PURIFICATION AND CHARACTERIZATION OF MICROBIAL CELLULOLYTIC ENZYMES

BY

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DEDICATED TO MY PARENTS
ACKNOWLEDGEMENT
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Amtul Jamil Sami
PREFACE

Cellulose, the most abundant organic compound on earth, can be degraded into glucose by acid and enzymatic hydrolysis. Enzymatic hydrolysis has a greater potential in the conversion of cellulose to glucose. A variety of microbial species are known to produce enzyme system for the efficient hydrolysis of cellulose into glucose. For the application of cellulases in the bioconversion of cellulose, it is necessary to purify and characterise these enzymes which include endo-1,4-B-D-glucanase, exo-1,4-B-D-glucanase and 1,4-B-D-glucan gluconohydrolase.

A research programme on the "Bioconversion of cellulosic materials" is going on in this laboratory and the work presented here forms a part of this programme. As a result of a screening programme on isolation of cellulase producing organisms a potent bacterial species Cellulomonas flavigena was isolated and conditions were optimised for the production of its cellulases. Different extracellular endo-glucanase (CMCases) were purified and characterised.

These studies along with the further work going on in this laboratory should contribute in developing a system for the efficient degradation of cellulose into glucose.
ABSTRACT
ABSTRACT

A locally isolated, mesophilic, cellulolytic, facultatively aerobic bacterium identified as *Cellulomonas flavigena* was studied for the production, purification and characterization of extracellular cellulolytic enzymes. When the microbe was cultivated in the presence of 0.5% Avicel as carbon source, it produced higher levels of CMCase as well as avicelase activities as compared to those produced in the presence of 0.5% CMC. Tween-80 when added to the culture medium at a concentration of 0.1% resulted in more than two fold increase in the enzyme activities in the presence of Avicel as substrate. Extracellular CMCase and avicelase activities increased with the increase in concentration of yeast extract upto a level of 0.2%, beyond which no further increase took place in the enzyme activities. Maximal CMCase and avicelase activities were 10.0 and 1.2 U/ml of the culture supernatant. These were obtained after 72 h of fermentation at which stage, the growth as determined in terms of cellular proteins, was maximal. Production of extracellular cellulases, both free and Avicel bound was studied in the presence of different concentrations of Avicel. It was found that both the enzyme activities in the culture supernatant were maximum in the presence of 0.5% Avicel. Beyond this concentration there was a decrease in the enzyme activities in the culture supernatant.
Characteristics of Avicelase and CMCcase activities were studied and it was found that Avicelase and CMCcase were most active at pH 6.5 and 50°C. End product of each of the enzyme activity was cellobiose along with glucose some short fragments of carbohydrates. Both the enzyme activities were stable in the pH range of 3-11 and 0-10°C and 100% activities were lost when incubated at 70°C for 30 minutes. Calcium and magnesium activated the enzymes when used in the concentration of 10-15mM, while NaCl had no effect on both the activities. Heavy metal ions i.e. silver and iron inhibited both the enzymes. Enzymes were inhibited in the presence of cellobiose and glucose upto 30 mM in the reaction mixture. β-mercaptoethanol strictly inactivated the enzyme. C.flavigena also secreted protease activity which showed two pH optima i.e. 6.5 and 8.9.

A substantial amount of cellulase activity bound to the residual avicel during fermentation. Conditions were optimized for the elution of the substrate bound cellulases from residual Avicel. Sonication or shaking of residual Avicel from the specific hours of fermentation medium with distilled water were equally effective for the elution of substrate-bound enzymes. Maximum CMCcase activities were bound to the substrate during initial stages of fermentation. Higher proportions of avicelase activity remained bound to be insoluble cellulosic substrate throughout the fermentation periods studied. Maximal total CMCcase activities (both the free and substrate bound activities) obtained with 2% Avicel as carbon source were 13 U/ml and for avicelase it was 3.0 U/ml of the culture medium.
A comparative study of substrate bound and free CMCase activities was done on PAGE followed by zymogram technique. It was found that free activities comprised of at least 7 CMCase bands while the bound activities showed only four major bands active against CMC.

For purification of CMCases a sequential procedure was employed including precipitation with 80% acetone, gel filtration, ion-exchange chromatography and preparative gradient PAGE. First two purification steps increased specific activities up to three times e.g. 330 U CMCase/mg protein and 36 U avicelase/mg protein. Each of the purified enzyme CMCase 1, CMCase 2, CMCase 3, CMCase 4, and CMCase 5 showed single band on native PAGE and SDS-PAGE.

Characteristics of all the purified CMCase were studied and it was found that all of them were active at 45-50°C and were only active against CMC. The major hydrolytic products of all the enzymes were oooligosaccharides and cellobiose. Each of the enzyme was inhibited in the presence of cellobiose and glucose when added to above 15 mM concentration. Enzymes were stable in the temperature range of 0 - 30°C and pH of 4 - 9.5. Ca++ and Mg++ has no considerable effect on the enzyme activities while iron and silver were proved to be their strict inhibitors. β-mercepto ethanol also inhibited these enzymes. The purified enzymes differed in their mobilities on non denaturing gradient PAGE as appeared on zymogram. pH optima of these enzymes ranged between 6.5 - 7.0 CMCase 1 showed a sharp pH optimum at 7.0 while CMCase 3 and 5 were most active at pH 6.5 - 7.0 and CMCase 4 exhibited maximum activities at pH 6.5. Molecular weights
of each of the enzymes were estimated by SDS-PAGE. CMCase 1 and CMCase 2 were both found to have molecular weight of 20,400, CMCase 4 showed a molecular weight of 40,000, while CMCase 3 and 5 had same molecular weight of 80,000. Km value of each of the purified enzyme was determined by using CMC as substrate. These were 0.83, 1.7, 1.54, 1.64, 1.6 g/l for CMCase 1, 2, 3, 4 and 5 respectively.
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ABBREVIATION

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CELLULOSE

Cellulose is the most abundant biological organic compound on terrestrial earth. It is a linear glucopolymer composed of anhydro-glucose units joined to each other by β 1-4 glucosidic bonds (Fig.1). The anhydrous glucose unit adopt the chain configuration shown in Fig.2 with the units rotated 180°C about the main axis in proportion to each other. The result is a strained linear configuration with minimum steric hinderance. Cellulose molecules are joined together by hydrogen bonds to give larger units which are ordered in crystalline manner. Moyer and Mish have proposed a structure for monoclinic unit cell of native cellulose (Fig.2). The model contains four glucose residues in each unit cell. These include two in the centre and one fourth of each of the 8 residues which are placed at the corner of the monoclinic cell, and share each of the four cells which meet at corners. Gardner and Blackwell supported this model by using X-ray crystallography and proposed a parallel chain model for cellulose, with all the molecules lying in the same direction, a hydrogen bonding network, with one intermolecular and two intermolecular bonds. This supports glycosidic bonds in maintaining the rigidity of the cellulose molecules. Molecules are joined to each other by Vander Waals forces (Fig.3).

HYDROLYSIS

Cellulose can be hydrolysed into its subunits by acid and enzymatic hydrolysis. Enzymatic hydrolysis has a potential to overcome many of the drawbacks of acid
Fig. 1 Conformational structure for cellulose.

Fig. 2 The monoclinic unit cell of native cellulose, according to Meyer and Misch (1937), but which the fibre axis designated as the c axis. The chains lying in the front right and hind left corners have not been drawn. Note that the central chain is in an antiparallel position. According to Gardner and Blackwell (1974) all chains are
Fig. 3 The hydrogen-bonding network in cellulose. Each glucose residue forms two intramolecular bonds (O3-H ... O5' and O6 ... H-O2') and one intermolecular bond (O6-H ... O3)
hydrolysis. The conversion is carried out under mild conditions, thus greatly reducing the cost of hydrolysis equipment. Sugars decomposition is avoided, thus eliminating this cause for loss in yield and producing clear sugar streams for further processing. Costly neutralization and purification equipment is unnecessary, and disposal of waste streams from acid neutralization are eliminated. Balancing these potential savings, extensive pretreatment to breakdown lignin and increase cellulose accessibility is required to achieve good yields. The cost of high activity cellulolytic enzymes solution is at present very high. Extensive research to overcome these problems is underway. Mutation and selection methods have been used to develop and isolate Fusarium, Phanerochaete and Trichoderma strains of high cellulolytic activities. The highest activity enzyme solution thus so far prepared are those of Trichoderma reesei Rut C30 isolated at Rutgers University.

Researchers are interested in the maximum yield of microbial cellulolytic enzymes by using waste cellulosic materials. Martin et al. have studied the production of cellulase along with the hydrolysis of steam exploded wood. Cellulases of T. reesei are also produced on waste cellophane and physically treated wheat straw. Comparison of different cellulolytic fungi for bioconversion of apple distillery waste has been studied. Enzyme Saccharification of agricultural residues by combined cellulolytic and pectinolytic system has also been reported. Srivastava et al.
have produced cellulases on Soyahean seed coat. Rajoka and Malik have compared different strains of Cellulomonas for the production of cellulolytic and xylanolytic enzymes from biomass produced on saline land. Successive cultivation of selected cellulolytic fungi on rice straw and wheat bran for economic production of cellulases and D-xylanases was reported. Activities of cellulase and other extracellular enzymes during lignin solubilization of Streptomyces viridosporus were also investigated. Production of amylase and cellulase from four fungal species have been studied. Cellulase production was enhanced by culture enrichment and optimization of fermentation conditions. Studies on development of a medium for hyper production of xylanase and β-glucosidase were reported. Effect of fatty acids on cellulase production by Penicillium funiculosum and its mutants was investigated. Choline was proved to be an activator for the production of cellulases in Trichoderma species. Knapp has studied the effect of different growth substrates and pH values on the production of cellulolytic enzymes by T. reesei. Induction of cellulases by L-Sorbose in T. reesi has also been reported.

Investigations are also going on, for isolating most potent cellulolytic microbes. Recently many new strains of cellulolytic microbes are introduced e.g. Bacillus, Bifidobacterium breve, Thielavia terrestris, Cellulomonas fermentans, Clostridium, an unidentified novel cellulolytic.
bacterium from marine shipworm\textsuperscript{30}, Sporolactobacilless\textsuperscript{31}, Aureobasidium\textsuperscript{32}, Acremonium persicinum\textsuperscript{33} Acremonium persicinum\textsuperscript{33}. Hall and faune \textsuperscript{34} have isolated a functional gene for cellobiose utilization in natural isolate of Escherichia coli. Isolation of a new thermophilic cellulolytic bacterium \textit{Acidothermus cellulolyticus} also reported\textsuperscript{35}.

**CELLULASES**

As discussed earlier cellulase has a compact structure and its hydrolysis require a complex system of cellulolytic enzymes for its breakdown into glucose units. There are three major groups of enzymes in cellulase system, endo-\(\beta\)-1,4-glucanase(3.2.1.4) exo-\(\beta\)-1,4-glucanase and \(\beta\)-1,4-glucosidase (3.2.1.91). Reese\textsuperscript{36} presented a detailed description of the criteria used to characterise the various components. In summary, the endo \(\beta\)-1,4-glucanase (alternatively called \textit{C}_{x}\', CMCase and endoglucanase) hydrolyze cellulose molecules in a random fashion with products retaining the \(\beta\)-configuration. These enzymes also have a preference for larger molecules. Exo-\(\beta\)-1,4-glucanase (avicelase, exoglucanase or cellobiohydrolase or \(G_{1}\)) removes cellobiose or glucose units from the non-reducing ends of the cellulose molecules with the monomer or dimer so released being converted to an \(\alpha\) configuration. \(\beta\)-1,4-glucosidases (3.2.1.21) are required for the conversion of cellobiose into glucose. \(\beta\)-1,4-glucosidases maintain the \(\beta\)-configuration in the products, prefer small oligomers, and appears to be more subjected to end product inhibition.
ADSORPTION

Adsorption of enzyme on the substrate is the first step in the enzymatic hydrolysis. Studies on the strength and nature of each adsorption are very important for understanding the mechanism of hydrolysis and the recovery of enzyme for its reuse. As it is well established now, that cellulases exist in a complex form, sometimes very large extracellular complex structures.

In addition to cellulolytic enzymes, the presence of an affinity or binding factor required for the attachment of the cellulase complex to the cellulose, has been proposed for cellulolytic bacterium *Ruminococcus albus*[^37][^38]. A variety of surface structures was also described for different cellulolytic bacteria including "glycocalyces" observed in rumen bacterium, fibrous and membranous structures noticed in *Bacteroides succinogens*[^40], spherical bodies vesicular structure, lobes[^38][^39][^41] and tube like appendages in *Ruminococcus albus*[^41]. In *Clostridium thermocellum* various cell surface structures including ruthenium red stained fibrous materials[^42] and more polycellulosomal protuberance which produced by the cells during growth were reported[^43]. It was found that cellulosome (cellulase complex) was intimately associated with the surface of the cellulosic substrate and a fibrous material forms contact corridors[^44]. Thus, these structures may represent large "poly saccharide protein supramolecular complexes helpful in the efficient degradation of microcrystalline cellulose[^45].
Reese\textsuperscript{46} stated that adsorption was a function of (1) the amount of enzymes present (2) available surface and its nature (3) physical properties of the enzymes (e.g. charge, size and solubility) (4) the environment (e.g. pH, salt concentration temperature). The presence of other substances such as lignin can affect adsorption\textsuperscript{47}. Of the physical conditions, pH has been found to have a little effect on adsorption in the range 3.5 - 5.5\textsuperscript{48-50}, although there is maximum adsorption at pH 4.8\textsuperscript{48}. An adjustment of pH 10 has been suggested as a possible method of enzyme recovery\textsuperscript{51}. The effect of temperature is not clear due to the rapid onset of the hydrolysis as the temperature increased. Endoglucanase adsorption appears to be more sensitive to temperature changes than exoglucanase adsorption. Water\textsuperscript{53} and 8M Guanidine hydrochloride\textsuperscript{54} were used for the elution of the adsorbed enzymes from the residual cellulose. Recently Marsden and Gray\textsuperscript{55} have drawn some general conclusions for adsorption of cellulases on the cellulosic substrates from the literature which are summarized below.

1. The total amount of cellulase enzymes adsorbed increases surface area\textsuperscript{56} and increasing crystallinity\textsuperscript{48}.

2. For more crystalline substrates endoglucanase activity is adsorbed preferentially\textsuperscript{57,58}. Reports on the preferential adsorption
of exo-glucanase enzymes$^{50,52,59}$ particularly in the early stages of
hydrolysis are most acceptable probably due
to the presence of free ends created by
the method of preparation of substrates$^{58,59}$.

3. With less crystalline substrates and
particularly these treated in such a way to
minimize free end formation (alkali treatment),
endoglucanase activity is preferentially
adsorbed$^{48,57}$.

4. As the hydrolysis proceeds adsorption of
exo-glucanase increases$^{57}$ e.g. as the
endoglucanase enzyme creates more free ends.
In a study into the kinetics of enzymatic
hydrolysis, Okazaki and Moo Young$^{60}$ found
that a high level of endocellulase activity
was required in the initial stages of
hydrolysis with greater level of exoglucanase
activity being necessary in the later stages.

5. Adsorption rates are determined initially
for the enzyme concentration and later by the
concentration of accessible substrates$^{52,57,60}$

6. The endoglucanase and exoglucanase enzymes
appear to be adsorbed competitively$^{59}$ and
in so doing exert a synergistic effect on
breakdown of the cellulose.
While B-glucosidase has not been adsorbed in most reports\(^{52,58}\) but there are reports which found some evidences of cellobiases being adsorbed\(^{64}\), although their activity towards cellobiose were diminished relative to that of soluble cellobiases\(^{64}\).

**ACTIVE SITES**

Cellulases catalyse the random hydrolysis of the glycosidic bonds of cellulose and related cellooligosaccharides. Through kinetic and chemical modification studies on cellulases, some workers have tried to identify the amino acids present at the active sites. Hurst et al.\(^{65,66}\) have reported the participation of carboxyl residues in the active site of a cellulase from *Aspergillus niger*. Tryptophan and histidine thought to be involved in the active site of a cellulase of *Penicillium notatum*\(^{67,68}\).

Two highly homologous forms of endoglucanase (EG 1, EG II) produced by a white rot fungus *Schizopyllum commune* have been characterized and differ only in their amino acid terminal sequence\(^{69}\). Ionizable carboxyl groups were essentially required for EG I activity\(^{70}\). Through sequence homology studies Yaguchi et al.\(^{71}\) suggested that enzymes follows the same pathway as hen egg white lysozyme. Two specific carboxyl groups of endoglucanase, Glu-33 and Asp-50 were postulated to participate in the catalytic site of the enzyme. Glu-33 was assumed to act as general acid catalyst while Asp-50 would be negatively charged and stabilize the
incipient carbonium ion of the substrate. Recent studies made on ultraviolet difference adsorption spectra of the cellulase from white rot fungus *S.-commune*, suggested the presence of tryptophan residues in the binding region of this enzyme\textsuperscript{72}. One residue appears to be involved in the binding of substrate, while second residue proposed to constitute an integral part of the catalytically sound active centre\textsuperscript{73}. Implication of histidine at the active site of exoglucanase from *Basidiomycetes* species has been reported recently\textsuperscript{74}.

Generally most of the carbohydrases have large active centres containing several subsites, same is the case with cellulases. A few workers have tried to identify the subsite structure. Halliwell and Vincent\textsuperscript{75} and Li *et al.*\textsuperscript{76} suggested a subsite structure consisting of a sequence of six affinity sites for binding of the glucoside residue of cellulose. Similarly, subsite structure accommodating five glu-copy-ranosyl moieties have been proposed for the binding region of the enzyme from *Aspergillus niger*, *Penicillium notatum*\textsuperscript{78} and *Myrothecium verrucaria*\textsuperscript{79}. Existence of different binding sites for cellobiase and p-Nitrophenyl-\(\beta\)-D-glucopyranosidase in *A.foetidus* was reported\textsuperscript{81}.

**MODE OF ACTION**

For understanding the mechanism of enzymatic hydrolysis (Fig.4)\textsuperscript{80} of cellulose two different approaches were considere by the workers e.g. kinetic approach and enzymatic approach. Kinetic approach emphasizes on the use of Michaelis-Menten kinetics to describe the mechanism and concentrate on the substrate factor rather than inhibition effects. While in
Fig. 4. Proposed mechanism of cellulose degradation by fungi. The numbered enzymes are: 1, cellobiose oxidase/dehydrogenase; 2, lactonase; 3, exoglucohydrolase; 4, B-glucosidase; 5, endoglucanase; 6, exocellulbiohydrolase; 7, glucose oxidase.
case of enzymatic approach amorphus components of the substrates were attacked first and followed by the hydrolysis of crystalline components at slower rates.

**Kinetic Approach**

Many workers have tried to explain the phenomena of cellulose hydrolysis on the basis of kinetic approach. The mechanism postulated by Rabinovich *et al.*$^{82}$ is widely accepted. Rabinovich *et al.*$^{82}$ has adopted a mechanism for the production of glucose from cellulose. They have showed that the rate of glucose formation from cellobiose during hydrolysis of carboxymethylcellulose(CMC) could not account for the amount of glucose being found. The excess of glucose was attributed to the action of exo-1,4-β-glucosidase whose action was rate limiting in the conversion of CMC to glucose. In a subsequent report$^{33}$ the effect of the exoglucosidase was shown to be more important with less crystalline substrates and during the early stages of hydrolysis. This reduced role for the exoglucosidase with time, was attributed to the kinetic properties of cellulase system rather than a change in the mechanism (Fig.5).

![Diagram](image)

**Fig.5** Mechanism for glucose production by two routes. The cellobiose route (—) takes place by the action of the cellobiohydrolase followed by the cellobiase, while the noncellobiose route(---) occurs by the action of the endoglucanases and the exoglucosidase.
Fig. 6  Mechanism for mechano enzymatic hydrolysis

Ci  The i/n component of cellulose
Pi  The product from i/n component
\((HC)_1\)  The intermediate formed from the component
\(a_i\)  The proportion of Ci converted to Pi via intermediate \(K_i, K_i',\) and \(K_i''\)
**SERIAL MECHANISM**

Crystalline Cellulose

\[ I_{C_1} \]

Amorphous Cellulose

\[ I_{C_x} \]

Reducing Sugar

**Fig 7**

- Original Cellulose
- Enzymatic Hydrolysis
- Mechano-Enzymatic Hydrolysis
Later, these results were confirmed by studying the kinetics of hydrolysis of ball milled cotton linters and microcrystalline cellulose using cellulases from twelve different microbial sources\textsuperscript{84}.

A model based on cellulose having "I" component to differing reactivity, which are converted to produce at different rates was postulated by Chang et al.\textsuperscript{85} (Fig. 6).

\[
\begin{align*}
C_i & \xrightarrow{K_i} P_i \\
(Eq. 1)
\end{align*}
\]

With pretreated substrates cellulose change of intermediate length were produced during the hydrolysis, such a substrate (pretreated cellulose with cellulase) did not obey the first order kinetics shown in equation I so the model was modified to that as shown in Fig. 6.

This model was termed as "mechano enzymatic hydrolysis". Since the changes were thought to be caused by the mechanical effects of the pre-treatments. These models are illustrated in Fig. 7. The model was tested with \( i = 1 \) using cotton linters and acid treated cellulose, a good correlation was reported.

**Enzymological approach**

Reese\textsuperscript{36,86} proposed a serial mechanism on the basis of enzymological approach. In which \( C_1 \) was taken to be distinct enzyme which acted by splitting a covalent linkage as well as hydrogen bonds. (Fig. 8)
A second mechanism named as parallel mechanism was postulated by Fan et al. in which amorphous and crystalline fractions were broken down by two different modes of attack (Fig.9). Such mechanism does not necessarily exclude the serial mechanism (and may in fact encompass it); but hopefully gives a more accurate representation of hydrolysis of complex cellulosic substrates.

Fig.9

\[ \text{Crystalline cellulose} \xrightarrow{\text{B-1,4-cellbiohydrolase}} \text{Reducing sugar} \]
\[ \text{Amorphous cellulose} \xrightarrow{\text{B-1,4-endoglucanase}} \text{Reducing sugar} \]

Ryu et al. explained a mechanism in which two enzymes interact in three ways:

1. The cellbiohydrolase speeds up the adsorbed endoglucanases in producing a free end for subsequent attack.
2. Two components already adsorbed affect each other and
3. The endoglucanase affects the action of adsorbed cellbiohydrolase. This is a serial mechanism in which the synergistic action resulted in the formation of more reactive products which were then broken down into glucose.

Marsden and Gray put forward a mechanism to explain the results for the hydrolysis of Sigma cell, Solka floc and
alkali and acid treated bagasse by *T. reesei* cellulases with differing amounts of the three component activities.

\[ \text{Cellulose} \]

\[ \text{Cellbiohydrolase } ( + \text{ endoglucanase}) \]

\[ (1) \]

\[ \text{Crystalline cellulose} \]

\[ \text{Amorphous cellulose} \]

\[ \text{Endoglucanases} \]

\[ (3) \]

\[ \text{Cellobiase} \]

\[ (2) \]

\[ \text{B-glucosidase (exoglucosidase)} \]

\[ (4) \]

\[ \text{glucose} \]

\[ (4a) \]

\[ \text{Cellobiose} \]

\[ \text{Celooligomers} \]

**Fig. 11  Mechanism of cellulose degradation**

Step 1 was the action of cellbiohydrolase enzyme either alone on free ends or synergistically with the endoglucanase enzymes. Step 2 was carried out by cellobiase enzyme. Step 3 represented probably a number of steps as the
endoglucanase enzymes randomly hydrolysed amorphous cellulose to produce soluble oligomers. Step 4 appears to be the least studied and understood and was attributed to the action of exoglucanase. 4a is the residual cellobiose from the action of the exoglucanase on the cellooligomers with the some minor contributions from cellobiohydrolase (Fig.11).

Okada et al.⁹⁰ have proposed a catalytic mechanism for the hydrolysis of cellulose. The illustrated model of substrate binding at the active centre provides for the cleavage of cellobiose from the nonreducing end of the molecules. A major type of reaction catalysed by the exoglucanase with other binding modes. Scheme would also serve for the hydrolysis of interior glycosidic linkages, predominantly catalysed by random type cellulase e.g. endo enzyme of Aspergillus niger. One carboxyl group in the protonated is assumed to be located above the glycosidic oxygen atom and to act as a general acid to protonate the oxygen atom. The protonation step is pictured as leading to formation of transient carbonium-ion-enzyme complex that is stabilized by a carboxylate anion located below the C-1 centre of substitution. A second step would affect break down of this complex by a directed attack of water at C-1 to form cellobiose of β-configuration as reported for this enzyme preparation.
PRODUCTION

There are many organisms which are capable of growth on insoluble cellulose, only a small number of these produce extracellular enzyme that can degrade cellulose. Bisaria and Ghose listed 13 species of fungi and a few bacteria which are capable of producing cellulases that can extensively degrade insoluble cellulose in vitro. The fungal species included T. reesi, T. viride, T. coningi, Penicillium funiculosum, Aspergillus wentii, Sporotrichum pulverulentum, Fusarium solani and Sclerotium rolfsii while the bacterial species included Cellulomonas Clostridium, Bacillus, Thermomonospora, Flavobacterium and Thermoactinomycetes. The screening and isolation of better mutants is continued. Of the most commonly used strain T. reesi, QM9414 strain gave increased cellulase production over its parent T. reesi QM9123 while T. reesi MCG77 mutant gave increased β-glucosidase activity over the QM9414 strain. T. reesi Rut-C30 is resistant to catabolite and retained the high cellulase productivity of its parent. T. reesi NG-14 which has been reported to have 100 times the level of cellobiohydrolase found in the cellulase from T. reesi QM9414 as reviewed by Marsden and Gray.

PURIFICATION AND CHARACTERIZATION

For understanding the mechanism of cellulose degradation by the enzyme, it is necessary to isolate, purify and characterise these enzymes. Different workers have used
a variety of techniques for this purpose i.e. gel filtration, ion-exchange chromatography, preparative polyacrylamide gel electrophoresis, high pressure liquid chromatography and preparative iso-electric focussing. A few workers have taken the advantage of selective binding of cellulases to their insoluble substrates i.e. crystalline cellulose, cellulose powder and natural sources of cellulose i.e. wheat straw and cotton linters. Celllobiohydrolases of *T. viridi* has been purified by apply the same phenomena\(^93\). Mart, Yanov *et al*\(^94\) have reported single step purification of cellulases by using adsorption chromatography on crystalline cellulose with 50 times purification from the original sample. Poulsen\(^95\) has purified an extracellular cellulose binding endoglucanase of *Cellulomonas* species by affinity chromatography on phosphoric acid swollen cellulose. Lamed *et al*.\(^96\) have purified and partially characterized a complete cellulase complex "Cellulosome" from culture supernatant of *Clostridium thermocellum* by using adsorption chromatography on microcrystalline cellulose and gel filtration on sepharose-\(\beta\). "Cellulosome" was homogenous on *SDS-PAGE* having 14 polypeptide chain arranged in a symmetrical fashion with a molecular weight of 210,0000. Beside this, workers have tried to isolate, purify and characterize cellulase components separately.

\(\beta\)-1,4-Endoglucanase

The endoglucanases account for 15-20% of extracellular proteins when *T. reesi* was grown in the presence
of cellulose. Two extracellular monomeric endoglucanases from *Cellulomonas fermentens* were purified on PAGE, chromatography on DEAE-Trisacryl and high pressure liquid chromatography. Enzymes have molecular weight of 40000 and 57000, respectively with similar mode of action. Boyer et al have reported purification and some properties of two endoglucanases of *Erwinia chrysanthemi*, optimum pH values for the enzymes were 7 and 5.5 with molecular weights of 45000 and 30000 respectively. Each of the enzymes was focussed at pH value at 4.5 and 8.2. Okada has reported purification of an endoglucanase from *Aspergillus niger* with chromatography on Amberlite CG-50, Bio-gel P-150 and Sephadex G-50. Enzyme was homogenous on SDS-PAGE with a molecular weight of 31000 and isoelectric point at 3.67. Creuzet and Pixon have purified an endoglucanase from a newly isolated thermophilic aerobic bacterium to apparent homogeniety by precipitating with 40% ammonium sulphate and chromatography on DEAE TRisacryl with purification factor of 3.8. Enzyme has a molecular weight of 91000-99000. Purification and characterization of two monomeric endoglucanases of *Thermomonospora fusca* was reported. Each enzyme has molecular weight of 94000 and 96000 respectively. Both enzymes were most active at pH value 6.5 and 6 with optimum temperature of 74°C and 58°C, respectively. Enzymes were focussed at pH values 3.5 and 4.5. Rao et al have isolated and purified five
endoglucanases from *Fusarium lini*. Enzymes were purified on PAGE and preparative isoelectric focussing. Molecular weights of the enzymes ranged between 40000 to 65000.

Upto six endoglucanases have been isolated from *Trichoderma cellulase complexes*[^106]. Five endoglucanases from *Sporotrichum* culture supernatant have been isolated, purified and characterized[^107]. Langsford et al.[^54] have reported the presence of at least 10 endoglucanases in the culture supernatants of *Cellulomonas fimi* in six days old culture. While Prasertan and Doelle[^108] have partially purified and characterized at least six endoglucanases from the culture supernatants of *Cellulomonas*. Presence of four endoglucanases resolved on PAGE from *Cellulomonas* sp. have been reported[^109]. Cellulase activity of *Clostridium thermocellum* has been displayed by a cellulase complex having fourteen polypeptide chains, eight of them were identified as CMCases[^110,111]. Different workers have tried to study the possible reasons for the multiplicity of endoglucanases. Covalent binding of carbohydrates to the enzyme proteins is one of the major reasons[^112,115]. Proteolysis of cellulases is also considered as a reason for the duplication of enzyme activity. Gong et al.[^150] reported finding of only one endoglucanase after six days of growth and four after fourteen days in the culture supernatant of *T. reesia*[^151]. In contrast to this report are published on the appearance of multiple forms of β-glucosidases
and endoglucanases during earlier stages of fermentation. It was considered that multiplicity is an inherent property\textsuperscript{116}. Langsford et al.\textsuperscript{54} have reported the presence of only two native endoglucanases instead of ten as reported in the culture medium\textsuperscript{54}, these were said to be the proteolytically degraded and deglycosylated product of the native enzyme. While in case of \textit{Clostridium thermocellum} ten sets of genes were isolated and cloned, specific for endoglucanases\textsuperscript{118,120}. Whatever, the reason for the multiplicity of the endoglucanases, the various enzymes displayed significant differences in their properties,\textsuperscript{114,121,122} such as differing degrees of randomness in their attack on cellulose, differing degrees of synergism with exoglucanases and differing ranges of hydrolytic product.

As far as the hydrolytic properties of endoglucanases are concerned, these are able to produce short fibres and reduce the tensile,\textsuperscript{121,122} strength considerably and showed significant activity against CMC\textsuperscript{114}. Endoglucanases are able to solubilize most of the less ordered cellulosic substrates producing soluble oligomers.\textsuperscript{99-105}.

\textbf{B-1,4-Exoglucanase}

When \textit{T. reesei} was grown in the presence of cellulose, 35-85\% of the extracellular proteins showed exoglucanase activity\textsuperscript{97,98} which may acts synergistically with endoglucanase enzymes in the break down of cellulose.
into reducing sugars. There are many reports on the purification of exoglucanases. An exoglucanase from Penicillium sp. has been purified from the culture supernatants by acetone treatment, chromatographies on DEAE-Bio-gel, CM-Bio-gel, PBE-94 and Bio-gel P-150. Enzyme was homogenous on SDS-PAGE. It was a glycoprotein with a molecular weight of 52000. Enzyme was most active at pH 4 and 60°C.\textsuperscript{117} Purification and properties of another exoglucanase from P. pinophilum has been reported.\textsuperscript{123} Mackenzie et al.\textsuperscript{124} have reported purification of B-glucosidase of Streptomyces flavogriseus by a sequence involving DEAE Bio-gel. A chromatography, gel permeation chromatography on Bio-gel-P 60, preparative isoelectric focusing and concanavalin-A affinity chromatography. Enzyme was homogenous on SDS-PAGE, with a molecular weight of 45000 and was focussed at pH 4.15. Exoglucanase of Cellulomonas uda was purified by ammonium sulphate precipitation, DEAE-Sephadex chromatography and Toyopearl HW-55-F gel filtration. The purified enzyme appeared homogenous on SDS-PAGE with a molecular weight of 66000 and was focussed at pH 4.\textsuperscript{125} Exoglucanase of Acetivibrio cellulolyticus was fractionated on PAGE, with a molecular weight of 38000.\textsuperscript{126}

The major characteristics of exoglucanase from Trichoderma species are discussed below.
It is generally accepted that Fungi produce at least two types of exoglucanases, differing in their amino acid sequence\textsuperscript{127} and immunological characteristics\textsuperscript{128,129}. Purification and characterization of four extracellular exoglucanases from \textit{T. viride} has been reported\textsuperscript{131}. Enzymes were similar with respect to ultraviolet adsorption, amino acid and amino sugar composition, heat stability, molecular weight, isoelectric point and carboxyl terminal residues\textsuperscript{131} with cross immunological reactivity. Enzymes were only differed in the contents and composition of covalently bound neutral carbohydrates and was said to be differentially glycosylated form of the same polypeptide. Effect of attached carbohydrate on cellulases of \textit{Trichoderma} has also been investigated\textsuperscript{131,132}. Multiplicity of exoglucanases has also been reported in the culture supernatants of \textit{Cellulomonas fermentans} showing at least four exoglucanases when resolved on PAGE\textsuperscript{133}.

Exoglucanases can extensively hydrolyze less ordered substrate with celllobiose as major end product and showed little activity against CMC and highly ordered cellulose\textsuperscript{134-136}. Exoglucanases have strong inhibition effect against celllobiose\textsuperscript{136-138}. Exoglucanases are unable to effect on tensile strength, short fibre formation\textsuperscript{139-141} and crystallinity of cellulose\textsuperscript{128}. The characteristics of Exoglucanases isolated from cellulase complex of other microbial species were reported to be similar to those of \textit{Trichoderma}\textsuperscript{129-131}. 

Alcaligenes faecalis β-glucosidase was purified 130 fold by series of Streptomycine and ammonium sulphate precipitation, gel filtration and ion exchange chromatography. Enzyme was most active at pH 6-7 and had a molecular weight of 120000. Purification of another β-glucosidase for A. faecalis \(^{140-141}\). Purification of β-glucosidase from candida molischiana was reported. Enzyme had a pH optima between 4-4.5 and was most active at 60°C with the molecular weight of 120000\(^{142}\). Two β-glucosidases from fungal bacterial and yeast origin were also purified and characterized\(^{143-147}\). Purification of intracellular β-glucosidase from Clostridium thermocellum with the recovery of about 5% of the initial enzyme activity in a 5 step procedure including ion-exchange chromatography on DEAE Cellulose, chromatographies on HA-ultro gel and DEAE-Sephadex, gel filtration on ACA 34 ultrogel and isoelectric focussing. Enzyme was most active at pH 6 and 65°C with a molecular weight of 50,000 and was focussed at pH 4.68\(^{148}\).

Cellobiases or β-glucosidases constitute about 1% of the extracellular proteins when T. reesei was grown in the presence of cellulose\(^{107,108}\) because most of the activity remains intracellular\(^{149-152}\). This component of cellulase complex is not extensively studied. Marsden and Gray\(^{55}\) have given two reasons. (1) Enzyme activity was required during the last step i.e. cellubiose Glucose(2)
T. reesei produced lower level of enzyme activity. Enzyme is able to hydrolyse cellubiose, p-nitrophenyl β-D-glucoside and Salicin. The physical and chemical functional properties of β-glucosidase of T. reesei are entirely different from β-glucosidases of other fungal species. Emert et al. have characterized β-glucosidases by transferase activity, configuration retention and greater activity against dimers, trimers relative to swollen cellulose, CMC or Avicel. Although β-glucosidase activity from Schizophilium rolfsii can hydrolyse acid swollen Avicel and CMC Candida wickerhamii produced β-1,4-glucosidase which has a unique ability of hydrolysing not only cellubiose but also oligomers of cellubiose upto the degree of polymerization upto seven. There are several reports on rapid hydrolysis of higher oligomers as compared to cellubiose.

GENETIC ENGINEERING

An area where there is potential for large increases in the efficiency of cellulase production is that of genetic engineering of improved strains. Different workers tried to clone the cellulase gene i.e. endoglucanase, exoglucanase and β-glucosidase genes of different potent cellulolytic microbes into other bacterial or yeast strain. Those strains which are used for this purpose are Escherica coli and Scchromyces cervisiae. There are reports on protoplast fusion and gene cloning of specific cellulase genes into other microbe genetic engineering.
Gene manipulation is an important tool for understanding the mode of action, regulation and biosynthesis of enzymatic proteins. Gene of cellobiohydrolase I (exoglucanase) from *Trichoderma* has been cloned\(^{169,170}\) in *E. coli*. It's primary structure coding 496 amino acids and the proceeding signal peptides have been demonstrated\(^{169}\). The N-terminal sequence\(^{171,172}\) and sequence of some internal peptides has also been investigated\(^{172}\). Penttila et al.\(^{173}\) have cloned and sequenced gene for endoglucanase I of *Trichoderma*. They deduce of homology between exoglucanase I gene and endoglucanase I gene of *Trichoderma* and suggested that the enzymes have arisen from a common ancestor by gene duplication. Gene sequence of exoglucanase II of *Trichoderma* has no homology with endoglucanase I and glucanase I genes as determined by Teeri et al.\(^{174}\). However, a short region of extensive homology was found in all *Trichoderma* cellulase genes characterized so far. Suggesting that this region is important for cellulose hydrolysis. Cloning of cellulase genes of *Bacillus* was reported by many workers\(^{174-179}\) because it's genomic sequence is well known. The B-glucosidase gene of *B-amyloliquefaciens*\(^{180}\) has homology with B-glucosidase gene from *B. subtilis*\(^{181-182}\). It has variability with B-glucosidase gene from other *B. subtilis* C 120\(^{176}\). Homology between cellulase gene of two strain of *Erwinia chrysanthemi* was also reported. Grepinet and Beguin\(^{184}\) reported the amino-terminal end of the gene for endoglucanase Ρ of *Clostridium thermocellum* sequence was similar to signal peptides described
for the other bacterial secretory proteins. The carboxy terminals ends of endoglucanase A and B appear to be remarkably homologus. A striking feature of the conserved region was that both proteins contain two reiterated stretches of 23 amino acids each, separated by nine residues.

Genetic Engineering of cellulases also used for the over production of enzymes. Overproduction of exoglucanase of Cellulomonas fimi, cellulase gene with a high guanosine plus cytosine content in E. coli has been reported. The recombinant exoglucanase gene was expressed in E. coli to a level that excrete 20% of the total cellular proteins with 250 times lower specific activity as compared to the parent strain.

By using recombinant DNA techniques mode of action of cellulases was established and their complete sequence was also determined. Cloning of DNA sequences involved in the over production of endoglucanase activity in Streptomyces lividans has been reported. Other celluloletic microbes investigated in recombinant DNA technology are Aspergillus nidulans, Thermomonospora fusca, Ruminococcus flavefeciens, R. albus, Saccharomyces cerevisiae, Bacteroides succinogens, Cellulomonas uda, Pseudomonas var. cellulosa, Clostridium thermocellum, C. acetobutylicus, Erwinia chrysanthemi, Rhodobacter capsulatus, Cellvibrio mixtus and Escherachia coli. Recently there is a report on expression of a Cellulase gene from C. uda in Zymomonas mobilis. While Hall and Gilbert have reported the nucleotide sequence of a carboxymethyl cellulase gene from Pseudomonas fluorescens cellulose.
APPLICATION OF CELLULASES

The major application of cellulases in industry is the large scale production of glucose and alcohol from cellulose and lignocellulose. Cellulases are currently used in cereal processing, brewing, alcohol production, plant extraction, fruit processing, wine manufacturing and waste treatment. In many of these processes cellulase supplements the effects of other enzyme. In brewing and cereal extraction, cellulase speeds up mass filtration and can increase extract yield. It may supplement the action of β-glucanases in alcoholic fermentation. Depending upon the raw material cellulase can increase the yield of alcohol produced, when used as a supplement to start degrading enzymes. Alcohol degrading yeast in general do not ferment cellobiose. So it is essential that the cellulase used degrade cellobiose. Enzymatic cellulose breakdown aids polysaccharide hydrolysis during cassava root liquefaction and saccharification to increase the alcoholic yield. Cellulase, used to supplement pectinase speed up colour extraction from skin of fruits and also has an important application as a part of an enzyme complex in total liquefaction or maceration of vegetables and fruits. Cellulases and β-glucanases are effective on skins and pomaces for production of pectin. The skins and pomaces are incubated with the enzymes for 206 hours at 20-45°C with agitation. Cellulases are also used for extraction, or refining of cereal proteins and for recovery of alginates from seaweed. Cellulases combined with
pectinases and hemicellulases are used to hydrolyse mucilage during coffee extraction. Use of *Trichoderma* cellulase in silage processes speeds up the process by more rapid release of fermentable sugars and nitrogen\(^5\). Recently there is a report on the application of fungal cellulases in the improvement of milk yield\(^2\)\(^7\).
MATERIALS AND METHODS
MATERIALS AND METHODS

Microorganism

The cellulolytic bacterial strain Cellulomonas was isolated from a soil sample locally and identified as Cellulomonas flavigena by the National Collection of Industrial and Marine Bacteria Limited, Aberdeen, Scotland. The organism was maintained on agar slant of the composition (g/l) yeast extract 2, carboxymethyl cellulose (CMC, low viscosity sodium salt, BDH) 5, NaNO₃ 0.5, K₂HPO₄ 1.0, MgSO₄ 7H₂O 0.5, FeSO₄ 7H₂O 0.1, KCl 0.5 and agar (Difco) 15. The pH of the medium was adjusted to 7.3.

Culture Methods

20 ml of the medium of composition as given above but without agar and CMC replaced with 0.2 ml of 5mM cellobiose (BDH), was taken in a 250 ml Erlenmeyer flask and inoculated with cells from a freshly grown slant. The flasks were incubated with cells from a freshly grown slant. The flasks were incubated in an orbital shaker at 30°C till the O.D.₆₁₀ reached 0.6 mm. 1 ml of this inoculum was used to inoculate 100 ml of the culture medium.

Fermentation were carried out in one litre Erlenmeyer flasks each containing 250 ml of the medium as given above. After inoculation the flasks were incubated in an Orbital Incubator Shaker at 150 rpm at 30°C. After specific hours of fermentation samples were removed from the flasks aseptically for further processing.
Determination of growth rate

For determining the growth rate of 5 ml sample was withdrawn from the cultures aseptically at specific hours of fermentation and centrifuged for 30 minutes at 12000 x g. The residue was incubated with 1 N NaOH at 100°C for ten minutes. The cell debris was separated by centrifugation at 10,000 x g for 10 minutes and proteins were estimated by Lowry's method\(^2\)). The amount of cellular proteins thus obtained was taken as an indicator of bacterial growth.

PRODUCTION

Effect of carbon source on the production of cellulases

Effect of CMC and Avicel (0.5%) was studied on the production of extracellular CMCase and avicelase activities of \(C. flavigena\) after specific hours of fermentation. Effect of different concentrations of Avicel in the range of 0.2 - 4.0% (W/V), was also studied on the production of free and substrate-bound cellulase activities

Effect of Tween-80

Effect of Tween-80 was studied by adding different concentrations of 0.02 - 0.2% in the culture medium.

Effect of yeast extract

Effect of yeast extract on the production of cellulases was studied by adding different concentrations of yeast extract 0.02 - 0.2% in the culture medium.
ISOLATION OF ENZYMES

Broth Soluble

After specific hours of fermentations culture broth was centrifuged 10,000 x g for 30 minutes at 4°C to remove residual cellulose and bacterial cells. The supernatant thus obtained was used as the source of free enzymes in the culture supernatant.

Substrate bound

Substrate-bound cellulases of C. flavigena were eluted from the residual cellulose when Avicel was used as a carbon source. The culture medium after specific fermentation periods was centrifuged at 500 x g for ten minutes to recover the residual cellulose. The residue was shaken for 30 min. or sonicated (5x15 sec. each) chilled distilled water at 4°C containing 20 μg phenyl-methyl-sulfonyl-flouride/ml (double the volume of broth sample), for thirty minutes and the clear supernatant thus obtained.

Protein estimation

Proteins were estimated by dye-binding method with a slight modification. To estimate the lower levels of proteins in the purified fractions of the enzymes, 450 mg of the Coomassie brilliant blue G-250 (Shandon) was dissolved in 50 ml ethanol and 100 ml orthophosphoric acid (BDH). Volume was made up to 1 litre with distilled water. The reagent was filtered twice. For estimating protein 1 ml of the dye reagent was mixed with 1 ml of protein solution prepared in 0.05M tris HCl buffer. Adsorption was read at 595 nm against the reagent blank after 5 min. The bovine serum albumin (Sigma) was used as the standard. This reagent can estimate 1-15 μg protein/ml (Fig.11). During the purification processes by column chromatography proteins in the fractions were monitored by taking O.D.280
Fig. 11 Standard curve for protein estimation by using modified dye binding method. 450 mg of Coomasie brilliant blue G-250 (Shandon) was dissolved in 50 ml ethanol and 100 ml ortho phosphoric acid and volume was made upto 1 l. 1 ml of protein solution was mixed 1 ml of dye and after 5 min. absorbance was read at 595 nm.
Enzymes assays

CMCase. One ml of the appropriately diluted enzyme sample was mixed with 1% CMC dissolved in 0.1M citrate buffer (pH 6.5) containing 20 mM CaCl₂ (BDH), and incubated for 10 minutes at 50°C in a shaking water bath. Reducing sugars thus released were estimated by dinitrosalicylic acid (BDH) reagent method. One unit of CMCase activity was defined as the amount of enzyme that liberated one µM of reducing sugars from CMC per minute under the assay conditions described.

Avicelase

One ml of the appropriately diluted enzyme sample was mixed with 1 ml of 5% Avicel (W/V) suspended on 0.1M citrate buffer (pH 6.5) and incubated for 10 minutes at 50°C in a shaking water bath. Residual Avicel was removed by centrifugation at 500 x g for 5 minutes and the reducing sugars in the supernatant were determined by the dinitrosalicylic acid (DNS) method. One unit of avicelase activity was defined as the amount of enzyme that released one µM of reducing sugar from Avicel per minute under the assay conditions described.

Protease

1 ml of the appropriately diluted enzyme sample was incubated with 1 ml 0.5% haemoglobin (E. Merck) dissolved in 0.1M citrate buffer (pH 6.5) and incubated for 30 minutes at 50°C. Residual proteins were precipitated
by adding 3 ml of 10% trichloroacetic acid (BDH) and centrifuged at 5000 x g for 10 min. The liberated amino acids and peptides were estimated by the modified dye-binding method. One unit of protease activity was defined as the amount of enzyme that produced an O.D_{595} of 1.0 under the assay conditions.

**Effect of pH, temperature and inhibition studies.**

Effect of pH on enzyme activities of *C. flavigena* was studied by using 0.05M buffer of pH value ranging from 3-12. For the pH value 3-5 acetate buffer, 5-7 citrate buffer, 7-9 tris HCl buffer and 9-12 NaOH-glycine buffer solutions were employed.

Effect of temperature on cellulase activities of *C. flavigena* was studied in the temperature range of 0-70°C.

Inhibition of cellulases was studied by adding glucose and cellobiose separately in the reaction mixture at a concentration of 0 - 20 mM.

**Effect of β-mercaptoethanol**

Inactivation of cellulases was studied by adding β-mercaptoethanol in a concentration range of 5 - 20 mM in the enzyme solution prior to enzyme assay.

**Effect of metal ions**

Activation of cellulases was studied by adding different concentrations of CaCl₂, MgCl₂, AgNO₃ and FeSO₄ ranging from 5 mM to 50 mM in the reaction mixture.
Stability of cellulases

Thermal stability of cellulases was checked by incubating the enzymes for 30 min. at different temperatures ranging from 0°C to 80°C. pH stability of the enzyme was checked by incubating the enzyme solution at 4°C with buffer solutions of desired pH values ranging from 3 to 12. The buffer used were acetate (pH 3-5) citrate (pH 5-7), tris-HCl (pH 7.1 - 8.9) and NaOH-glycine (pH 9-12) strength of each buffer was 0.05M.

Substrate specificity of cellulases

Substrate specificity of the purified cellulases was checked by using 1% solutions of CMC and xylan and 5% suspensions of Avicel and filter paper.

Effect of substrate concentration on cellulases

Effect of substrate concentration was studied by using different concentrations of substrate ranging from 0.1 - 2% in the reaction mixture.

Identification of hydrolytic products

 Sugars produced by the action of cellulases were identified by paper chromatography on Whatman filter paper No.1. 1 ml of the enzyme solution alongwith the 1 ml of buffer substrate was incubated for 24 hours at 50°C and 10 μl of the hydrolyzate was spotted on the paper. About 1 μg of each reference sugar (glucose and cellobiose) was also applied. The solvent system contained
n-butanol:pyridine: water (6:4:3 V/V). Chromatogram was run at room temperature for 20 hours. Products were identified by dipping the chromatogram in AgNO₃ solution (0.1 ml saturated aqueous solution diluted to 20 ml with acetone and water added dropwise until AgNO₃ redissolved). Chromatogram was dried and sprayed with 0.5N NaOH in aqueous ethanol. Carbohydrates appeared immediately as black spot on light brown background.

**Purification of cellulases**

**Acetone precipitation:** G. flavidus was grown for 72 hrs. of fermentation. Supernatant was obtained after centrifugation and precipitated with 30% acetone at 0°C. pH of the solution was 7.5. Solution was incubated overnight at -20°C and the precipitates were separated by centrifugation at 10,000 x g for 30 minutes in a Beckman Model J2-21 centrifuge. The pellet thus obtained was dissolved in minimum volume of chilled distilled water and lyophilized. This crude enzyme preparation was stored -20°C in an air tight bottle till further use.

**Gel filtration:** 200 mg of the crude enzyme preparation in 1 ml of 0.025M of tris-HCl buffer pH 8.3 and was subjected to molecular sieve chromatography on swollen Sephacryl Superfine-300 packed in a column(1.6 x 44 cm). Column was equilibrated with the same buffer at 4°C. Proteins were eluted at the flow rate of 0.5 ml/min. at the same temperature.
A total of 100 fractions of 3 ml each were collected. Proteins were monitored at 280 nm in each fraction. CMCase activity was also determined in each fraction. Fractions having enzyme activity were pooled together and lyophilised if needed.

**Ion-Exchange chromatography:** 4 g of DEAE-Sephadex-A-50 was swollen in 0.05M tris-HCl buffer containing 0.05M NaCl at 100°C in a boiling water bath for 5 hours. A column (1.6 x 20 cm) was packed with the same gel using the same buffer at 4°C. The concentrated enzyme solution (passed through a Sephacryl column) was loaded on the column and proteins were eluted at flow rate of 0.2 ml/min. at 4°C using a salt gradient in the range of 0.05M - 1.0M. Column was washed with the eluent buffer with one column volume to elute any loosely bound proteins and then gradient 0.05 - 0.7M NaCl was applied. Finally column was washed with 1M NaCl. A total of 150 fractions (3 ml each) were collected. Enzyme activity and protein concentrations were estimated in each fraction. Active fractions were pooled and subjected to further purification processes.

**Electrophoresis:** Proteins were electrophoresed on a 5-20% gradient polyacrylamide gel using 5 - 10 μg protein or 0.05 - 0.2 unit/10 μl of CMCase activity with a constant current supply of 4 mA/cm of the gel (14 x 7 x 0.15 cm), using a constant buffer system of 0.1M, pH 8.3 tris-borate-EDTA(21.6, 11 and 1.86 g/l),
respectively. Gradient gel were prepared by gradually mixing two solution containing 5% and 20% acrylamide and bisacrylamide. The composition of 5% solution was 4.7 g acrylamide (Sigma) 0.3 g bisacrylamide (Sigma) 15 mg ammonium per sulphate (Sigma), 50 μl N N’ N’ N’-tetramethylene diamine (Sigma) and 0.9 g sucrose (Sigma) in 100 ml of 0.1M tris borate EDTA buffer. Solution of 20% acrylamide had the same composition except that it contained 18.6 g acrylamide and 1.3 g bisacrylamide and 3.6 g sucrose per 100 ml.

For preparative gradient PAGE 3 mm thick gel was used and 300 μg of the protein sample was loaded.

Detection of proteins: After electrophoretic run gel was fixed in 10% trichloroacetic acid for 30 minutes and then stained with 1.0% Coomassie brilliant blue R-250 (Shandon) in 20% methanol (BDH), and 10% acetic acid (BDH) and 2% glycerol (BDH) for three hours. Gel was destained electrophoretically in the destaining solution having the same composition except the dye. Proteins appeared as blue bands against clear background.

Identification of CMCase band: For identifying the CMCase bands after an electrophoretic run, the gel was overlayed on a previously dried CNC - agar plate (10 x 25 x 0.2 cm) containing 0.05% CNC and 2% agar dissolved in 0.05M citrate buffer pH 6.5 and incubated for 20 minutes at 50°C. After incubation gel was removed and the agar plate was washed
with 1M NaCl and stained with freshly prepared 0.5% aqueous solution of congo red (BDH) for ten minutes. Gel was washed with 1M NaCl to remove the excess dye for ten minutes. CMCase bands appeared yellow against red background. Intensity of the bands was increased by washing the plate with 5% acetic acid. Zymogram was photographed on a pan chromatic Kodak 200 film against transmitted light using a green light.

**Elution of proteins from the gel:** After preparative electrophoresis proteins were eluted from the polyacrylamide gel. The gel was cut into 0.3 mm pieces with a sharp razor. Each slice was crushed by passing through a 5 ml disposable syring with 3 ml of distilled water under pressure. Gel matrix was further broken down by freezing it at -20°C, and then proteins were allowed to diffuse at 4°C over night. Each fraction was analysed for CMCase activity and protein concentration.

**Molecular weight determination:** For molecular weight determination proteins were electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS (Shandon) using tris borate EDTA buffer as described by Lammeli. Samples of proteins purified containing 2μg/10 μl were loaded on the gel along with 10μg/10 μl of the standard markers as supplied by Pharmacia Fine Chemical, Uppsala, Sweden.
RESULTS
RESULTS

Effect of carbon sources on the production of extracellular cellulases

Effect of different Avicel and CMC on the production of cellulases of *C.flavigena* was studied. Bacterium was grown in the salt medium supplemented with 0.2% yeast extract along with 0.1% Tween-80 and 0.5% of the carbon source. As shown in Fig. 12 maximum yield of CMCase as well as avicelase activities were obtained after 72 hours of fermentation when maximum growth was also observed, in each case. There was a decline in enzyme yield at 96 hours of fermentation but rapid increase was observed at 120 hours of fermentation, when cell lysis was noticed (Fig. 12). Increase in the yield of soluble proteins (as determined by modified dye-binding method) and reducing sugars was also observed (Fig. 12).

A comparative study between the two carbon sources showed that Avicel was better for the production of CMCases as well as avicelase activities of *C.flavigena*. Maximum yields obtained was 5.0 and 0.6 U/ml of CMCAse and avicelase activities, respectively when 0.5% Avicel was used as a carbon source. When CMC was used as a carbon source the maximum CMCase activities obtained were 2.5 and 0.3 U/ml of the culture medium, respectively. Reducing sugars produced in the case of CMC as a carbon source was 100 µM/100 ml of the culture medium at 24 h of fermentation. In case of Avicel as carbon source lower levels of reducing sugars were
Fig. 12  Cultivation of *C. flavigena* in the presence of 0.5% Avicel (a), CMC (b). Cell protein (▲), soluble protein (●), CMCase (○), avicelase (●), Reducing sugars (△).
obtained throughout the fermentation period. Greater amount of proteins were obtained during fermentation when Avicel was used as carbon source. At 120 h of fermentation 15 mg/100 ml of the soluble proteins were present in culture medium when Avicel was used as a carbon source while in case of CMC as a carbon source, this amount was 10 mg/100 ml.

Avicel was therefore used as a carbon source for the production of extracellular cellulases. The effect of different percentages of Avicel was studied for the production of substrate bound and free CMCases and avicelase activities. As shown in Fig.13 a and b, when 0.5% of Avicel was used in the culture medium along with 0.2% yeast extract and 0.1% Tween-80, maximum yield of free CMCase and avicelase activities obtained were 12 and 1.4 U/ml of the culture supernatant, respectively. Below and above this concentration there was a decline in the yield of each of the enzyme activity (Fig.13,a and b).

Avicel being an insoluble cellulosic substrate can easily adsorb cellulases, after these are secreted by the microbe. Conditions were standardized for the elution of substrate bound cellulases from the residual Avicel. Objective of this experiment was also to determine the total amount of cellulases produced by C.flavigena in the culture medium under the standardized conditions. For optimization of the elution condition C.flavigena was grown in the presence of 4% Avicel. After specific hours of
Fig. 13 Effect of Avicel concentration on the production of extracellular free cellulases. CMCase (a), avicelase (b). 0.2% (●), 0.5% (○), 1.0% (△), 2% (▲).
fermentation residual Avicel was separated by centrifugation at 500 x g for 10 minutes at 4°C. The sedimented Avicel was shaken with distilled water, using twice the equivalent volume of broth, containing phenyl methyl sulphonyl fluoride (PMSF) 0.5 mg/ml, for 30 min. at 4°C. As shown in Fig.15a when residual Avicel was washed 5 times with water nearly all the cellulase activities were recovered in the supernatants. Subsequent washes did not release any detectable enzyme activities.

Enzymes were also desorbed by sonication. As shown in Fig.14b by sonicating the residual Avicel in the presence of distilled water containing PMSF and removing the supernatant by centrifugation, approximately same amounts of enzymes were eluted as obtained by shaking. Elution of the bound enzymes in the subsequent experiments was done by sonication due to its convenience.

For studying the effect of Avicel concentration on the production of cellulases, fermentations were done in the presence of 0.5%, 1.0%, 2.0% and 4.0% Avicel. Results of these studies are summarized in Tables 1 and 2. As shown in Table 1 greater proportion of the enzymes were bound to the residual substrate at earlier stages of fermentation. With the passage of time the proportion of the free enzyme activities increased. Maximum yield of CMCase activity obtained was 13 U/ml of the culture medium after 72 h. of fermentation, when 2% Avicel was used as carbon source. Below and above this Avicel concentration lesser amounts of CMCase activity was obtained.
Fig. 14 Elution of Avicel bound cellulases of *C. flavigena*

(a) by shaking the residual Avicel with double the volume of distilled water of the broth.

(b) Sonicating the residual Avicel in the presence of distilled water doubled the volume of broth.

CMCase ●

Avicelase ■
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### Table 1: Proportion between bound and free chymase activity of C. flavescens

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Cultivated in the presence of different "ages" of Alyce in the culture medium.
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<th>U/100 ml</th>
<th>Bound:Free</th>
<th>Total</th>
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<td>2.0</td>
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<td>2.0</td>
<td>2.0</td>
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Table 2: Proportion between bound and free enzymatic activity of C. jeikeium
Maximum yield of avicelase was obtained after 72 h. of fermentation when 2% Avicel was used as a carbon source (Table 2). In contrast to CMCase activity maximum avicelase activity remained bound to the residual Avicel till the later stages of fermentation, possibly due to the availability of binding sites on the substrate, as CMCase action would result increased substrate fragments for avicelase activity. The total avicelase activity as produced by C. flavigena with 2% Avicel was 3.1 U/ml of the culture medium, which is the highest yield as reported for any Cellulomonas strain, 15, 125. Above this concentration there was a decrease in the production of total cellulase activities (Tables 1 and 2).

Production of reducing sugars in the presence of different percentages of Avicel in the culture supernatant was also determined. As shown in Fig. 15 lower levels of reducing sugars were obtained when 0.5 - 2.0% Avicel was used as carbon source in the culture medium. But in case of 4% Avicel higher levels of reducing sugars were obtained after 72 h. of fermentations i.e. 210 μM/100 ml. Reduction in the cellulase yields with 4% Avicel was possibly due to the higher production of reducing sugars which inhibited the production of cellulases. It was also found that the amount of reducing sugar decreased at 96 h. of fermentation and after cell lyses it increased again (Fig. 15).
Fig. 15 Production of reducing sugars at different concentrations of Avicel.
0.5% (●), 1.0% (○), 2% (■), 4% (△).
Effect of yeast extract on the production of cellulases

Bacterial strains belonging to genus *Cellulomonas* require thiamine for their growth. So, the effect of yeast extract which contain vitamin B complex was studied on the production of extracellular cellulases of *C.flavigena*. Different concentrations of yeast extract used were 0.05%, 0.1%, 0.2% and 0.3%. As shown in Fig.16 maximum enzyme activities were obtained when culture medium was supplemented with 0.2% yeast extract. Above this concentration there was no significant increase in the enzyme yield. Maximum CMCase and avicelase activities obtained in the presence of 0.2% yeast extract were 10 and 0.9 U/ml of the culture medium, respectively.

Effect of Tween-80 on the production of cellulases

Effect of Tween-80 was studied on the production of extracellular cellulases of *C.flavigena*. Different concentrations used for the study were 0.01%, 0.05%, 0.1%, 0.15% and 0.2%. It was found that 0.1% Tween-80 was optimum for the production of CMCase as well as avicelase activities of *C.flavigena* (Fig.17 a,b). Above this concentration there was no significant increase in the enzyme activities. Maximum yields obtained after 72 h of fermentation were 12 U/ml for CMCase and 1.2 U/ml for avicelase activities. There was thus about two fold increase in the yields of cellulase activities.
Fig. 16 Effect of yeast extract on the production of extracellular free cellulases of *G. flavigena*. (a) CMCase, (b) avicelase 0.02% (▲), 0.05% (△), 0.1% (○), 0.2% (●).
Effect of Tween-80 on the production of soluble cellulases (a) CMCase, (b) avicelase 0% (△), 0.05% (△), 0.15% (○), 0.18% (●).
Production of proteolytic enzymes

Production of proteases during the fermentation period using Avicel as carbon source was also studied (Fig. 19). A study on the effect of pH on the protease activity of C. flavigena showed that the enzyme had two pH optima i.e. 6.5 and 8.9 (Fig. 18). Protease with pH optima 6.5 was produced in maximum quantity at 48 h. of fermentation and then decreased later. The production of proteases with pH optima of 8.9 however increased gradually until 120 h. of fermentation (Fig. 18). Maximum soluble proteins were also obtained at 120 h. of fermentation (Fig. 12).

Acetone Precipitation

500 ml of the culture supernatants of C. flavigena after 24, 48, 72 and 96 h. of fermentation were precipitated and lyophilized. Approximately 500 mg of the powder with two times increase in specific activity was obtained in each case. About 80% of the enzyme activities were recovered.

For substrate-bound cellulases maximum enzyme activities were obtained at 24 h. of fermentation having 15 times higher specific activity as compared to the unbound activity in the culture supernatant. Using Avicel at a concentration of 2.0% about 100 mg powdered sample was obtained from the residual Avicel of 200 ml culture.
Fig. 18  Effect of pH on extracellular protease activity of *C. flavigena*
Fig. 19  Production of proteases of C. flavigena.
ph 8.9 (△), ph 6.5 (○)
Comparative study of substrate-bound and free CMCCase activities.

Each lyophilized sample of *C. flavigena* (Crude enzyme preparation) containing 0.005 - 0.05 U of CMCCase/10 ul was electrophoresed on 5-20% gradient polyacrylamide gel and replica was made. As shown in Fig.20 free enzyme showed at least 7 CMCCase bands and as the fermentation period increased additional bands appeared. In case of substrate-bound enzymes only four major active CMCCase bands were detected. The bound CMCCase coincided with the fast moving CMCCase fractions of the culture supernatant. Three slow moving bands of the free enzymes were absent from the bound-enzyme activity. These enzymes formed a fraction of total cellulase activity as detected on zymogram Fig.20. Since the major CMCCase bands of the bound and the free fractions appeared to be common.

The free enzymes of the culture produced at 72 h of fermentations were subjected to further purification. Comparative study of substrate bound enzymes at different fermentation periods was also made. As shown in Fig.21 at 48,72 and 96 h of fermentation similar enzyme patterns were obtained. These fractions have an additional slow moving band which was absent in 24 h sample. Condition standardized for the broth enzymes and the bound enzymes eluted from Avicel at 24 h fermentation was not suitable for the separation of bound enzymes of 48,72 and 96 h samples. To obtain the best results these samples were electrophorised on 5-20% gradient polyacrylamide gel with constant current supply of 0.5 mA/cm of the gel.
Fig. 20  Analysis of the extracellular Avicel bound and free CMCase of C. flavigena.
A - 72 hours, B - 48 hours
C - 24 hours, D - Bound 24 hours CMCases
Fig. 21  Analysis of the extracellular avicel bound CMCase activities produced in the cultures of C. flavigena at different fermentation periods by gradient (5-20%)PAGE.
A  96 hours ,  B  72 hours
C  48 hours , and D after 24 hours
overnight. It was also seen that the bound CMCases after resolution showed very little activity on the zymogram as compared to the broth free enzymes. Thus, for having a clear picture both the amount of the enzyme activity and incubation period were increased upto 0.5 U and 40 minutes respectively.

**Electrophoresis of C. flavigena proteins.**

Protein pattern of 24, 48, 72 and 96 h of fermentation broth samples was also studied after electrophoresis. As shown in Fig.22 number of fast moving bands increased with the passage of time and amount of a few bands were increased in the culture supernatants as the fermentation period proceeded.

**Characteristics of cellulases**

**Optimum pH**

Optimum pH for both the avicelase and CMCase activities were determined as shown in Fig.24. Both the enzyme activities had broad pH optima i.e. pH 6.0 - 7.5.

**Optimum temperature**

Optimum temperature for both the CMCase and avicelase activities of C. flavigena was 50°C, as shown in Fig.25. There was an increase till 50°C but abrupt decline in enzyme activity was noticed when reaction mixtures were incubated above 50°C.

**Effect of substrate concentration**

Effect of substrate concentration on the CMCase and avicelase activity was studied. A concentration of 0.5% CMC gave maximum rate of reaction (Fig.26). For avicelase activity 3% Avicel showed maximum rate of reaction. Above these concentrations there was no considerable increase in the enzyme activity (Fig.27).

**Inhibition studies**

Reducing sugars are considered as the end product
Fig. 22  Resolution of extracellular proteins in the cultures of C. flavigena at different fermentation periods.
A  96 hours, B 72 hours, C 48 hours and D 24 hours.
Fig. 24  Effect of pH values on pH values on cellulases of C. flavigena

CMCase  •  Avicelase  ○
Fig. 25 Effect of temperature on cellulases of C. flavigena

CMCase • Avicelase o
Fig. 26  Effect of substrate concentration on CMCases of *C. flavigena*
Fig. 27 Effect of substrate concentration on avicelase activity of *C. flavigena*
avicelases. Glucose inhibited 80% CMCase activities till 20 mM concentrations while cellobiose inhibited 70% activity at the same concentration. (Fig.28-31). These two sugars probably compete with the substrate for the active sites of the enzyme. Thus they are said to be the competitive inhibitors for both the enzyme activities (Fig.28-31).

Effect of metal ions

Effect of different ions e.g. Fe\(^{++}\), Ca\(^{++}\), Mg\(^{++}\) and Ag\(^{++}\) in the concentrations of 5 - 50 mM was studied. Ca\(^{++}\) at lower concentrations, i.e. 2-5 mM, inhibited the CMCase activities while a higher concentrations i.e. 10 mM activated the enzymes. A decline in the reaction rate was observed when calcium chloride was added in higher concentrations (Fig.32a). However, Ca\(^{++}\) showed no significant effect on the reaction rate of avicelase activity (Fig.32 a). NaCl had no effect on the avicelase and the CMCase activities.

As shown in Fig.32, enzyme activities were greatly inhibited by Ag\(^{++}\) when added in the form of AgNO\(_3\). Fe\(^{++}\) also inhibited enzyme activities (Fig.32 c,d)

Effect of reducing agent

β-mercaptoethanol, a reducing agent, for the S - S bonds of the proteins, was seen that β-mercaptoethanol greatly was found to inhibit both the enzyme activities, indicating the involvement of S - S bonds in the active sites of the enzymes (Fig.33).
Fig. 28 Effect of glucose on CMCase of *C. flavigena*

Fig. 29 Effect of glucose on Avicelase activity of *C. flavigena*
Fig. 30 Effect of Cellulobiose on CMCase of *C. flavigena*

Fig. 31 Effect of Cellulobiose on avicelase activity of *C. flavigena*
Fig. 32 Effect of different metal ions on the extracellular cellulase activities of *C. flavigena*

a) CaCl₂  b) MgCl₂  c) FeSO₄  d) AgNO₃

- CMCase
- Avicelase
Fig. 33 Effect of β-mercaptoethanol on CMCase of *C. flavigena*

CMCase • Avicelase ○
Stability

Thermal stability of both the enzymes was checked. As shown in Fig.34 both the enzymes were stable in the range of 0 - 30°C and lost 100% activity when heated at 70°C for 30 minutes (Fig.34).

Both the enzymes were stable in the pH range of pH 4 - 10. On either side of these pH values there was a decline in enzyme stability.

Identification of hydrolytic products

The major end product against Avicel and CMC were cellobiose and glucose along with some other short oligosaccharide fragments of cellulosic substrates (Fig.34b).

Purification of cellulases

Gel filtration

One ml of the sample containing 750 mg of the crude enzyme preparation after acetone precipitation at 72 h of fermentation was subjected to partial purification on Sephacryl S-300 column (1.6 x 44 cm). Proteins were eluted just after the elution of the void volume. Five peaks were obtained for proteins (Fig.35). First peak was comprised of fraction Nos.10-13, while second peak along with its shoulder eluted in the fractions 15-29. Second peak was the major peak having its absorption maxima in fraction 20. Third, and fourth peaks comprised of fractions 33-35 and 37-42 respectively.
Fig. 34 a) Thermal stability of cellulases of *C. flavigena*.
CMCase (●), Avicelase (○)

Fig. 34 b) Identification of hydrolytic products of CMCase and Avicelase by paper chromatography.
- A. Hydrolytic products of CMCase
- B. Hydrolytic products of Avicelase
- C. Cellobiose
- D. Glucose
Fig. 35 Gel filtration of acetone precipitated crude preparation of broth soluble extracellular cellulases on Sephacryl 3-300 (1.6 × 40 cm). Proteins were eluted at flow rate of 0.5 ml/min. at 4°C. Each fraction comprised of 5 ml of the eluent. Proteins were eluted with 0.05 M tris-HCl buffer pH 7.5.

CMCase ■ Avicelase □

A 280 ▲
Fraction showing CMCase activity were pooled together and analysed for total protein concentration and enzyme activity. CMCase activity was compared with the starting material on non-denaturing gradient PAGE followed by replica plating (Fig.37). Specific activity for CMCase was 303U/mg protein and for avicelase 36U/mg protein in this preparation. This preparation was further purified on ion-exchange chromatography and preparative gradient PAGE.

Ion exchange chromatography on DEAE Sephadex A-50

Cellulase activity as obtained after gel filtration on Sephacryl column was subjected to ion exchange chromatography column (1.6 x 30 cm) packed with the DEAE-Sephadex A-50. As shown in fig.36 proteins were eluted with 0.05M NaCl resulting in the elution of first peak for CMCase activity named D₁. Second peak was eluted in the salt concentration of 0.12 - 0.20 M NaCl. This peak was larger than the first peak. Second peak was named as D₂. The third peak, D₃ was eluted with the salt concentration of 0.5 - 0.75M NaCl. This was a rather broad peak. The fraction D₃ contained the major amount
Fig 36. Ion-exchange chromatography, through a DEAE-Sephadex A-50 column (1.6 x 30 cm), of the endoglucanase activity obtained after gel filtration. Elution was done at the rate of 0.2 ml/min. with linear salt gradient.
of CMCase activity. A total of 30% CMCase activity was recovered after fractionation. D₁ contributed 5%, D₂ 37% and D₃ 57% of the total eluted enzyme activity (Table 3).

The fraction D₃ was subjected to further purification. Composition of each of these fractions was also studied by electrophoretic and zymogram methods.

Electrophoretic pattern of fractions D₁, D₂ and D₃

As shown in Fig.38 when each of the fraction was electrophoresed on 5-20% polyacrylamide gel and replica was made. It was seen that D₁ comprised of at least two slow moving bands of CMCases. They formed only a minor portion of the total enzyme activity. D₂ fraction showed four major CMCase bands on the zymogram. These bands correspond to the fast moving bands of the bound enzyme sample and from the residual Avicel of 24 hours fermentation. Fraction D₃, the major contributor to the total CMCase activity comprised of only two bands which were very close to each other on the zymogram. These bands seemed to coincide with the fast moving bands of the Avicel bound CMCases of a 24 h culture of C.flavigena. (Fig.20,37)

Gel filtration of D₃

D₃ fraction after concentration through lyophilization was passed through a Sephadryl column (1.6 x 40 cm) at flow rate of 0.5 ml/min. Single peak was obtained for D₃ comprised of fraction 18-23 (Fig.38).

Preparative electrophoresis of D₃ fraction

Efforts to separate the two bands of the D₃ fraction by ion exchange chromatography using narrow salt gradients
Fig. 37  Analysis of CMCase activity on 5-20% gradient PAGE after
E acetone precipitation, D Gel filtration on Sephacryl S-300, C, B and A after ion-exchange chromatography.
A : D3, B : D2 and C : D1
Table 3: Percentages of CMCase activity in different fractions after ion-exchange chromatography.

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<tr>
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</table>
Fig. 38. Gel filtration of D3 fraction on Sephacryl S-300 (1.6 x 40 cm) at flow rate of 0.5 ml/min. at 4°C. Each fraction comprised of 5 ml of the eluent.
and gel filtration but were not successful. Separation of these fractions was done by preparative gradient gel electrophoresis. 300 μg protein of D₃ sample was loaded on a 3 mm thick 5-20% polyacrylamide gel and was electrophoresed for 17 h. at 4°C at a constant current supply of 2.0 mA/cm of the gel. After electrophoretic run proteins were eluted from the gel slices. Enzyme assay was done for each fraction. As shown in Fig.39 CMCase activities were shown by fraction 28-30, 33 and 38.

Similarly two peaks were obtained for proteins by the same fractionation method. First peak was named as CMCase 1, second peak was named as CMCase 2. CMCase 1 was a larger peak as compared to CMCase 2. CMCase 1 comprised of 70% of the eluted activity while CMCase 2 showed only 30% of the recovered activity. Each of these fractions when electrophoresed on 5-20% polyacrylamide gel showed a single protein band.

As shown in Fig.40 each fraction showed single band on zymogram after electrophoretic run. CMCase 2 had less mobility as compared to the CMCase 1. These two single bands were comparable with the 24 hrs substrate bound activity (Fig.41).

**Molecular weight determination**

Molecular weight of the purified enzyme was determined by SDS-PAGE (Fig.41). It was a single polypeptide chain having molecular weight of 20,400 (Fig.41).

Both the purified CMCases 1 and 2 had same molecular weight as determined by SDS PAGE (Fig.41). Each CMCase 1 and 2 move very close to each other on 5-20% gradient polyacrylamide gel as seen on zymogram (Fig.41).
Fig. 39 Resolution of D₃ fraction into its components by using gradient (5-20%) acrylamide gel electrophoresis. After electrophoretic run gel was cut into 3 mm slices and proteins were eluted after crushing the gel slice. Each fraction was tested for CMCCase activity and protein concentration.
Fig. 40: Location of CMCase activity on CMC agar plate.

A  CMCase 2
B  CMCase 1
C  D₃
D  72 hours free CMCase activity
E  24 hours bound CMCase activity
Fig. 41  SDS-PAGE for molecular weight determination

A   CMCcase 1
B   CMCcase 2
C   Molecular weight markers
Characteristics of CMCase 1

Substrate specificity: Substrate specificity of purified enzyme was checked against Avicel, xylan, cellobiose, salisin, filter paper and CMC. It was found that enzyme was only active against CMC. It showed no activity against any other substrate.

Effect of substrate concentration: Effect of substrate concentration was studied on the reaction rate of CMCase 1. It was found that maximum enzyme activity was shown when 1.0% CMC was used as substrate in the reaction mixture. Above this concentration, there was no linear increase in the reaction rate. Km value of the enzyme was calculated from the Lineweaver Burk plot (Fig. 42) was 0.83 g/l.

End product: End product of the CMCase 1 was identified and it was found that enzyme produced oooligosaccharides and cellobiose as hydrolytic products.

Inhibition studies: CMCase 1 was inhibited in the presence of 10 - 15 m... of glucose or cellobiose when added into the reaction mixture.

Optimum pH: As shown in Fig. 43 enzyme was most active at pH 7. Purified enzyme has a sharp pH optima.

Optimum temperature: CMCase 1 was most active at 50°C. Enzyme had a sharp temperature optima. Above 50°C there was an abrupt decline in the rate of reaction (Fig. 44).
Fig. 42  Line weaver burk plot for purified CMCase for C. flavigena
**Fig. 43** Effect of pH on purified CMCase 1 activity.

**Fig. 44** Effect of temperature on purified CMCase 1 activity.

**Fig. 45** Effect of temperature on stability of purified CMCase 1.

**Fig. 46** Effect of pH on stability of purified CMCase 1.
Thermal stability: As shown in Fig.45, enzyme was stable from 0-30°C and there was a gradual decrease till 70°C at which enzyme lost 100% activity.

pH stability: pH stability of CMCase 1 was studied and it was found that enzyme was stable in the range of 3.10 pH value. Below and above 10 pH enzyme activities were gradually lost (Fig.46).

Effect of calcium chloride: As shown in Fig.47 calcium chloride inhibited 30% of enzyme activity till 10 mM concentration in the reaction mixture. Above this concentration inhibition was also observed but to a lesser extent.

Effect of silver nitrate and Ferrous sulphate: As shown in Fig. 48 silver nitrate inhibited CMCase when added in the concentration of 5 mM or above in the reaction mixture. Ferrous ions also inhibited the enzyme activity (Fig.49).

Effect of B-mercaptoethanol: B-mercaptoethanol strictly inactivate the enzyme activity when added into the reaction mixture even in mM (Fig.50).

Characteristics of CMCase 2

Substrate specificity: Substrate specificity of purified enzyme was checked against Avicel, xylan, cellobiose, salicin, filter paper and CMC. It was found that enzyme was only active against CMC. It had no activity against any other substrate. Km value of the enzyme was 1.7 g/l line weaver burk plot was plotted for the enzyme (Fig.51).

Identification of hydrolytic products: Hydrolytic product of CMCase were oligosaccharides and cellobiose when CMC was used as substrate
**Fig. 47** Effect of CaCl₂ on purified CMCase₁ of *C. flavigena*

**Fig. 48** Effect of AgNO₃ on purified CMCase₁ activity

**Fig. 49** Effect of FeSO₄ on purified CMCase₁ activity

**Fig. 50** Effect of β-mercaptoethanol on purified CMCase₁ of *C. flavigena*
Fig. 51  Line weaver burk plot for purified CMCase 2 of C. flavigena
Fig. 52 Effect of pH on purified CMCase 2.

Fig. 53 Effect of temperature on purified CMCase 2.

Fig. 54 Effect of pH on the stability of purified CMCase 2.

Fig. 55 Effect of temperature on the stability of purified CMCase 2.
Optimum pH and optimum temperature: Enzyme has optimum pH of 6.5 and optimum temperature of 45-50°C (Fig. 52, 53).

Stability: Enzyme was stable in the pH range of 5-10 (Fig. 54) and in the temperature range of 0-25°C (Fig. 55).

Effect of calcium chloride: Different concentration of calcium chloride was added in the reaction mixture to study its effect. It was found that calcium chloride did not activate the enzyme but in lower concentrations it inhibited the enzyme activity (Fig. 56).

Effect of silver nitrate and ferrous sulphate: Silver nitrate when added to the reaction mixture it strictly inhibited the enzyme activities (Fig. 58). Ferrous ions also inhibited the enzyme activity (Fig. 59).

Effect of B-mercaptoethanol: As shown in Fig. 57 B-mercaptoethanol strictly inactivate the enzyme activity.

Purification of D₂ fraction

D₂ fraction was concentrated through lyophilization and was loaded on Sephacryl S-300 column (1.6 x 44 cm). As shown in Fig. 60a D₂ fraction was eluted in two peaks named S₁ and S₂. For CMCase activity S₁ was the larger peak as compared to S₂. While for proteins S₂ was the major peak. Further purification of S₁ was done on preparative gradient PAGE.
Fig. 56 Effect of CaCl$_2$ on purified CMCase 2 activity of C. flavigena

Fig. 57 Effect of β-mercaptoethanol on purified CMCase 2 activity of C. flavigena

Fig. 58 Effect of AgNO$_3$ on purified CMCase 2 activity of C. flavigena

Fig. 59 Effect of FeSO$_4$ on purified CMCase 2 of C. flavigena
Preparative electrophoresis of $S_1$

$S_1$ fraction after concentration by lyophilization was analysed by 5-15% gradient PAGE. After electrophoretic run, gel was sliced and proteins were eluted from each slice. As shown in Fig.60a two CMCase activity fractions were obtained. These were named as $F_1$ and $F_2$.

Electrophoresis of $F_1$ and $F_2$

$F_1$ and $F_2$ were electrophorised on 2 - 20% PAGE (Fig. 60b) and activity was located on zymogram. Each showed single band of zymogram (Fig. 61b) and on SDS-PAGE (Fig. 61a) named as CMCase 3 and CMCase 4.

Characteristics of CMCase 3

**Substrate specificity:** Enzyme was only active against CMC. It showed no activity against any other cellulosic substrate.

**Effect of substrate concentration:** Purified CMCase 3 exhibited maximum activity when incubated with 2% CMC in the reaction mixture. Till 1.2% CMC there was a linearity in the reaction rate and above this concentration no linear increase was observed in the reaction rate (Fig. 62).

Kinetics of the enzyme was also studied and Lineweaver Burk plot of the enzyme was also plotted. $K_m$ value of the enzyme was 1.5g/l.

**Identification of hydrolytic product:** Enzyme was able to produce oligosaccharides and cellobiose as end products.
Fig. 60 a) Gel filtration of the fraction obtained after ion-exchange chromatography through a Sephacryl S-300 column (1.6 x 45 cm) at a flow rate of 1 ml/min.
Fig. 60b) : Preparative gradient polyacrylamide gel electrophoresis of S1 fraction on 5-20% gel. After electrophoretic run gel was divided into 3mm slices, and proteins were allowed to elute through diffusion. Each slice was checked for CMCase activity and protein concentration.

CMCase •

Gradient -

A280 □
**Fig. 61(a).** SDS-PAGE for molecular weight determination.

a) CMCase 3
b) CMCase 4
c) Molecular weight markers
d) CMCase 5
Fig. 61(b) Location of CMCase activity on zymogram.

a  CMCase 3,  b. CMCase 4,

b  CMCase 5  d. 72 hrs. broth CMCase
Fig. 62. Line weaver burk plot for purified CMCase 3 \textit{C. flavigena}
Inhibition studies

CMCase 3 was inhibited by both glucose and cellobiose. When 20 mM of glucose was added in the reaction mixture 100% inhibition was noticed while same concentration of cellobiose inhibited 80% of the total enzyme activity.

Optimum temperature: Enzyme was most active at 45-50°C. Below 45°C and above 50°C there was a gradual decrease in enzyme activity. (Fig.63).

Optimum pH: Purified CMCase 3 exhibited maximum activity at pH values 6.5 - 7.0. Below 6.5 and above 7.0 there was a decline in enzyme activity (Fig.64).

Stability: Enzyme was stable between 4.5 - 9.5 pH values. CMCase 3 gradually lost its activity below 4.5 and above 9.5 pH values (Fig.65). Thermal stability of the purified CMCase 3 was also checked and it was found that enzyme was stable till 0 - 30°C and lost 100% activity when incubated at 70°C for 30 min. (Fig.66).

Effect of metal ions: Effect of different metal ions e.g. Na⁺, Ca²⁺, Fe³⁺, and Ag⁺⁺ was studied. It was seen that Na⁺ has no effect on the enzyme activity. Ca²⁺ was found to slightly inhibited the enzyme activities. At lower concentrations there was less decline in enzyme activity as compared to higher concentration (Fig.72). Heavy metal ions Ag⁺ and Fe³⁺ were found to be the strong inhibitor for the enzyme activity.
Fig. 64 Effect of pH on purified CMCase 3

Fig. 63 Effect of temperature on purified CMCase 3

Fig. 65 Effect of pH on stability of purified CMCase 3

Fig. 66 Effect of temperature on stability of purified CMCase 3.
even in lower concentrations. 7 mM of Fe$^{++}$ was sufficient for inhibiting 100\% enzyme activity while Ag$^{+}$ at 7 mM concentration affected complete inhibition (Fig.70.71).

**Effect of β-mercaptoethanol:** β-mercaptoethanol at lower concentration completely inhibited purified CMCase 3 as shown in Fig.69.

**Molecular weight determination:** Purified CMCase 3 was a monomeric polypeptide having molecular weight of about 80,000 as determined by SDS-PAGE on a 10\% polyacrylamide gel. (Fig.61a)

**Characteristics of CMCase 4**

**Substrate specificity:** Purified CMCase 4 showed activity only against CMC. It showed no activity against any other cellulosic substrates including Avicel, salicin, filter paper, tissue paper, xylan and cellulobiose.

**Effect of substrate concentrations:** Purified CMCase 4 showed maximum activity when 2\% CMC was used as substrate, linearity in rate of reaction was observed till 1.2\% CMC and above this concentration there was no regular increase in the rate of reaction. Enzyme has Km value of 1.69/l. Lineeweaver Burk plot for the purified enzyme was also plotted (Fig.73).

**End products identification:** Enzyme produced oligosaccharides and cellulobiose as end products against CMC substrate.
Fig. 69 Effect of β-mercaptoethanol on purified CMCase 3 activity.

Fig. 70 Effect of AgNO₃ on purified CMCase 3 activity.

Fig. 71 Effect of FeSO₄ on purified CMCase 3.

Fig. 72 Effect of CaCl₂ on purified CMCase 3 activity.
Fig. 7.3 Line weaver burk plot for purified CMCase † for C. flavigena
Inhibition studies: Purified CMCase 4 was inhibited by both glucose and cellobiose. When 30 mM of glucose was added in the reaction mixture approximately 100% inhibition of the enzyme was noticed while 20 mM of cellobiose was sufficient for the same purpose.

Optimum temperature: It was found that enzyme CMCase 4 was most active between 40-50°C. Below 40°C and above 50°C there was a decline in the rate of reaction (Fig. 74).

Optimum pH: Purified CMCase 4 had a sharp pH optima at pH 6.5. Below and above this pH value there was a decrease in rate of reaction. At pH 8.5 only 15% activity was exhibited by the enzyme.

Stability: Purified CMCase 4 was stable in the pH range of 5-9.5. Below pH 5 and above pH 9.5 there was a gradual decline in enzyme stability. Thermal stability of the enzyme was also checked and it was found that enzyme was stable at 30°C and lost 100% activity when incubated at 70°C for 30 min. (Fig. 76, 77).

Effect of metal ions: Effect of different ions was studied on CMCase 4 activity. Na⁺ had no effect on the enzyme activity. Ca⁺⁺ inhibited enzyme activity specially when used in lower concentrations (Fig. 81). Ag⁺⁺ and Fe⁺⁺ strictly inhibited the enzyme activity (Fig. 78, 80).
Fig. 74 Effect of temperature on purified CMCase 4.

Fig. 75 Effect of pH on purified CMCase 4.

Fig. 76 Effect of pH on stability of purified CMCase 4.

Fig. 77 Effect of temperature on purified CMCase 4.
Fig. 78 Effect of AgNO$_3$ on purified CMCase 4.

mM AgNO$_3$

Fig. 79 Effect of B-mercaptoethanol on purified CMCase 4.

μM B mercaptoethanol

mM FeSO$_4$

Fig. 80 Effect of FeSO$_4$ on purified CMCase 4.

Fig. 81 Effect of CaCl$_2$ on purified CMCase 4.

mM CaCl$_2$
Effect of β-mercaptoethanol: As shown in Fig. 79, β-mercaptoethanol was found to be a strong inhibitor for the pured enzyme. 10 μM was sufficient for 100% inactivation of the enzyme.

Molecular weight determination: Purified enzyme CMCase has molecular weight of 40,000 as determined by gradient SDS PAGE (Fig. 6th).

Purification of D₁ fraction

Concentrated D₁ fraction was subjected to gel filtration on Sephacryl-300 column (1.6x44 cm). As shown in Fig. 82 enzyme activity was eluted as a single sharp peak corresponding to its maxima in fraction 22 where protein concentration was also maximum (Fig. 82).

Preparative electrophoresis of D₁ fraction

Sample was concentrated through lyophilization and subjected to 5-15% gradient PAGE. Proteins were eluted from the gel slices and it was found that only 1 peak for CMCase activity was obtained on electrophoreogram which consisted of fraction 17-19 (Fig. 83). This fraction was electrophoresed on 5-20% gradient PAGE and replica was made on an agar CM plate and it was found that this peak was comprised of a single band active against CMCase. It was named as CKase 100%.
Fig. 8.2 Gel filtration of D₁ fraction on Sephacryl S-300 column (16 x 25 cm). Proteins were eluted at flow rate of 1 ml/min at 4°C. Each fraction comprised of 0.5 ml of the eluent.

CMCase activity •

A₂₈₀ □
Fig. 83 Electrophoresis of D1 fraction on 5-15% gradient polyacrylamide gel. After electrophoretic run gel was divided into 3 mm slices and proteins were allowed to elute through diffusion. Each fraction was tested for CMCCase activity and protein concentration.

CMCase ●  A280 □

gradient —
Characteristics of CMCase 5

**Substrate specificity:** Enzyme was only active against CMC. It showed no activity against any other cellulosic substrates including Avicel, salicin, xylan, tissue paper, filter paper and cellobiose.

**Effect of substrate concentration:** Effect of different concentration of CMC was studied and it was found that enzyme showed maximum activity when 2% CMC was used as substrate. Upto a 1.2% CMC concentration a linear increase in the rate of reaction was observed. Above this concentration no regular increase in rate of reaction was noticed. Line weaver burk plot was plotted for the enzyme (Fig.85). CMCase 5 had a kM value of 1.6 g/l.

**Identification of hydrolytic product:** Enzyme was able to produce ooligosaccharide and cellobiose as end products against CMC substrate.

**Inhibition studies.** CMCase 5 was inhibited by both glucose and cellobiose at a concentration of 10 mM or above.

**Optimum pH.** It was found that enzyme has a broad pH optima range of 6-8, centered at pH 7 (Fig.86).

**Optimum temperature:** Optimum temperature of the purified CMCase 5 was 45°C (Fig.87). Between 40 - 50°C enzyme was quite active but below these concentrations less rate of reaction was observed.

**Thermal stability:** Thermal stability of the CMCase 5 was studied and it was found that enzyme was stable from 0-30°C and gradually loss its activity when incubated for 30 min. from 70°C enzyme lost
Fig. 85 Line weaver burk plot for purified CMCase 5 of *C. flavigena*
Stability: Stability of purified CMCase 5 was checked at different pH values and it was found that enzyme was stable in the pH range of 4-8.5 (Fig.88). Regarding thermal stability, the enzyme was stable in the range of 0 - 38°C, and lost 100% activity when incubated at 70°C for 30 min. (Fig.88,89).

Effect of metal ions: Effect of different metal ions including Na⁺, Fe⁴⁺ and Ag⁺⁺ was studied on the CMCase 5. It was found that Na⁺ has no effect of enzyme activity. Ca⁺⁺ inhibited enzyme activity from 10 - 100 mM concentrations in the reaction mixture. (Fig.90). Heavy metal ions Ag⁺⁺ and Fe⁺⁺ strongly inhibited the enzyme activity even at lower concentration (Fig.91,92).

Effect of reducing agent: β-mercaptoethanol inactivated the enzyme activity even when added in uM concentrations. (Fig.93).

Homogeneity of enzyme protein: Enzyme was homogeneous on 5-20% SDS-gradient PAGE and was comprised of a single polypeptide chain (Fig.61b).

Molecular weight determination: Purified CMCase 5 had molecular weight of 80,000 as determined by gradient SDS PAGE (Fig.61 a).
Fig. 86 Effect of pH on purified CMCase 5.

Fig. 87 Effect of temperature on purified CMCase 5.

Fig. 88 Effect of pH on the stability of purified CMCase 5

Fig. 89 Effect of temperature on the stability of purified CMCase 5.
Fig. 90 Effect of CaCl₂ on purified CMCase 5 activity.

Fig. 91 Effect of AgNO₃ on purified CMCase 5.
Fig. 92 Effect of FeSO₄ on purified CMCase 5 activity.

Fig. 93 Effect of β-mercaptoethanol on purified CMCase 5 activity.
Specific activities of the purified components of Cellulase system.

There was no increase specific activities of the purified CMCases. But there was increase in specific activity of the cellulase system when this system was purified by acetone precipitation and gel filtration. There was 3 times increase in specific activities of CMCase and avicelase e.g. 330 U/mg protein for CMCase and 36 U/mg for avicelase.
DISCUSSION
DISCUSSION

Present study deals with the production, purification and partial characterization of extracellular cellulolytic enzymes produced by a locally isolated cellulolytic mesophilic and facultatively aerobic bacterium identified as *Cellulomonas flavigena*. Studies were undertaken to optimize the production of extracellular cellulases by this organism. The enzymes were then isolated and purified and their characteristics were studied.

The microbe when cultivated in the presence of 0.5% Avicel as carbon source, produced higher levels of CMCcase as well as avicelase activities as compared to those produced in the presence of 0.5% CMC. Maximal activity of each of the enzyme i.e. 5.5 and 0.45 U/ml of the culture supernatant were obtained after 72 hours of fermentation at which stage the growth as determined in the term of cellular proteins, was maximal (Fig.12). Tween-80 when added to the culture medium at a concentration of 0.1% resulted in more than two fold increase in activities of both CMCcase and avicelase in the culture supernatants (Fig.17). This could be due to the effect of surfactant in hindering the immobilization of the enzyme on the substrate by reducing the strength of adsorption and also increasing permeability of the bacterial cell wall and thus facilitating the release of enzyme into the medium. Extracellular CMCcase and avicelase activities
increased with increase in the concentration of yeast extract up to a level of 0.2%, beyond which no further increase took place in the enzyme activities in the culture supernatants. (Fig. 16).

A study on the effect of Avicel concentration on the production of extracellular cellulases of *C. flavigena* in culture supernatants showed that maximum free activities of both the enzymes were produced in the culture containing 0.5% of the substrate (Fig. 13). Increase in the concentration of Avicel above 0.5% decreased the cellulases in the culture supernatant possibly due to the specific binding of the enzymes to the substrate. Gilkes et al.\textsuperscript{186} reported binding of all extracellular cellulases produced by *C. fimi* when > 3 of Avicel was used as carbon source in the medium. Nakamura and Kitamura\textsuperscript{135} found maximum yield of CMCase and avicelase activities by *C. uda* after 48 hours of fermentation in the presence of 2% Avicel. Maximum CMCase and avicelase activities obtained after 72 hours of fermentation with 0.5% Avicel as carbon source, were 11.0 and 1.2 U/ml of the culture supernatant (Fig. 13). As previously reported adsorption affinity and structural properties of Avicel, being crystalline cellulose has significant effect on adsorption of cellulases\textsuperscript{49}. Adsorption of cellulases on cellulosic substrate has been studied by a few workers\textsuperscript{50-57}. In case of *C. flavigena* the proportion of enzyme activities in the culture supernatants and those bound to the residual Avicel in the culture medium was studied in the presence of different concentrations of
Avicel. The enzyme activities bound to Avicel were eluted by shaking the residual Avicel with twice the broth volume of distilled water for 30 minutes at 4°C, five times successively. Further washing did not elute any detectable levels of cellulase activities. Sonication was also used for the elution of bound enzymes from the residual Avicel. As shown in Fig.14 a,b sonication desorbed approximately same amount of enzyme as eluted by shaking. It has advantages over the shaking method in that it was quicker. Carloz used sonication to increase the saccharification of cellulosic substrates. There are reports on elution of cellulases from the insoluble cellulosic substrates with 8M Guanidine hydrochloride, detergent, water and organic solvents. pH variations were also used for the elution of enzymes from the insoluble cellulose. Adjustment at pH 10 was found most successful for this purpose. Elution with plain water has obvious advantages, as the cellulase protein molecules are likely to retain their native form.

Proportion between bound and free CMCase activities when cultivated in the presence of different percentages of Avicel as shown in Table 1. The maximal total CMCase activities did not vary appreciably with change in Avicel concentrations between 0.5 - 4.0%. However, the proportion between Avicel-bound and free activities differed significantly not only at different fermentation stages of the same culture
medium but also with the change in the concentrations of Avicel in the culture medium. In general the proportion of the bound activities was greater during the initial periods of fermentation and this proportion decreased significantly along with the fermentation. Hydrolysis of amorphous regions of the substrate in the culture medium would reduce the binding sites for the enzymes with concomitant increased proportion of soluble cellulase activities. This also explains a much higher proportion of bound activities in the presence of higher percentage of Avicel. For avicelase, most of the activity remained to the residual Avicel, during fermentation period studied (Table 2) possibly due to the availability of binding sites, produced by the action of endoglucanase (CMCase) activity. The total activities of both CMCase and avicelase produced in the presence of 2.0% Avicel were 13.2 and 3.0 U/ml of the culture medium, respectively. The total avicelase activity produced by \textit{C. flavigena} is however, greater than any other \textit{Cellulomonas} species reported previously. Above this concentration there was a decrease in the total cellulase activity at 72 hours of fermentation, possibly due to the higher production of reducing sugars in the culture medium which resulted in the end product inhibition of the enzyme (Fig.15).

A comparative study on CMCase activities, both free and Avicel bound was made by using 5-20% gradient PAGE zymogram technique. As shown in Fig.20, at least eight fractions could be detected in the culture supernatants of \textit{C. flavigena} at all
stages of fermentation studied i.e. 24, 48 and 72 hours. CMCase activity bound to residual Avicel after 24 hours of fermentation, at which stage maximum enzyme was bound to the substrate only four major activity bands were detectable on zymogram. Bound enzyme samples of 48, 72 and 96 hours of fermentation were also compared with 24 hours bound CMCases on 5-20% gradient PAGE (Fig. 21). It was seen that at least five CMCases were detectable on zymogram. Each of the sample has one slow moving band which was absent from the 24 hours bound enzyme sample. It was also seen that the bound enzymes although have only those enzymes which are nearer to the native form of the enzymes, lost most of their activities during resolution as noticed on zymogram, so that for having a clear picture of enzymes 10 times higher activities were required to load on the polyacrylamide gel as compared to the free enzyme and time of incubation on CMC-agar plate was also increased. Possibly this was due to the removal of carbohydrate molecules from the enzyme molecules during resolution which stabilized the molecule structure, as reported previously, in case of carbohydrases.
Multiplicity of cellulases is considered as a general phenomena in microbes. In case of *C. Flavigena*, multiple forms of CMCase activity were demonstrated on 5-20% gradient PAGE followed by zymogram technique (Fig. 20, 21). At least seven bands were shown for free activity at all the stages of fermentation while for substrate bound activity at least four different fractions were identified for CMCase activity at early stage of fermentation (Fig. 20, 21). In literature there are different reasons for the multiplicity of cellulases. Marsden and Gray have summarised the possible reasons for the multiplicity of cellulases e.g. proteolytic degradation of cellulases, glycosylation, deglycosylation and the expression of more than one gene specific for cellulases. For *C. Flavigena* although it secretes considerable levels of protease activity (Fig. 18, 19) it was unable to produce proteolytically degraded low molecular weight active fragments of CMCase activity (Fig. 20, 21) as appeared on zymogram. Multiple forms appeared at early stages of fermentation in substrate bound activity are considered as the native forms of the enzymes. As reported by Akhtar et al., six DNA fragments encoding for CMCase activity in *C. Flavigena* have been isolated. The multiplicity in early stages of fermentation could be due to the expression of multiple genes for cellulase activity. During later stages of fermentation the appearance of slow moving bands on zymogram for CMCase activity could be the result of glycosylation of the cellulases with partially hydrolysed soluble carbohydrate fragments which hindered their mobility on PAGE (Fig. 20, 21).
Characteristics of extracellular cellulases of C. flavigena after 80% acetone precipitation were undertaken. Studies on the partial characterization of extracellular cellulases of C. flavigena showed that both the CMCase and avicelase activities were most active at pH 6.5 (Fig. 24) and 50°C (Fig. 25). Other workers have reported pH optima of endoglucanase of Cellulomonas between 6-7 \(^9\) and 5.5 - 6.5 \(^9\). The optimum temperature have also been reported to be 40-50°C for both the activities \(^9\), \(^9\). It was found that CMCase showed a linear increase in rate of reaction when 0.5% CMC was added in the reaction mixture (Fig. 26), while for avicelase 3% Avicel was sufficient for the same purpose (Fig. 27). End products of each of the crude enzyme activity was identified and it was found that both enzyme activities were able to produce glucose and cellobiose as their major (Fig. 34b) hydrolytic products. Inhibition studies were also undertaken which showed that 20-30 mM glucose and cellobiose can inhibit approximately 100% activity. Both the cellulases were stable at 10°C while nearly all the activity of each of the enzymes was lost when incubated at 70°C for 30 minutes (Fig. 34a.). Both the enzymes were stable in the pH range of 3-11. Below pH 3 and above pH 11 there was a gradual decrease in enzyme stability. Effect of different metal ions of enzyme activities was also studied. It was found that magnesium and calcium inhibited enzyme activities when used in lower concentration but there was an increase in enzyme activities when 10-12 mM of each of the ions were added into the reaction.
mixture. Above these concentrations there was a gradual decrease in enzyme activities (Fig. 32 a, b). NaCl has no effect on both the activities. Heavy metal ions like Ag⁺⁺ and Fe⁺⁺ strongly inhibited both the enzyme activities (Fig. 32 c, d) β-mercaptoethanol proved to be a strict inhibitor for both the enzyme activities. In case of \textit{C. flavigena} cellulases possibly there is involvement of sulphydral groups at the active sites or the reduction of S-S bonds, present in the enzyme molecules lead to the inactivation of the enzymes.

This sample was passed through a gel filtration column and cellulase activities appeared as a single sharp peak (Fig. 35). After acetone precipitation and gel filtration there was three times increase for specific activities of CMCase and avicelase enzymes e.g. 330/mg protein and 35 U/mg protein respectively. Electrophoretic pattern of CMCases after acetone precipitation and gel filtration were compared. It was found that in each case all the CMCase bands were present (Fig. 37). After gel filtration cellulase system of \textit{C. flavigena} was further resolved on DEAE-Sephadex A-50 column using a linear salt gradient. This technique was helpful in group separation thus three fractions named D₁, D₂ and D₃ were obtained, comprising of at least 4, 3 and 2 CMCase bands as appeared on zymogram after gradient PAGE (Fig. 36, 37). Efforts were made to resolve these fractions into their components by using narrow salt gradient but were unsuccessful. D₃ being the major contributor (Table 3) was eluted with high salt
concentration e.g. 0.5 - 0.75% (Fig. 36). D$_3$ was resolved into CMCase 1 and CMCase 2 after 5-20% gradient preparative PAGE while D$_2$ and D$_1$ were resolved into CMCase 3, CMCase 4 and CMCase 5 after 5-15% preparative gradient PAGE (Fig. 40, 61). Each of the enzyme appeared as single band on SDS PAGE (Fig. 41). CMCase 1 and CMCase 2 had different mobility on native gel as appeared zymogram (Fig. 40) and had same molecular weight of about 20 K. It is possible that these two cellulases have arisen from a common ancestor. CMCase 3 and CMCase 5 had molecular weight of 80 K while CMCase 4 had molecular weight of about 40 K. All the purified CMCases appeared as single band on SDS PAGE and had different mobilities on the PAGE.

After purification characteristics of each purified enzyme were studied and a comparative study was made. It was found that all the CMCases were only active against carboxymethylcellulose. The culture supernatant of _C. flavigena_ after 72 hours of fermentation showed considerable level of avicelase activity (Fig. 12) e.g. 1.2 U/ml. There are examples on the action of purified CMCases on other cellulosic substrates some workers have claimed slight contamination of other cellulase activities along with the purified enzymes which gave activity against the other cellulosic substrates.

Purified endoglucanases of _Erwinia chrysanthemi_ and _S. pulverulentum_ were only active against CMC. Generally, it is accepted that CMCases have endo-type of mode of action and they may give some activity against crystalline cellulose. Hydrolytic products of each of the enzyme was glucose along with cellobiose. The Km value for CMCase 1, 2, 3, 4 and 5 were 0.83, 1.7, 1.54, 1.64 and 1.6 g/l. CMCase 1, has lower Km
Km value as compared to the other purified CMCases. All the enzymes were stable in the pH range of 4.0 - 9.0 and 0-30°C. Effect of different metal ions was also studied and it was found that calcium and magnesium both inhibited the enzyme activities when used in lower concentration i.e. 2 - 5mM in the reaction mixture above these concentration less inhibition was noticed. In case of crude CMCases both the metal ions were said to be the inhibitor in lower concentration but activated the enzyme when used in higher concentration i.e. 10 - 15mM. Heavy metal ions like silver and iron strictly inhibited these enzymes. β-mercaptoethanol as a reducing agent proved to be a strictly inhibitor for all the purified CMCases. The molecular weight of each purified enzyme was estimated on 10% SDS PAGE. CMCase 1 & have same molecular weight i.e. 20400 daltons. These two enzymes have very close electrophoretic mobility on 5-20% gradient PAGE (fig.40). CMCase 3 and 5 had molecular weight of 80,000 while CMCase 4 had molecular weight of 40,000 (Fig.61a).

After acetone precipitation and gel filtration there was three fold increase in both the cellulase activities i.e. 330 U/mg protein for CMCase and 36 U/mg protein for avicelase activities. However, the cellulase system was resolved into its components i.e. CMCases 1,2,3,4 and 5 there was no further increase in specific activities. It is possible that after resolution of the cellulase system into its components some of them may be lost during purification procedure and there was a loss of synergistic effect. The other possibility is the removal of carbohydrate moieties which could result in instability of the enzymes.
These studies will be helpful in finding a way for the efficient degradation of cellulose by cellulases. Gene characterization of CMCase 1 and CMCase 2 (two low molecular weight cellulases) will provide a ground to find out the origin and homology between the two. This will reveal the active part of the gene which is responsible for the cellulose degradation. Studies on the isolation and characterisation of cellulase genes of *C. flavigena* are already in progress in this laboratory.
REFERENCES
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Production of free and substrate-bound cellulases of Cellulomonas flavigena

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Conditions for the production of extracellular carboxymethyl cellulase (CMCase) and avicelase activities by a locally isolated Cellulomonas species identified as C. flavigena were optimized. The microbe produced maximal levels of free cellulases after 72 h of fermentation, when cultivated in the presence of 0.25% yeast extract, 0.5% Avicel, and 0.15% Tween 80 at 30°C. Conditions for the elution of substrate-bound cellulases from the residual Avicel were optimized. Higher proportions of the extracellular cellulases were bound to the substrate when higher concentrations of Avicel were used. When 2.0% Avicel was used as a carbon source, maximal levels of extracellular CMCase and avicelase produced were 13.2 and 3 U ml⁻¹ of the culture medium, respectively. Both the cellulases were most active at pH 6.5 and 50°C and were inhibited by β-mercaptoethanol. Calcium chloride activated CMCase but had no effect on avicelase activity. Each of the enzymes lost activity when incubated at 70°C for 30 min. A comparative study of free and substrate-bound CMCase on gradient polyacrylamide gel electrophoresis (PAGE) showed that free CMCase activity was composed of at least seven active fractions, while bound activity had four major bands of CMCase activity. Since a considerable level of protease was secreted by C. flavigena, the additional fractions in the free CMCase activity could be a result of proteolysis.

Keywords: CMCase; avicelase; Cellulomonas flavigena

Introduction

Enzymes involved in the breakdown of cellulose to glucose fall into three basic groups:¹ endo-1,4-β-D-glucanase (1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4), exo-1,4-β-D-glucanases (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), and 1,4-β-D-glucan glucohydrolase, EC 3.2.1.74), and β-D-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21). A large variety of fungal and bacterial species have been reported to produce one or more of these enzymes. A number of reviews dealing with the nature of cellulose substrates,²—⁵ production and properties of cellulases, and their action on cellulose hydrolysis⁶—¹⁰ have been published.

Amongst a variety of bacterial species reported for cellulolytic activity, Cellulomonas species have received considerable attention. Kim and Wimpenny¹¹ studied the growth and cellulolytic activities of C. flavigena. Grass-based lignocellulosic substrates have been reported to enhance cellulase production by this organism.¹² A strain of C. uda was reported to be an efficient producer of crystalline cellulose hydrolysing activity when grown in the presence of crystalline cellulose.¹³ Stopkop et al.¹⁴ studied the formation, location, and regulation of endo-β-1,4-glucanase and β-glucosidases by C. uda in the presence of different carbon sources. Production of cellulases by C. uda was studied using printed newsprint as a carbon source.¹⁵

Béguin and Eisén¹⁶ reported the purification of three extracellular cellulases from the culture of Cellulomonas. One of these was found in the culture supernatant, while the other two were found to be bound to the cellulose added as a carbon source. Two extracellular endoglucanases were purified and partially characterized from culture supernatants of C. fermentans.¹⁷ Six major components exhibiting endoglucanase activity were partially purified from culture filtrates of a Cellulomonas species.¹⁸ Langsford et al.¹⁹ reported that the cellulase system of C. fimi was composed of only three enzymes, which had great affinity for, and were stabilized by binding to, an insoluble

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cellulosic substrate used in the fermentation medium. Free enzymes in the culture medium, however, were subject to limited proteolysis and deglycosylation, which generated a variety of products, some of which stained enzymatic activity.

As a result of screening of the locally isolated organisms, a highly potent cellulolytic bacterial strain, named as *Cellulomonas flavigena*, was studied for the reduction of free and substrate-bound extracellular cellulases.

**Materials and methods**

**Microorganism**

The organism was isolated locally and the preliminary identification, by the National Collection of Industrial Marine Bacteria Ltd., Aberdeen, Scotland, showed it to be *Cellulomonas flavigena*. The organism was maintained on agar slant of the following composition (g l\(^{-1}\)): yeast extract (Difco) 2, carboxymethyl cellulose (CMC, low viscosity, BDI) 5, NaNO\(_3\) 0.8, KH\(_2\)PO\(_4\) 1.0, MgSO\(_4\) • 7H\(_2\)O 0.5, FeSO\(_4\) • 7H\(_2\)O 0.1, and agar (Difco) 15. The pH of the medium was adjusted to 7.3 before adding the agar and autoclaving. All the chemicals, unless otherwise stated, were of nautical grade reagent.

**Culture methods**

Twenty milliliters of the medium of the composition given above, but without agar, and with CMC replaced with 0.2 ml of 5 mm cellulose (BDI), was taken in a 50-ml Erlenmeyer flask and inoculated with cells from a freshly grown slant. The flasks were incubated in an orbital incubator shaker at 30°C until the absorbance at 610 nm reached 0.6. One milliliter of this inoculum was used to inoculate 100 ml of the culture medium.

Fermentations were carried out in 1-l Erlenmeyer flasks, each containing 300 ml of the medium as given above. After inoculation, the flasks were incubated in an orbital incubator shaker at 150 rev min\(^{-1}\) at 30°C. After specific hours of fermentation, samples were removed from the flasks aseptically for further processing.

The supernatants were obtained by centrifugation at 4000g for 15 min. When *C. flavigena* was grown in the presence of Avicel (microcrystalline cellulose, E. I. du Pont de Nemours & Co.), the culture medium after specific fermentation periods was centrifuged at 5000g for 10 min to recover the residual cellulose. The residue was shaken with distilled water at 4°C for 30 min and the clear supernatant was obtained by centrifugation at 12000g for 15 min. Further washings were similarly obtained until the enzyme activities were not detectable in the supernatant. All the washings were mixed for further processing.

The cell growth was determined in terms of the cellular proteins, which were extracted by a modified method of Huang et al. A 5-ml culture sample, withdrawn aseptically at specific hours of fermentation, was centrifuged for 30 min at 4°C. The residue was heated with 1 ml NaOH in a boiling water bath for 10 min. Proteins in the supernatant, as obtained by centrifugation at 10000g for 10 min, were estimated by the method of Lowry et al.

**Protein estimation**

Proteins in the samples, other than those described above, were estimated by the dye-binding method.

**Enzyme assays**

CMIase. One milliliter of the appropriately diluted enzyme sample was mixed with 1% CMC dissolved in 0.1 M citrate buffer (pH 6.5) containing 20 mm CaCl\(_2\) (BDI) and incubated for 10 min at 50°C in a shaking water bath. Reducing sugars thus released were estimated by the dinitrosalicylic acid (BDI) reagent method. One unit of CMIase activity was defined as the amount of the enzyme that liberated 1 μg of reducing sugar as glucose from CMC per minute under the assay conditions described.

Avicelase. One milliliter of the enzyme was mixed with 1 ml of 5% Avicel (w/v) suspended in 0.1 M citrate buffer (pH 6.5) and incubated for 10 min at 50°C in a shaking water bath. Residual Avicel was removed by centrifugation at 5000g for 5 min and the reducing sugars in the supernatant were estimated as given above. One unit of avicelase activity was defined as the amount of the enzyme that released 1 μg of reducing sugar as glucose from Avicel per minute under the assay conditions described.

Protease. One milliliter of the appropriately diluted enzyme sample was incubated with 1 ml of 0.5% hemoglobin (E. Merck) dissolved in 0.1 M citrate buffer (pH 6.5) and incubated for 30 min at 50°C. Residual proteins were precipitated by adding 3 ml of 10% trichloroacetic acid (BDI) and centrifuged at 5000g for 10 min. The liberated amino acids and peptides were estimated by the dye-binding method. One unit of protease activity was defined as the amount of the enzyme that produced an absorbance at 595 nm of 1.0 under the assay conditions.

**Electrophoresis**

The enzyme samples were first precipitated at 4°C with 80% acetone. The precipitates were separated by centrifugation at 10000g at 4°C and dissolved in a minimum volume of water. The concentrated enzyme solutions thus obtained were electrophoresed on a 5–20% polyacrylamide (BDI, Electran) gradient gel (0.1 x 5 x 12 cm) for 5 h at 4°C with a constant current supply of 4 mA cm\(^{-2}\) of the gel slab using tris-borate-EDTA (all BDI) buffer (pH 8.3), containing 20.6, 11.0, and 1.86 g l\(^{-1}\), respectively. After the electrophoretic run, the gel was incubated on CMC-agar plate (0.5% CMC, 2% agar, and 5 mm CaCl\(_2\) dissolved in 0.05 M citrate buffer, pH 6.5) for 20 min. The agar plate was stained at 30°C until the absorbance at 610 nm reached 0.6. One milliliter of this inoculum was used to inoculate 100 ml of the culture medium.

The supernatants were obtained by centrifugation at 3000g for 15 min. When *C. flavigena* was grown in the presence of Avicel (microcrystalline cellulose, E. I. du Pont de Nemours & Co.), the culture medium after specific fermentation periods was centrifuged at 5000g for 10 min to recover the residual cellulose. The residue was shaken with distilled water at 4°C for 30 min and the clear supernatant was obtained by centrifugation at 12000g for 15 min. Further washings were similarly obtained until the enzyme activities were not detectable in the supernatant. All the washings were mixed for further processing.

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with 0.5% congo red (BDH) for 10 min and then washed with 1 M NaCl. CMCase activity appeared as lighter bands against a red background.

### Results and discussion

*C. flavigena* produced higher levels of CMCase as well as avicelase activities in the presence of 0.5% Avicel as a carbon source when compared to those produced in the presence of 0.5% CMC. Maximal activities of both the enzymes, i.e., 5.5 and 0.45 U ml⁻¹ of the culture supernatant, were obtained after 72 h of fermentation, at which stage the growth, as determined in terms of the cellular protein, was maximum (Figure 1). Tween-80, when added to the culture medium at a concentration of 0.1%, resulted in more than twofold increase in activities of both CMCase and avicelase in the culture supernatants (Figure 2). This could be due to the effect of surfactant in hindering the immobilization of the enzyme on the substrate by reducing the strength of adsorption, and also increasing permeability of the bacterial cell wall, thus facilitating the release of the enzyme into the medium. Extracellular CMCase and avicelase activities increased with increase in the concentration of yeast extract up to a level of 0.2%, beyond which no further increase took place in the enzyme activities (Figure 3).

A study on the effect of Avicel concentration showed that maximum free activities of both the enzymes were produced in the cultures containing 0.5% of the substrate (Figure 4). Increase in the concentration of Avicel above 0.5% resulted in decrease of cellulases in the culture supernatants, possibly due to the specific binding of the enzymes to the substrate. Gilkes et al. reported binding of all extracellular cellulases produced by *C. fimii* when >3% of Avicel was used as carbon source in the medium. Nakamura and Kitamura found maximum yield of CMCase and avicelase activities by *C. uda* after 48 h of fermentation in the presence of 2% Avicel. Maximal CMCase and avicelase activities, obtained after 72 h of fermentation with 0.5% Avicel as carbon source, were 11.0 and 1.2 U ml⁻¹ of the culture supernatant (Figure 4). As previously reported, adsorption affinity and structure properties of Avicel, which is crystalline cellulose, have significant effects on adsorption of cellulases. Adsorption of cellulases on cellulosic substrates has been studied by a few workers. In the case of *C. flavigena*, the proportion of the enzyme activities in the culture supernatants to those bound to the residual Avicel in the culture medium was studied in the presence of different concentrations of Avicel. The enzyme activities bound to Avicel were eluted by shaking the residual Avicel with twice the broth volume of distilled water for 30 min at 4°C, five times successively. Further washings did not elute any detectable levels of cellulases activities. Other workers have reported elution with 8 m guanidine hydrochloride, detergent and plain water, and organic solvents. Elution with plain water has the obvious advantage that the cellulases protein molecules are likely to retain their native form.

![Figure 1](image1.png)  
**Figure 1** Cultivation of *C. flavigena* in the presence of (a) 0.5% Avicel and (b) CMC. (A) Cell protein; (○) soluble protein; (△) CMCase; (●) avicelase; (△) reducing sugars

![Figure 3](image3.png)  
**Figure 3** Effect of yeast concentration on the production of (a) extracellular free CMCase and (b) avicelase activities by *C. flavigena*. (A) 0.02%; (△) 0.05%; (○) 0.1%; (●) 0.2%

![Figure 2](image2.png)  
**Figure 2** Effect of Tween 80 concentration on the production of (a) extracellular free CMCase and (b) avicelase activities by *C. flavigena*. (A) 0%; (△) 0.05%; (○) 0.075%; (●) 0.1%

![Figure 4](image4.png)  
**Figure 4** Effect of Avicel concentration on the production of (a) extracellular free CMCase and (b) avicelase activities by *C. flavigena*. (A) 0.2%; (○) 0.5%; (△) 1.0%; (●) 2.0%
Table 1. Proportion between bound and free CMCase activity of C. flavigena cultivated in the presence of different percentages of Avicel in the culture medium

<table>
<thead>
<tr>
<th>Fermentation period</th>
<th>0.5</th>
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<th>2.0</th>
<th></th>
<th>4.0</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Bound</td>
<td>Free</td>
<td>Total</td>
<td>Bound</td>
<td>Free</td>
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<tr>
<td>24</td>
<td>453</td>
<td>40:60</td>
<td></td>
<td>457</td>
<td>51:49</td>
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</tr>
<tr>
<td>48</td>
<td>832</td>
<td>20:80</td>
<td></td>
<td>880</td>
<td>26:74</td>
<td></td>
<td>915</td>
</tr>
<tr>
<td>72</td>
<td>1216</td>
<td>9:91</td>
<td></td>
<td>1284</td>
<td>19:81</td>
<td></td>
<td>1320</td>
</tr>
<tr>
<td>96</td>
<td>1073</td>
<td>9:91</td>
<td></td>
<td>1091</td>
<td>19:81</td>
<td></td>
<td>1179</td>
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</tbody>
</table>

Table 2. Proportion between bound and free avicelase activity of C. flavigena when cultivated in the presence of different percentages of Avicel in the culture medium

<table>
<thead>
<tr>
<th>Fermentation period</th>
<th>0.5</th>
<th></th>
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<tr>
<td></td>
<td>Total</td>
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<td>Total</td>
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<tr>
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<td>28</td>
<td>75:25</td>
<td></td>
<td>35</td>
<td>78:22</td>
<td></td>
<td>34</td>
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<tr>
<td>48</td>
<td>105</td>
<td>60:40</td>
<td></td>
<td>137</td>
<td>66:34</td>
<td></td>
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</tr>
<tr>
<td>72</td>
<td>196</td>
<td>38:62</td>
<td></td>
<td>259</td>
<td>44:56</td>
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</tr>
</tbody>
</table>

The proportion of bound and free CMCase activities when cultivated in the presence of different percentages of Avicel is shown in Table 1. The maximal total CMCase activity did not vary appreciably with change in Avicel concentration between 0.5 and 4%. However, the proportion between Avicel bound and free activities differed significantly, not only at different fermentation stages of the same culture, but also with the change in concentration of Avicel in the medium. In general, the proportion of bound activity was greater during the initial periods of fermentation, and this proportion decreased significantly along with the fermentation. Hydrolysis of amorphous regions of the substrate in the culture medium would reduce the binding sites for the enzyme with concomitant increased proportion of soluble cellulase activity. This also explains a much higher proportion of bound activities in the presence of higher percentages of Avicel. In general, a similar picture for avicelase activity was also found, although the proportion of bound enzyme activities was always greater during the fermentation period than those in the case of CMCase (Tables 1 and 2). The total activities of both CMCase and avicelase produced in the presence of 2.0% Avicel were 13.2 and 3.0 U ml⁻¹ of the culture medium, respectively. Above this concentration, there was a slight decrease in the total cellulase activity at 72 h of fermentation, possibly due to the higher production of reducing sugars in the culture medium, which resulted in the end-product inhibition of the enzymes (Figure 5).

Amongst the most potent cellulase producers reported previously are Trichoderma reesei Rut-30,¹¹ a mutant of Thermomonospora curvata,²² and Cellulomonas uda,¹³ which have been reported to produce extracellular CMCase activities of over 100, about 20, and 32 U ml⁻¹ of the culture supernatants, respectively. The C. flavigena produced higher levels of CMCase activity than most of the other cellulolytic organisms studied. Further, C. flavigena produced greater avicelase activity than C. uda, which, at about 1 U ml⁻¹ of the culture supernatant,¹³ was reported to be a potent Cellulomonas species for the production of this activity.

The extracellular protease activity produced by C. flavigena showed two pH optima at 6.5 and 8.9. Protease activities at both of these pH values were therefore determined in the culture supernatants at different fer-
mentation periods. As shown in Figure 6, activities at both pH values increased along with the fermentation period. The increase was more rapid after 72 h of fermentation due to cell lysis, as indicated by a decrease in cellular proteins (Figure 1) and examination under a microscope.

Studies on the partial characterization of extracellular cellulases of C. flavigena showed that both the CMCase and the avicelase activities were most active at pH 6.5 (Figure 7) and 50°C (Figure 8). Other workers have reported pH optima of endoglucanase of Cellulomonas between 6 and 7 and 5.5 and 6.5. The temperature optima have also been reported to be 40-50°C for both the activities. Both the cellulase activities were stable at 10°C, while nearly all of the enzyme of each of the enzymes was lost when incubated at 70°C for 30 min (Figure 9). Ten millimolar CaCl₂ in the reaction mixture increased CMCase activity up to 40%, while it had no effect on avicelase activity (Figure 10). NaCl had no effect on cellulase activities. β-Mercaptoethanol inhibited activities of both the enzymes (Figure 11), possibly due to the involvement of sulfhydryl groups at the active site of the enzymes present in the enzyme molecules.

A comparative study on CMCase activities, both free and Avicel-bound, was made by using gradient PAGE and zymogram techniques. As shown in Figure 12, at least seven fractions could be detected in the culture supernatants of C. flavigena at all the stages of

Figure 6. Production of extracellular protease activity in the culture of C. flavigena. (Δ) Activity at pH 8.5; (○) activity at pH 6.5

Figure 7. Effect of pH on cellulase activities produced by C. flavigena. (○) CMCase; (○) avicelase

Figure 8. Effect of temperature on cellulase activities produced by C. flavigena. (○) CMCase; (○) avicelase

Figure 9. Thermal stability of the cellulases produced by C. flavigena. (○) CMCase; (○) avicelase

Figure 10. Effect of CaCl₂ on the cellulases produced by C. flavigena. (○) CMCase; (○) avicelase

Figure 11. Effect of β-mercaptoethanol on the cellulases produced by C. flavigena. (○) CMCase; (○) avicelase
fermentation studied, i.e., 24, 48, and 72 h. CMCase activity bound to the residual Avicel after 24 h of fermentation showed only four major activity bands on the zymogram. The appearance of a greater number of bands in the culture supernatants, as compared to those bound to Avicel, was possibly due to proteolysis of the CMCase enzymes after these were released by the cells. The release of considerable amounts of protease activity has already been demonstrated (Figure 6). Langsford et al.19 found more than 10 CMCase in the culture supernatants of C. flavigena that were said to be the proteolytically degraded and glycosylated products of the native enzyme. However, the number of CMCase fractions shown in a culture supernatant of C. flavigena would also depend on the conditions under which it was handled. CMCase enzymes specifically bound to Avicel are likely to represent the forms in which these were secreted by the cells.

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Abstracts

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School of Medicine
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281 MODULATION OF RAT LIVER CYTOSOLIC PROTEIN TURNOVER KINETICS ACTIVITY. D. Munnik and J. K. Bonsall, University of Sheffield, Sheffield.

Soluble protein tyrosyl kinase(s) activity can be isolated from rat liver cytosol by affinity chromatography on immobilized poly (Glutyl 8:1). However, subsequent purification procedures are generally accompanied by loss of activity, for example at 70% of the activity can be lost by simple gel filtration on Ultrogel AcA 200, or ammonium sulphate precipitated extract. This activity can be increased by the addition of fractions containing little or no TX activity. It is also possible to demonstrate the presence of inhibitors of the enzyme activity. There appear to be at least two activators and one inhibitor of the TX activity in rat liver cytosol.


Endo-1,4-3-B-glucanase of Cellulomonas Flavigena was resolved by 5-20% gradient PAGE and identified by tryptophan technique. Micromolase grown in the presence of crystalline cellulose. Substate bound cellulase was removed by desalination. The broth soluble activity showed the presence of two fractions while substate-bound activity comprised of 4 fractions at early phase. Rubber and intensity of slow moving fractions increased with time. At least 3 of the bound cellulases were comparable of the soluble or free activity. This suggested that the appearance of slow moving fraction was the result of glycosylation of cellulase which hindered its mobility. Multiplicity could be a result of expression of more than one gene for cellulase.

283 A CORRELATION BETWEEN PROTEIN THERMOSTABILITY AND RESISTANCE TO DEHNATURATION IN AQUEOUS ORGANIC MIXTURE SOLVENT SYSTEMS. R. K. Ojaya and D. A. Cowan, Department of Biochemistry, University College London, Gower Street, London, UK.

Biophysical transformations of water insoluble substrate can be readily performed in organic solvents providing that enzymes with appropriate organic solvent stabilities are available. Using protein extracts from 11 microorganism, thermostable and extremely thermostable bacteria (at growth temperature range, 20°C to 97°C), we have established a strong positive correlation between protein stability with respect to denaturation in various organic solvents and protein thermostability in aqueous solution. This correlation has been demonstrated using a range of purified enzymes. We conclude that organisms from high temperature environments should be valuable sources of enzymes suitable for organic solvent biocatalysis.

284 IN VITRO GLYCATON OF MOVING SPERM ALBIRIN. H. Alwood and J. L. Forth, The Open University, Oxford, Research Unit, Parks Hill, Oxford, OX1 3UD.

Protein glycation is thought to promote many of the undesirable effects of aging and diabetes. The most lasting consequences involve crosslinking between elminated protein and adjacent molecules. The rate and extent of albumin glycation was assayed fluorometrically by the periodate oxidation method. Glycation rates were faster for glucose than for fructose. Electrophoretic mobility on SDS gel was reduced by glycation to different extents, depending on the sugar used.

The effects of glycation inhibitors and of crosslinking will be discussed.
β-glucanase bands were detected by staining with 1% (w/v) Congo Red. Excess dye was removed by washing with 1 M NaCl. It was seen that the substrate-bound activity appeared as very faint bands compared with free activity by a comparative study of bound and free endo-1,4-β-glucanase, ten times higher bound activity was found on the gel. The incubation period for transferring enzyme activity to the ymogram from the gradient gel was doubled, which may have led to the mixing of two very close moving bands. As shown in Fig. 1, bound activity comprised at least two bands at the early stage of fermentation, while free activity comprised at least seven fractions throughout the fermentation period. In case of substrate-bound activity, the number and intensity of slow-moving bands increased with the passage of time and were comparable with that of the free cellulase bands. The appearance of slow-moving fractions led to the conclusion that this could be the result of glycosylation of the cellulases with partially hydrolysed soluble carbohydrate fragments which hindered their mobility on PAGE. Although C. flavigena secretes a considerable level of protease activity with two optimum pH values (Sant et al., 1988), it was unable to produce endo-1,4-β-glucanase fragments which remained active after degradation. Langford et al. (1984) reported multiplicity of cellulases due to proteolytic degradation, deglycosylation and glycosylation of native enzymes in C. flavigena. As shown in Fig. 1, at least three of the bound enzyme fractions were comparable with the free enzyme activity at all the fermentation times studied. The bound endo-1,4-β-glucanase activity which appeared at early stages of fragmentation possibly represents the native form of the enzyme in which they were secreted by the microbe and free from post-secretional modification. Multiplicity of cellulases in the earlier stages of fermentation could be the result of the expression of more than one gene specific for endo-1,4-β-glucanase activity. Purification and characterization of native endo-1,4-β-glucanase will be helpful in elucidating the mode of action of each fraction and should aid in defining the course of the evolution of multiple forms of cellulases in microbes.


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Thermostable microbial protein stability in aqueous: organic two-solvent phase systems

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Introduction

Protein stability in immiscible solvent systems is important in the fields of organic solvent phase biocatalysis (Laane et al., 1987a) and oil emulsion technology (Dickinson, 1987). A reasonable level of enzyme stability is an essential prerequisite for applications in organic solvent phase biocatalysis. The presence of organic solvent usually leads to a reduction in protein stability. The addition of surface active exogenous proteins and surfactants reduce enzyme inactivation by blocking the aqueousorganic solvent interface. Enzyme stability may also be enhanced by immobilization or chemical modification (Laane et al., 1987a). An alternative approach would be the use of intrinsically stable enzymes and whole cells for biocatalysis in aqueousorganic two-solvent phase systems.

Proteins from thermophilic micro-organisms often show greater resistance to heat denaturation. This appears to be associated with an equally enhanced stability towards other denaturants (e.g. urea and surfactants) and to polydispersity (Amehunen & Murdock, 1978; Daniel et al., 1982).

We experimentally examined possible correlations between thermostability and stability in aqueousorganic two-solvent phase systems using whole cell-free protein extracts from a range of mesophilic, thermophilic and extreme thermophilic micro-organisms. Experiments were also performed using purified proteins.

Methods

Mesophiles (Pseudomonas aeruginosa, ATCC 15442; Escherichia coli, ATCC 4157 and Bacillus subtilis, ATCC 23350); thermophiles (Bacillus steatorrhophilus, ATCC 12979 and Streptomyces thermovulgaris, ATCC 12924); extreme thermpophiles (Thermus strain) 351 (Cowens & Daniel, 1982) and a Thermus-like isolate from New Zealand thermal water samples (D.A. Cowan, unpublished work); and thermococcal archaeabacteria (Desulforococcus mobilis. Thermococcus celer and Thermoproteus tenax) were grown on appropriate liquid culture media for 24–48 h at 37°C, 50°C, 70°C and 84°C, respectively.

Cells were harvested by centrifugation (19000 g, for 20 min) washed in NaHPO₄ buffer (0.1 M, pH 7.0) and sonicated. Whole cell-free protein extracts were prepared by centrifugation (40000 g, for 2 h) to remove particulate matter and stored at -80°C until use.

Protein thermostability was measured by following the percentage residual soluble protein concentration after incubation at a range of temperatures for 60 min. Stability in aqueousorganic solvents was measured as the residual soluble protein after whole cell-free protein extracts had been shaken with a range of water-saturated organic solvents for 60 min. Protein concentration was determined by the modified Lowry method (Peterson 1977). Initial protein concentration did not exceed 0.5 mg/ml.

Experiments were also performed using commercial protease preparations (Fig. 1B) and proteins purified in our laboratory: caldolysin (Cowen & Daniel, 1982) and Thermo proteinase (D.A. Cowan, unpublished work), where enzyme activity loss as was used as the criterion for denaturation. Enzyme activity was determined spectrophotometrically using azocasein as substrate (Cowen & Daniel, 1982). Enzyme protein concentration was <0.3 mg/ml.

Results and discussion

Proteins from micro-organisms grown at high temperatures were more thermostable than proteins from low-temperature species. Significant correlations between microbe growth temperature and thermostability were observed in experiments at 50–90°C. The correlation coefficient (r) values observed ranged from 0.64 to 0.88 (P = 0.05–0.001) with an optimum r value at 70°C.

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The LTR1 probe was used to screen a T. conglobata genomic cDNA library and a clone containing a 1.8 kb insert was isolated. Two oligonucleotides (Omers) derived from conserved regions of glutathione reductase (GR) & TTR hybridised to LTR1, suggesting that the insert contained at least 10% of the protein-coding region. Sequence analysis of LTR1 revealed similarity to both the T. conglobata TR sequence & a tryptic peptide of C. fuscata TR (Fig. 1). The LTR1 insert contains a polyadenylated sequence at one end and a 600 nucleotides stretch which corresponds to the 3'-untranslated region of the mRNA. Current work is aimed at expressing the full-length TR gene in a high-level expression vector to obtain biologically active enzyme for structural and functional studies involving site-directed mutagenesis, N-tran crystallography and molecular modelling. Comparison of the structures of TR and GR should provide useful information for the design of specific drugs.

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Multiplicty of the endo-1,4-β-D-glucanase activity in Cellulomonas flavigena

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Multiplicty of microbial cellulases has been accepted as a general phenomenon (Marston & Gray, 1986). Endo-1,4-β-D-glucanases are known as some of the most heterogeneous enzymes among the cellulases. Different workers have tried to explain this phenomenon using different approaches (Langford et al., 1984; Marston & Gray, 1986). Here we report the isolation of insoluble substrate (Avicel)-bound and free endo-1,4-β-D-glucanases and a study of their comparative structure using 5-20% gradient polyacrylamide-gel electrophoresis (PAGE) during different stages of fermentation. This approach may be helpful in determining the native forms of the enzymes along with the possible reasons for multiplicity.

Cellulomonas flavigena was grown in the presence of crystalline cellulose (Avicel) as the substrate in salt medium, as previously reported (Sami et al., 1988). Free cellulase activity was isolated by centrifugation, while substrate-bound activity was eluted by sonication. The sonication method was compared with the previously reported shaking and washing method (Sami et al., 1988). After fermentation for specific times, residual Avicel was removed by centrifugation at 10,000 g and suspended in a volume of distilled water, that was double the volume of the culture. Avicel was then subjected to sonication at amplitude 20 in an MSE sonicator three times, 20 s each. Supernatant was removed after centrifugation and Avicel was washed similarly three times. It was found that the sonication method was much more efficient and less laborious than the shaking and washing method. Almost the same amount of enzyme activity was recovered in less than one-fifth of the time. Carroz (1986) has successfully used sonication for increasing the rate of hydrolysis of cellulose by cellulases. At earlier stages of fermentation when most of the cellulase activity was bound to the substrate (Sami et al., 1988), substrate-bound activity showed 15 times higher specific activity compared with the free activity. After fermentation for specific times, both substrate-bound and free activities were subjected to 5-20% non-denaturing PAGE (Sami et al., 1988) and enzyme activity was located on 0.05% CM-cellulose and 2.0% (w/v) agar plate. Endo-1,4-

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Abbreviation used: PAGE, polyacrylamide-gel electrophoresis

Fig. 1. Location of extracellular endo-1,4-β-D-glucanases of C. flavigena on 0.05% (w/v) CM-cellulose and 2.0% (w/v) agar plate after resolution on 5-20% (w/v) non-denaturing PAGE

Gradient gel was incubated for 20 min on an agar plate at 50°C and then stained with 0.2% (w/v) Congo Red. Excess dye was removed by washing with 1.0 M NaCl. Lanes A, B and C, free cellulase activity after 24, 48 and 72 h of fermentation, respectively; lane D, substrate-bound activity after 24 h of fermentation.

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147 Purification and Characterization of Three Extracellular CNases of Cellulomonas Stigmatica


The three extracellular CNases of Cellulomonas stigmatica were purified from a complex system of cellulases. A sequential purification procedure was employed involving precipitation with ammonium acetate, gelling, and gel chromatography and preparative gradient polyacrylamide gel electrophoresis. (PACE). CNase 1 and CNase 2 had the same molecular weight of about 20,000, but had different mobilities on SDS-polyacrylamide gel electrophoresis. (PAGE). The characteristics of the CNase 1 and CNase 2 were studied. Both the cellulases exhibited crystalline cellulase (substrate) based on well-stained activity during all the purification steps.

150 ULTRABIOCHEMISTRY OF TISSUE CULTURES OF OIL SEED RAPE

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Oil-seed rape, by far the most important source of edible fat in Northern Europe. This seed produces a hydrolyzed mixture which is dominated by high amounts of fat. In order to understand the regulation of oil quantity and quality, we have been studying lipid synthesis in tissue cultures from oilseed rape, especially in the leaves, which are the major site of cell division and maturation. Using such cultures, the lipid composition for growth at 15°C and 25°C has been analyzed. Incorporation of radioactive brassinolide (14C) into the seed has been followed in tissue cultures in the absence and presence of brassinolide. The results will be reported in detail.

153 THE BIOSYNTHESIS OF AMINOACETULATE IN HIGHER PLANTS

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Aminoacetulate (AUA) is the first common precursor of tetracyclic biosynthesis in various organisms. In animals AUA is synthesized via succinyl-CoA but in plants and many bacterial species it is synthesized from glutamate by a distinct pathway which involves the irreversible transamination of glutamate to succinyl-CoA (GLS). GLS aminoacetulate (AUA) was partially purified using an S-100 gel filtration column followed by two affinity columns and finally by non-denaturing gel electrophoresis. Inhibition studies with D-galactose and two other "keto" inhibitors of GABA aminotransferase, acetyl-CoA-GABA and vinlo-CABA will be described. This study of the enzyme's properties has been facilitated by a novel method of synthesizing GLS in large quantities.