enable the bacteria to degrade native cellulose. It would be interesting to uncover the structure and components of the polysaccharide material from this particular strain of Aeromonas. Unfortunately the standard extraction procedure used in this study did not yield material of sufficient purity to carry out these studies.

Additionally, if pure exopolysaccharide could be obtained, the action of the 39kDa enzyme on this material could be more closely examined. It is possible that the endoglucanase present as part of the cellulose synthase modifies the exopolysaccharide material by cleaving/rejoining it at particular junctions. This information may be important in studying host/pathogen interactions.

The exopolysaccharide from Aeromonas sp. YS3 is expected to be more chemically complex than cellulose, as exopolysaccharide material from various Aeromonas spp. has been found to contain other sugar monomers along with glucose (Ueda et al. 1981; Bricknell et al. 1997; Xu et al. 2004). It is possible that either this exopolysaccharide is produced from genes separate from the bcs operon, or that the different structure of bcs genes in Aeromonas spp. result in a modified cellulosic polymer rather than the cellulose seen in Salmonella spp. If the latter is the case, it is interesting to speculate what role the different polysaccharides play in pathogenesis. Bacteria with the same bcs operon structure as Salmonella typhimurium include the human pathogens E. coli and Yersinia pestis as well as the plant pathogen Erwinia carotovorum. It is possible that cellulose protects such bacteria from host immune responses whilst the more complex EPS material of Aeromonas spp. is more readily recognized. If this is the case, a greater understanding of the bcs genes and their products should help combat these pathogens.

If the β-1,4-endoglucanase of Aeromonas sp. YS3 is required for exopolysaccharide synthesis and therefore biofilm formation, studies examining the regulation of this process may be important
when considering the pathogenicity of this bacterial genus in general. Environmental *Aeromonas* spp., found to be closely adherent to aquatic plants, were shown to possess beta-haemolytic activity, a potentially cytotoxic behaviour (Shukla et al. 1995). Particular *Aeromonas* spp. cause diseases in fish resulting in severe losses in production farms (Braun et al. 2002). The ubiquitous nature of *Aeromonas* in aquatic habitats worldwide and their ability to tolerate temperature ranges from 4-42°C is a concern when the bacteria have been associated with various human diseases such as gastrointestinal infections, septicaemia, endocarditis and peritonitis (Martinez-Murcia et al. 1992). The ability of the common *Aeromonas* spp. to form biofilms may have implications on colonization of water distribution systems or food processing systems (Bechet and Blondeau 2003).

Alternatively, the biofilm forming abilities of *Aeromonas* spp. has been seen to be advantageous when it enables the bacteria to remain in close contact with plant roots. *Aeromonas hydrophila* was classified as a plant growth-promoting rhizobacteria, as it increased plant growth and development when inoculated onto soybean roots (Zhang et al. 1997). Additionally, *Aeromonas caviae* has been shown to be effective as a biocontrol agent, as it can prevent fungal infections of roots, utilizing chitinase enzymes (Inbar and Chet 1991). Therefore, understanding the regulation of biofilm formation, which is seen to be variable in *Aeromonas* spp. (Bechet and Blondeau 2003), will be valuable in taking advantage of the plant growth promoting ability.

The aim of discovering novel cellulose enzymes/microorganisms was not fulfilled with this work, but the closer examination of the appearance of a previously unstudied phenotype in an *Aeromonas* sp. was surprisingly illuminating. The glycoside hydrolase family 8 endoglucanase enzymes may have been thought to be of little use industrially as they are produced in small quantities and are not catalytically efficient. However, this is unsurprising if the natural function of the enzymes is not to degrade polysaccharide material but to assist in the synthesis of such materials. Understanding the function of these enzymes more, will hopefully help describe in detail the biofilm forming behaviours of bacteria. Such behaviours, seen here with the environmental isolate *Aeromonas* sp. YS3., (for example, ‘fruiting body-like structures’) are
indeed very complex and more work needs to be done to understand the regulation and functioning of the enzymes involved.
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Appendix 1. Blastn results with sequenced 16s rDNA PCR products as query.

Isolate: YS3

The query sequence consisted of 741 bases.
The closest match in the NCBI databases was: *Aeromonas veronii* clone 3 16S ribosomal RNA gene, partial sequence. Accession number: AF418211.1
The two sequences aligned between position 304-1043 of the subject sequence, with 740/741 identities (99% similar).

Isolate: YS5

The query sequence consisted of 741 bases.
The closest match in the NCBI databases was: *Aeromonas caviae* 16S rRNA gene, strain NCIMB 13016. Accession number: AC16SRDN
The two sequences aligned between position 331-1071 of the subject sequence, with 741/741 identities (100% similar).

Isolate: CH3

The query sequence consisted of 781 bases.
The closest match in the NCBI databases was: *Cellvibrio ostraviensis* partial 16S rRNA gene, type strain LMG 19434T. Accession number: AJ493583
The two sequences aligned between position 282-1062 of the subject sequence, with 764/781 identities (97% similar).

Isolate: BC03.1

The query sequence consisted of 601 bases.
The closest match in the NCBI databases was: *Pigmentiphaga kullae* strain K24 16S ribosomal RNA gene, partial. Accession number: AF282916
The two sequences aligned between position 173-773 of the subject sequence, with 585/601 identities (97% similar).

Isolate: BC03.3

The query sequence consisted of 1048 bases.
The closest match in the NCBI databases was: *Bacillus cereus* strain 213 16S ribosomal RNA gene, partial sequence. Accession number: EF593043
The two sequences aligned between position 63-1110 of the subject sequence, with 1036/1048 identities (98% similar).

Isolate: BC06

The query sequence consisted of 727 bases.
The closest match in the NCBI databases was: *Chryseobacterium indologenes* 16S ribosomal RNA gene, partial sequence. Accession number: FVBR16SD
The two sequences aligned between position 1277-551 of the subject sequence, with 710/727 identities (97% similar).
Isolate: EV1

The query sequence consisted of 854 bases. The closest match in the NCBI databases was: *Acidovorax delafieldii* 16S rRNA gene, strain DSM 50263. Accession number: [AJ420323](https://www.ncbi.nlm.nih.gov/nuccore/AJ420323). The two sequences aligned between position 297-1148 of the subject sequence, with 846/854 identities (99% similar).

Isolate: EV7

The query sequence consisted of 944 bases. The closest match in the NCBI databases was: *Sphingomonas capsulata* DNA for 16S ribosomal RNA. Accession number: [D16147](https://www.ncbi.nlm.nih.gov/nuccore/D16147). The two sequences aligned between position 276-1219 of the subject sequence, with 934/944 identities (98% similar).

Isolate: EV8

The query sequence consisted of 858 bases. The closest match in the NCBI databases was: *Sphingomonas capsulata* DNA for 16S ribosomal RNA. Accession number: [D16147](https://www.ncbi.nlm.nih.gov/nuccore/D16147). The two sequences aligned between position 281-1138 of the subject sequence, with 849/858 identities (98% similar).
Appendix 2. Analysis of clone no.28

Sequence data obtained by amplifying the insert region of pUC19 with M13 primers:

GACTGGTACGGGACTGCAGGTCGGCAGGCAGCCAGACTCGAGTGTCGCGTCGCTAGGAGAACGAGAACAGCGCCGACCAACTGCTCTACACGACGAGTGCCGCTGCTACTACCTATTGCTGCTGCTGGTGCTGCTGGCCGTCGCCCTGATCCTGCTCGGGCTCACCACCGCAGCCTCCACCGCAGCTCGCGCCACCTCTACAAGCTCGCCACCTTCGATCAGCTGACCGGGCTGCCCAACCGGCGCGCCCTGCTGGAGAGACTGGGTCAGCAGTGGCTGCAAGAAAACCCCCTTCAAGCTCAGTTCATCCATCCGGATCAGCCGGTTGCCATCGGCGACGAATCTCTCCTGGGCCCGTACATCTTGTCGGCAAACAGCCGCTGCATCTGCTCGATGCAGCCCTCGTGAACACACG

This sequence was used in a Blastx search with the following results:

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Accession number</th>
<th>Identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGDEF [Shewanella sp. PV-4]</td>
<td>ZP_00836998</td>
<td>79/177 (44%)</td>
</tr>
<tr>
<td>Putative diguanylate cyclase (GGDEF domain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Pseudomonas fluorescens PfO-1]</td>
<td>YP_350394</td>
<td>76/176 (43%)</td>
</tr>
<tr>
<td>Putative GAF sensor protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Alkalilimnicola ehrlichii MLHE-1]</td>
<td>YP_742336</td>
<td>77/172 (44%)</td>
</tr>
<tr>
<td>GGDEF [Frankia sp. EAN1pec]</td>
<td>ZP_00571766</td>
<td>75/174 (43%)</td>
</tr>
<tr>
<td>GGDEF [Halorhodospira halophila SL1]</td>
<td>ZP_01152047</td>
<td>72/180 (40%)</td>
</tr>
<tr>
<td>FOG: GGDEF domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Magnetospirillum magneticum AMB-1]</td>
<td>YP_423778</td>
<td>73/172 (42%)</td>
</tr>
<tr>
<td>Diguanylate cyclase (GGDEF domain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Rhodopseudomonas palustris BisB18]</td>
<td>YP_532029</td>
<td>76/173 (43%)</td>
</tr>
<tr>
<td>GGDEF [Marinobacter aquaolei VT8]</td>
<td>ZP_00816878</td>
<td>72/175 (41%)</td>
</tr>
<tr>
<td>GGDEF [Chloroflexus aurantiacus J-10-1]</td>
<td>ZP_00767779</td>
<td>72/173 (41%)</td>
</tr>
<tr>
<td>Diguanylate cyclase with PAS/PAC sensor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Sphingopyxis alaskensis RB2256]</td>
<td>YP_616741</td>
<td>67/159 (42%)</td>
</tr>
<tr>
<td>GGDEF [Acidothermus cellulolyticus III]</td>
<td>ZP_01136306</td>
<td>67/158 (42%)</td>
</tr>
<tr>
<td>GGDEF domain [Acidovorax sp. JS42]</td>
<td>ZP_01382142</td>
<td>71/176 (40%)</td>
</tr>
<tr>
<td>Sequence name</td>
<td>Accession number</td>
<td>Identities</td>
</tr>
<tr>
<td>---------------------------------------------------</td>
<td>------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Putative diguanylate cyclase (GGDEF domain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Thiobacillus denitrificans ATCC 25259]</td>
<td>YP_315781</td>
<td>71/166 (42%)</td>
</tr>
<tr>
<td>GGDEF [Magnetococcus sp. MC-1]</td>
<td>ZP_00605766</td>
<td>69/169 (40%)</td>
</tr>
<tr>
<td>FHA domain/GGDEF domain protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Myxococcus xanthus DK 1622]</td>
<td>YP_629777</td>
<td>64/164 (42%)</td>
</tr>
<tr>
<td>ChyY like receiver and GGDEF domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Wolinella succcinogenes DSM I740]</td>
<td>NP_907964</td>
<td>71/171 (41%)</td>
</tr>
</tbody>
</table>


Figure 6.2 (Alignment of GH family 8 sequences from selected gamma proteobacteria)
<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BcsC E.coli K12</td>
<td>AAC76556</td>
</tr>
<tr>
<td>BcsZ E.coli W3110</td>
<td>BAE77763.1</td>
</tr>
<tr>
<td>WssD Pseudomonas fluorescens</td>
<td>AAL71844.1</td>
</tr>
<tr>
<td>WssD Pseudomonas syringae</td>
<td>AAO54560.1</td>
</tr>
<tr>
<td>BcsZ Salmonella typhimurium</td>
<td>CAC44017.1</td>
</tr>
<tr>
<td>BcsC Salmonella typhimurium</td>
<td>AAL22477.1</td>
</tr>
<tr>
<td>BcsZ Xanthomonas campestris</td>
<td>CAJ25372.1</td>
</tr>
</tbody>
</table>

Figure 6.4 (BcsB genes from Gram neg. bacteria)
<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yersinia intermedia BcsB</td>
<td>ZP_00832010</td>
</tr>
<tr>
<td>Yersinia frederiksenii BcsB</td>
<td>ZP_00830672</td>
</tr>
<tr>
<td>Salmonella enterica BcsB</td>
<td>YP_152595</td>
</tr>
<tr>
<td>E.coli BcsB</td>
<td>ABJ03014</td>
</tr>
<tr>
<td>Salmonella typhimurium BcsB</td>
<td>NP_462519</td>
</tr>
<tr>
<td>Shigella boydii BcsB</td>
<td>YP_409843</td>
</tr>
<tr>
<td>Shigella sonnei BcsB</td>
<td>YP_312634</td>
</tr>
<tr>
<td>Shigella flexneri BcsB</td>
<td>YP_690893</td>
</tr>
<tr>
<td>Shigella dysenteriae BcsB</td>
<td>YP_405912</td>
</tr>
<tr>
<td>Pectobacterium carotovora BcsB</td>
<td>YP_052457</td>
</tr>
</tbody>
</table>

Figure 6.5 (BcsC genes from Gram neg. bacteria)
<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yersinia intermedia BcsC</td>
<td>ZP_00832008</td>
</tr>
<tr>
<td>Yersinia frederiksenii BcsC</td>
<td>ZP_00830670</td>
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<tr>
<td>Salmonella typhimurium BcsC</td>
<td>NP_462517</td>
</tr>
<tr>
<td>Salmonella enterica BcsC</td>
<td>YP_152593</td>
</tr>
<tr>
<td>E.coli BcsC</td>
<td>ZP_00717982</td>
</tr>
<tr>
<td>Shigella boydii BcsC</td>
<td>YP_409841</td>
</tr>
<tr>
<td>Pectobacterium carotovora BcsC</td>
<td>YP_052459</td>
</tr>
</tbody>
</table>
Appendix 4. Sequences obtained for YS3 endoglucanase gene (BcsZ)

Using primers (degenerate) bcsZ forward and reverse (see p152), the following section of the gene was amplified and sequenced:

Using perfect match egl-R and a degenerate primer designed to BcsB sequences, the following sequence data was obtained:

Perfect match BcsB-F primer position