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Biodegradation of carboxymethyl celluloses by *Agrobacterium* CM-1

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A Gram-negative rod tentatively identified as an *Agrobacterium* sp. was isolated from activated sludge with carboxymethyl cellulose (CMC) with a degree of substitution as the of 0·6 sole carbon and energy source. Not only did CMC DS (degree of substitution) 0·60 support the growth of this strain but also CMC DS 0·75, CMC DS 1·12, glucose and cellobiose. *Agrobacterium* CM-1 did not utilize cellulose. The metabolism of CMC is initiated by hydrolytic reactions catalysed by free and cell-bound endoglucanases. CMCs are only partially degraded by *Agrobacterium* CM-1. The extent of biodegradation of CMC DS 0·60, CMC DS 0·75 and CMC DS 1·12 determined in chemostat cultures increased with decreasing DS. The monomer compositions of CMCs and their biodegradation products revealed that only glucose and, remarkably, monosubstituted 6-glucose units are mineralized. As a consequence biodegradation of CMC by *Agrobacterium* CM-1 results in the formation of carboxymethylated oligomers with DS values greater than those of the original CMC. The DS of the original CMC also determined the chain length of the resulting oligomers.

INTRODUCTION

Water-soluble polymers can be divided into three groups: natural, synthetic and semisynthetic like cellulose ethers. The cellulose ethers are the most abundant of the semisynthetic polymers, with sodium carboxymethyl cellulose (CMC) as the most important representative. CMC is a colourless-to-cream coloured material, produced by the reaction of cellulose hydroxyls with chloroacetate. The average number of hydroxyls in the glucose unit which have reacted is called the degree of substitution (DS) and CMCs are categorized accordingly (Stelzer & Klug, 1980). Because it is only partially carboxymethylated, CMC may be regarded as a copolymer of eight monomers: unsubstituted glucose, monosubstituted (2-, 3- and 6-), disubstituted (2,3-, 2,6- and 3,6-) and trisubstituted (2, 3, 6-) glucose (Reuben, 1986).

CMC, a protective colloid, emulsifying and binding agent, is used in many fields including coatings, cosmetics, detergents, foods, textiles, pharmaceuticals and the oil industry. Because of the water-solubility of the compound, it eventually finds its way into waste streams and ultimately into surface water. Therefore, knowledge about the biodegradability of CMC is desirable in order to assess the impact on the environment.

The initial biodegradation of CMC probably involves the enzymatic hydrolysis of the glycosidic linkages by cellulases (Batelaan *et al.*, 1992). The enzyme system can be roughly subdivided into three enzyme groups: β-D-glucosidases or cellobiases (EC 3.2.1.21), cellobiohydrolases or exoglucanases (EC 3.2.1.91), and 1,4-D-glucanohydrolases or endoglucanases (EC 3.2.1.4). β-Glucosidases hydrolyse cellobiose and some soluble cello-oligosaccharides to give glucose. Exoglucanases release terminal cellobioses from the non-reducing end of the cellulose chain and are active on the crystalline parts whereas the endoglucanases hydrolyse amorphous cellulose randomly to give glucose, cellobiose and cellodextrins (Béguin & Aubert, 1994; Wood & Garcia-Campayo, 1994).

The carboxymethylation of cellulose makes the material soluble in water. Carboxymethylation influences the mode of action of cellulases, enabling only endoglucanases to act on the CMC chain. The degree of substitution is the predominant parameter governing the enzyme reaction, whereas the degree of polymerization of the cellulose derivatives does not significantly affect enzymatic hydrolysis. The presence of one carboxymethyl group on each glucose unit makes the molecule

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completely resistant to enzymatic attack (Reese *et al.*, 1950; Reese, 1957; Wirick, 1968). Wirick (1968) found that enzyme-induced chain scission occurred only at sites with three or more adjacent unsubstituted glucose units. Kalsulke *et al.* (1988) extended this finding by demonstrating the enzyme attack takes place at sequences of three or more unsubstituted glucose units, leaving CMC chains ending with unsubstituted glucose units.

All these investigations are based on work with partially purified cellulases usually derived from fungi. The purpose of this study was to elucidate a possible mechanism of CMC breakdown by isolated bacteria. To this end a bacterium capable of degrading CMC was isolated and characterized, and the initial biodegradation of CMCs varying in degree of substitution was studied. Finally, an attempt was made to correlate the extent of CMC biodegradation by the isolated bacterium with the theoretically calculated frequencies and distribution of substituted and unsubstituted glucose units.

MATERIALS AND METHODS

Chemicals

CMC DS 1·12, 0·75 and 0·60 were supplied by Akzo Nobel Functional Chemicals, Amersfoort, The Netherlands. The other chemicals were obtained from Janssen Chimica, Beerse, Belgium and Sigma Chemical Co., St. Louis, USA.

Crystalline cellulose (ex Sigma) was swollen in 10% NaOH in water for 5 h. Next the water was partially removed using a Büchner funnel. The residue in the funnel was suspended in 200 ml of 10% H_2SO_4 and neutralized. Finally, the resulting product was filtered through a 12-μm paper filter.

Growth media, isolation and maintenance of the bacterium

The liquid mineral salt medium contained per litre of deionized water: 0·85 g of $NaH_2PO_4{\cdot}2H_2O$, 1·55 g of K_2HPO_4, 0·5 g of NH_4Cl, 0·1 g of $MgSO4{\cdot}7H_2O$, 0·01 g of $FeSO_4{\cdot}7H_2O$, 0·01 g of $CaCl_2{\cdot}2H_2O$, 0·01 g of NaEDTA·$2H_2O$, and 0·1 ml of a trace element solution (Vishniac & Santer, 1957). All growth media were prepared by adding 1 g l^{-1} growth substrate to the mineral salt medium with the exception of the CMCs (0·4 g l^{-1}). Agar plates were prepared by the addition of 4 g l^{-1} of CMC DS 0·6 and 15 g l^{-1} of agar to the above mineral salt medium. The ph of the medium was approximately 7·0.

Isolation of CMC-degrading bacteria was carried out by aerobically incubating activated sludge obtained from a plant treating domestic waste water in liquid mineral salts media containing CMC DS 0·60 as sole carbon and energy source. After three successive transfers in fresh medium, serial dilutions of the enrichment culture were streaked onto agar plates. One strain was isolated from the agar plates. The bacterium was routinely grown in chemostats (Applikon, Schiedam, The Netherlands). The chemostat cultures were supplied with mineral salts medium to which CMC DS 0·60, CMC DS 0·75 or CMC DS 1·12 had been added at a dilution rate of 0·04 h^{-1}. The temperature was maintained at 30°C.

Bacterial characterization

The bacterium was characterized by Gram staining and by determining the biochemical properties using a commercially taxonomic kit, API NE 20 (Biomèrieux, Marcy-l'Étoile, France).

Growth experiments

The ability of the isolated strain to utilize various substrates for growth was tested in 100 ml Erlenmeyer flasks containing 20 ml of mineral salt medium with growth substrate. After inoculation, the Erlenmeyer flasks were incubated for 20 days at 30°C. Growth was determined visually by comparing the increase of turbidity of the cultures with a control without substrate. Growth rates of the isolated strain at 30°C were determined by following the increase of turbidity using a nephelometer (Hach, Loveland, USA).

Preparation of washed cell suspension and supernatant

The isolated bacterium was grown on CMC DS 0·60 and glucose. Cells were harvested in the exponential growth phase by centrifugation (Suprafuge 22, Heraeus Sepatech GmbH, Osterode, Germany) at 10,000 ***g*** at 4°C for 15 min, washed with phosphate buffer (pH 7·0, 50 mM) and suspended in the same buffer.

Oxygen uptake experiments

The oxygen uptake rates of washed cell suspensions were measured using a biological oxygen monitor (Yellow Springs Instruments Inc, Yellow Springs, USA) equipped with an oxygen electrode. Endogenous oxygen uptake by washed cells (total volume, 5 ml) was measured for 5 min at 30°C. Subsequently 100 μl of a substrate solution (1 g l^{-1}) was added and the substrate-dependent respiration was monitored for at least 5 min.

Enzymatic assays

Endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) activities were measured in both supernatant and pellet from a culture grown on 4 g l^{-1} of CMC DS 0.60. A portion (20 ml) of the culture was centrifuged (15 min, 10,000 ***g***, 4°C), after

which the pellet was suspended in 20 ml of phosphate buffer (pH 7·0, 50 mM).

Endoglucanase activity was determined by measuring reducing sugars from the hydrolysis of 1 ml of 0·5% CMC DS 0·60 solution. A quantity of 0·02% sodium azide was added as a preservative to inhibit the utilization of sugars released by endoglucanase activity. Portions (1 ml) of the resuspended pellet were incubated at 30°C for 1 h. After the incubation period the reaction was stopped by placing the mixture tubes in ice water. The amount of reducing sugars was determined using the 3,5-dinitrosalicylic acid method of Miller (1959). The enzyme activities in the supernatant were determined in the same way.

Exoglucanase and β-glucosidase activity were determined according to the method of Deshpande *et al.* (1984) with some modifications. *p*-Nitrophenyl-β-D-cellobioside (*p*NPC) and *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) were employed for the determination of exoglucanase and β-glucosidase activity, respectively. The assay mixtures contained 0·9 mg ml^{-1} of *p*NPC or *p*NPG in phosphate buffer (pH 7·0, 50 mM) and 0·2 ml of culture fluid in a total volume of 2 ml. Exoglucanase activity was determined in the presence of D-glucono-1,5-δ-lactone (0·5 mg ml^{-1}) to inhibit β-glucosidase activity. Incubation conditions were the same as described above. The amounts of *p*-nitrophenol released were calculated using the molar extinction coefficient of 18·5 ml μmol^{-1} cm^{-1} for *p*-nitrophenol at 410 nm.

Preparation of degradation products

The extent as well as the mode of biodegradation of CMC was determined by analysing the original CMCs and the biodegradation products formed in chemostat cultures. To prepare the degradation products for analysis, 2 l of effluent of the chemostat cultures were collected and centrifuged (15 min, 10,000 *g*, 4°C). Next the supernatant was concentrated by evaporation using a Rotavap (Heidolph, model W2000) and finally the product was freeze-dried (Virtis Company, New York, USA).

Analyses

The non-purgeable organic carbon (NPOC) contents of the influent and effluent of the chemostat cultures were determined using a Dohrmann DC-190 TOC apparatus (Rosemount Analytical Inc., Santa Clara, USA). Prior to injection all samples were acidified to enable removal of carbon dioxide by purging.

Gas–liquid chromatography (GLC) analysis was used to determine the substituent distribution in CMCs and their biodegradation products. The samples were methanolysed in an acidified methanol solution. During the methanolysis the β-1,4-glucosidic linkages were broken, resulting in a mixture of carboxymethylated glucose residues and glucose. GLC analyses were carried out on a Perkin-Elmer Model 8410 gas chromatograph equipped with an SE-30 bounded-phase, fused silica capillary column (25 m × 0·32 mm I.D. Pierce) and a flame ionization detector connected to a Shimadzu C-R3A integrator. The temperature of the column was set from 150 to 250°c with an increase of 4°C min^{-1}, and kept at 250°C for 5 min.

High-performance size exclusion chromatography (HPSEC) was used to determine the relative molecular weight distribution of CMC. Prior to analysis the CMC samples were dissolved in 0·2 M ammonium acetate and filtered (Spartan 30/B, 0·45 μm, Schleicher & Schüll). HPSEC was performed on an ultrahydrogel linear column (300 × 7·8 mm, Waters) with a flow rate of 0·7 ml min^{-1}. The eluent was monitored with a Waters 411 refractive index (RF: 32 × 20) detector. The temperature of the column was 30°C and the running time was 45 min. The amount of cell protein in the cell suspensions was determined using the bicinchoninic acid method, with serum albumin as a standard (Pierce, Rockford, USA).

RESULTS

Isolation and characterization

CMC-degrading bacteria were enriched from secondary activated sludge of a sewage plant treating predominantly domestic waste water. Serial dilutions of the enrichment culture were streaked onto CMC plates. A colony excessively present was isolated and designated as CM-1. Strain CM-1 was a Gram-negative motile rod, which formed round, slimy cream-coloured colonies when plated on solidified medium containing 1 g l^{-1} of glucose. The strain was able to oxidize cytochrome c, to reduce nitrates, to hydrolyse aesculin and *p*-nitrophenyl-β-D-galactopyranoside (PNGP), but not gelatin. It produced neither indole, arginine dihydrolase nor acid from glucose. Strain CM-1 assimilated arabinose, mannose, mannitol, *N*-acetylglucosamine, citrate, phenylacetate, adipate, caprate, acetate, lactose, maltose, mannose, and starch. On the basis of the above characteristics the organism was tentatively classified as an *Agrobacterium* sp.

Growth and whole cell studies

CMC DS 0·60, CMC DS 0·75 and CMC DS 1·12 serve as growth substrates for *Agrobacterium* CM-1. The doubling time on CMC DS 0·60 amounts to approximately 4 h. Other cellulose ethers i.e. hydroxyethylcellulose MC 1·2 DS 0·9 and methylcellulose DS 0·9 also support growth but celluloses, i.e. Avicel and Sigmacell type 100 did not. Moreover, no growth was observed on cellulose pretreated with NaOH to improve

the bioavailability. The strain was capable of growing on glucose and cellobiose. The generation time on glucose and cellobiose is 1·5 h.

In addition, the respiration by strain CM-1 pregrown on glucose and on CMC DS 0·60 was measured. Comparing the oxygen uptake it is clear that CM-1 pregrown on glucose medium is not able to respire CMCs (Table 1). The oxygen uptake of substituted glucose was not tested because such compounds were not available.

Chemostats were supplied with influent containing CMCs with different DS. The NPOC concentration of the influent and steady-state effluent of the chemostat cultures and the calculated removal percentages are presented in Table 2. Biodegradation expressed as removal percentage decreases with increasing DS.

Table 1. Rates of oxygen uptake by washed cell suspensions of *Agrobacterium* CM-1 grown on glucose and CMC DS 0·60

Substrate	O_2 nmol min^{-1} (mg $protein^{-1}$)	
	Glucose	CMC DS 0·60
Glucose	28·5	12·9
Cellobiose	14·9	20·4
CMC DS 0·60	0·0	10·5
CMC DS 0·75	0·0	11·7

Exoglucanases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.4)

The inability of the isolated bacterium to grow on cellulose may be explained by the lack of exoglucanases. Indeed, no exoglucanase activity was observed. Subsequently, the assumption that biodegradation of CMC proceeded by an initial hydrolytic reaction catalysed by endoglucanases was investigated. Endoglucanases are extracellular enzymes either bound or free (Wood & Garcia-Campayo, 1994). At least part of the endoglucanases are anchored to the cell surface because a washed cell suspension of CMC-grown bacteria was capable of oxidizing CMC (Table 1). Therefore the endoglucanase production by *Agrobacterium* CM-1 growing on CMC DS 0·60 in a batch culture was measured in supernatant and pellet cultures. The total endoglucanase activity which increased during growth, was distributed over the pellet and the supernatant of the culture. During growth of *Agrobacterium* CM-1 in the batch culture 70–80% of the endoglucanase activity was found in the pellet (Fig. 1). The endoglucanase activity of the pellet was approximately 20 μmol glucose mg cell $protein^{-1}$ h^{-1}.

The utilization of cellobiose for growth suggested β-glucosidase activity. β-glucosidase activity was detected using *p*-nitrophenol-β-D-glucopyranoside as substrate. The estimated β-glucosidase activity of the pellet was 0·4 μmol mg cell $protein^{-1}$ h^{-1}. In the supernatant no β-glucosidase activity was detected.

Identification of the degradation products

Glucose and cellobiose released by endoglucanase activity are metabolized by *Agrobacterium* CM-1 (Table 1). The utilization of these carbohydrates probably results in the formation of carboxymethylated oligomers. This assumption agrees with the incomplete biodegradation of CMCs in the chemostate cultures (Table 2). To study and identify these oligomers, CMC DS 0·60, CMC DS 0·75 and CMC DS 1·12 were degraded in chemostat cultures. The CMCs and their degradation products were characterized using gas–liquid chromatography (GLC) and high-performance size exclusion chromatography (HPSEC). The DS of the CMC before and after subjection to biodegradation was calculated using the measured monomer composition. Biodegradation of CMC resulted in an increase of the DS (Table 2).

Assuming that the di- and tricarboxymethylated glucose units are resistant to biodegradation, degradation of glucose and monosubstituted glucose can be estimated with the di- and trisubstituted glucoses as internal standard. The biodegradation of glucose and monosubstituted glucoses expressed in percentages is shown in Table 3. Although primarily glucose was utilized during the biodegradation of CMCs, it is clear that also monosubstituted 6-glucose units were utilized by *Agrobacterium* CM-1. 12 and 15% of the carbon removed by biodegradation in the chemostats fed with CMC DS 0·60 and CMC DS 0·75, respectively, is derived from the monosubstituted 6-glucose units. The calculated biodegradation percentages of all CMCs are

Table 2. The NPOC concentrations of influent and steady-state effluent of chemostat cultures fed with different CMCs

CMC	NPOC Influent ($mg l^{-1}$)	NPOC Effluent ($mg l^{-1}$)	Biodegradation (%)	DS of the biodegradation products
DS 0·60	1268·4	1060·2	16·4	0·68
DS 0·75	1304·6	1141·9	12·5	0·84
DS 1·12	1293·6	1232·2	5·0	1·16

The removal percentages are calculated from the NPOC values.

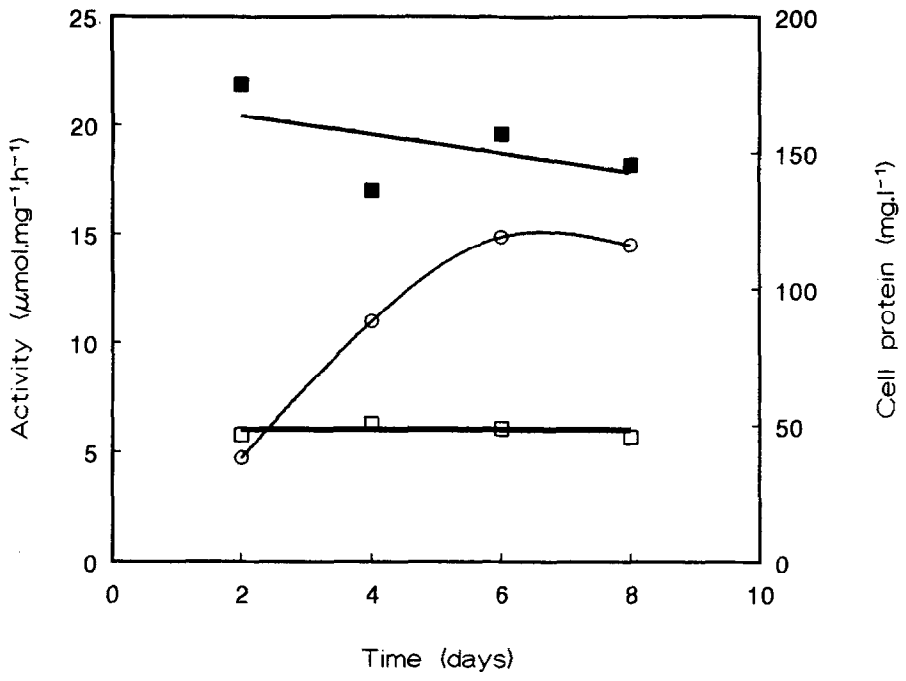

Fig. 1. Growth of *Agrobacterium* CM-1 measured as an increase of cell protein (○) and endoglucanase activity in the supernatant (□) and bound to the cell (■).

Table 3. Mol percentages of glucose and monosubstituted glucose of CMC DS 0·60, CMC DS 0·75 and CMC DS 1·12 and the mol percentages degraded by *Agrobacterium* CM-1

Sample	Glucose mol (%)	Mono-3	Mono-2	Mono-6
CMC DS 0·60	50·0	8·5	18·0	14·0
Monomers of CMC DS 0·60 degraded	14·2	0·2	0·5	2·3
CMC DS 0·75	41·0	10·0	20·0	14·0
Monomers of CMC DS 0·75 degraded	11·1	0·0	0·0	1·7
CMC DS 1·12	23·0	11·0	24·0	12·0
Monomers of CMC DS 1·12 degraded	4·0	0·6	0·3	0·6

in agreement with the removal percentages in the chemostat cultures (Table 4).

In order to investigate the depolymerization of the CMCs by *Agrobacterium* CM-1, the elution patterns and the average molecular number (Mn) of the degradation products were determined. The CMCs used as substrate have the same degree of polymerization. The elution patterns of the degradation products show that with decreasing DS the average elution time substantially increases. Absolute average molecular weights cannot be estimated because the hydrodynamic volume of the anionic CMC is not equal to the cello-oligomers and the dextran standard used in the calibration plot (Hamacher & Sahm, 1985). However, an estimate of the ratio of the number of chain scissions can be made:

$$Mn_{DS\ 0\cdot6}/Mn_{product\ DS\ 0\cdot60}:Mn_{DS\ 0\cdot75}/Mn_{product\ DS\ 0\cdot75}:$$
$$Mn_{DS\ 1\cdot12}/Mn_{product\ DS\ 1\cdot12} = 3\cdot4:\ 2\cdot6:\ 1\cdot0.$$

Table 4. Predicted degradation percentages assuming that glucose or glucose and monosubstituted 6-glucose are liberated according to the model of Kalsulke *et al.* (1988) in comparison with the percentages determined with GLC and NPOC

CMC	Predicted		Experimental	
	Glucose	Glucose and mono substituted 6-glucose	GLC	NPOC
DS 0·60	13	26	16	16
DS 0·75	7	13	13	13
DS 1·12	1	4	4	5

The polymer chain lengths of the degradation products demonstrate that cleavage by *Agrobacterium* CM-1 was less effective at higher DS values leaving fragments with a higher degree of polymerization. This is consistent with the results found in the carbon and GLC analyses.

DISCUSSION

Agrobacterium CM-1 used in this study was isolated with CMC DS 0·6 as sole carbon and energy source from sludge taken from a plant treating domestic waste water. *Agrobacterium* CM-1 was chosen because it was excessively present in the enrichment culture. The growth rate of the bacterium on CMC DS 0·6 and glucose is 0·2 and 0·5 h^{-1}, respectively. Comparing these generation times, it is conceivable that the liberation of carbohydrates is the rate limiting step in the biodegradation of CMC. Oxygen uptake rates monitored for glucose and cellobiose (Table 1) demonstrate, as postulated by Bhattacharjee & Perlin (1971), that these compounds are utilized as carbon and energy sources during growth on CMC.

Agrobacterium CM-1 cannot grow on cellulose. The absence of exoglucanase activity accounts for the inability to grow on cellulose. This is consistent with other observations because most bacteria producing cellulases appear to release only endoglucanases which show little activity to crystalline cellulose (Wood & Garcia-Campayo, 1994).

Biodegradation of CMCs by strain CM-1 is initiated through hydrolysis catalysed by endoglucanases existing in bound and in free form (Fig. 1). The endoglucanases involved in the hydrolysis of CMCs are inducible because washed cell suspensions of glucose-grown *Agrobacterium* CM-1 do not respire CMCs. Finally, only bound β-glucosidase was detected. Wood & Garcia-Campayo (1994) stated that there may be certain situations where cell-bound enzymes will be more efficient in the degradation of macromolecules. This includes the situation where the microorganisms are in an aquatic environment such as waste water treatment plants. Biodegradation of the CMCs in chemostat cultures was followed by determining the NPOC content of the influent and the effluent. All CMCs are partially degraded and the extent of biodegradation of the CMCs depends upon the DS of these substrates. The polymer stability increases with increasing DS. This relationship was also established in previous studies with fungal cellulases (Philip & Stscherbina, 1992).

To identify the organic carbon in the effluent of the chemostat cultures, the monomer composition and the substituent distribution over the monomeric units of the degradation products were determined using GLC analysis. The DS of the biodegradation products of the CMCs is greater than the original CMCs (Table 2). The extent of biodegradation of CMC reflects the degradation of glucose and monosubstituted 6-glucose units, resulting in an increase of the DS. The carboxymethylated oligomers formed vary in the degree of polymerization due to biodegradation of varying amounts of glucose and monosubstituted 6-glucose (Table 3).

An explanation of the mode of biodegradation of CMC by *Agrobacterium* CM-1 may be obtained by correlating the measured amount of unsubstituted glucose units with a statistical analysis. If a random distribution is assumed for the unsubstituted glucose units in CMC, it is possible to predict the probable frequency of two or more adjacent unsubstituted glucose units. In studies with fungal cellulases the probability of three adjacent unsubstituted glucose units is an indication of the extent of hydrolysis of CMC (Wirick, 1968). In view of this, Kalsulke *et al.* (1988) developed a model to predict the release of unsubstituted glucose. The release of unsubstituted glucose was predicted by assuming that the enzyme attack takes place at sequences of three or more unsubstituted glucose units, leaving CMC chains ending with unsubstituted glucose units. The predicted release of unsubstituted glucose from the CMC samples is compared with the measured degradation percentages. It is obvious that the former are much too low. However, not only unsubstituted glucose units were degraded but also monosubstituted 6-glucose units (Table 3). On the basis of the assumption that all monosubstituted 6-glucose units are viewed as unsubstituted glucose by exoglucanases, the release of unsubstituted glucose and monosubstituted 6-glucose can be predicted using the model developed by Kalsulke *et al.* (1988). Although the predicted releases of CMC DS 0·75 and CMC DS 1·12 are similar to those actually found, this mode of attack is not valid because a much higher release is predicted for CMC DS 0·60 than actually measured (Table 4). The mode of biodegradation by *Agrobacterium* CM-1 cannot be predicted using the model developed by Kalsulke *et al.* (1988). It appears that for the liberation of glucose and monosubstituted 6-glucose, the presence of special groupings is required.

Although carboxymethylated oligomers persist in pure cultures of *Agrobacterium* CM-1, these compounds are subject to biodegradation by other microorganisms. A high biodegradation percentage achieved in a prolonged Closed Bottle test suggests complete mineralization of CMC DS 0·7 (van Ginkel & Stroo, 1993). In further studies we intend to assess the biodegradation of CMC in simulation tests of waste water treatment plants and to determine the toxicity of CMC and the biodegradation products to aquatic organisms.

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