Pergamon

PII: S0043-1354(97)00050-X

Wat. Res. Vol. 31, No. 9, pp. 2137–2145, 1997

Printed in Great Britain
0043-1354/97 $17.00 + 0.00

MOLECULAR WEIGHT DISTRIBUTION OF HYDROLYSIS PRODUCTS DURING THE BIODEGRADATION OF MODEL MACROMOLECULES IN SUSPENDED AND BIOFILM CULTURES. II. DEXTRAN AND DEXTRIN

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(Received March 1996; accepted in revised form February 1997)

Abstract—To improve wastewater treatment models, it is important to consider that wastewater is composed of a variety of complex molecules, many molecules having large molecular weights. Previous experiments have shown that hydrolytic enzymes are cell-associated and that hydrolytic fragments accumulate in bulk solution during the degradation of a model polysaccharide (dextran) in pure culture. These results indicate that incompletely hydrolyzed macromolecules are released into solution prior to their complete degradation. The authors wanted to determine whether the release of incompletely degraded molecules was specific to dextran degradation by pure cultures or whether it could be generalized to mixed culture systems and the degradation of other polysaccharides. To accomplish this, both pure and mixed (wastewater) cultures were used to examine the degradation of dextran and another macromolecular polysaccharide, dextrin, in batch suspended culture, continuous suspended culture and fixed-film reactor systems. Membrane ultrafiltration was used to monitor the molecular weight distribution of polysaccharides in solution during degradation. In all reactor configurations, and for all substrates and inocula investigated, small-molecular-weight (< 1000 amu) oligosaccharides accumulated in solution during polysaccharide degradation. These results, in conjunction with results of enzyme studies, support a generalized model for macromolecular degradation by cells that features cell-bound hydrolysis of polysaccharides and the subsequent release of hydrolytic fragments back into bulk solution. This hydrolysis and release is repeated until fragments are small enough (< 1000 amu) to be assimilated by cells. Essential features of this model are that polysaccharide diffusivity changes during its degradation and that different enzymes, with different methods of operation and different kinetic characteristics, may be used in successive hydrolytic cleavages. These features are particularly important to consider in evaluating macromolecule degradation by aggregates and biofilms and in understanding overall uptake kinetics in bioreactors. © 1997 Elsevier Science Ltd

Key words—wastewater, biofilms, macromolecules, carbohydrates, hydrolysis

INTRODUCTION

Laboratory biodegradation studies have most often focused on degradation of small-molecular-weight substrates such as glucose, acetate or amino acids. However, the behavior of systems metabolizing simple substrates may not accurately reflect the more complex situations in engineered wastewater treatment systems or natural aquatic systems where the dissolved organic matter (DOM) consists of a wide range of molecules and macromolecules (Levine *et al.*, 1985). Metabolism of macromolecules is different than small-molecular-weight compounds as a consequence of physical and biological factors. Diffusion coefficients of macromolecules are low, which can limit transport to unattached cells and aggregates in suspended growth reactors and to biofilm surfaces and to cells within the biofilm matrix in fixed-film reactors (Bailey and Ollis, 1977; Logan *et al.*, 1987a,b; Confer and Logan, 1991). Polypeptides and polysaccharides greater than six or seven monomeric units (~ 1000 amu) cannot be assimilated by bacteria directly as can small-molecular-weight compounds. All compounds with molecular weights in excess of approximately 1000 will be defined here as macromolecular, since these large molecules must be hydrolyzed into monomers or smaller polymers by extracellular enzymes before they can be transported across the bacterial cell wall (Ferenci, 1980; Law, 1980).

Classical bacterial models, such as the Monod bacterial growth model and the Michaelis–Menton enzyme kinetic model, assume that the rate of substrate utilization is only a function of enzyme and

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substrate concentration and the rate coefficients of the system under study. For slowly diffusing substrates, these models may not accurately reflect the physics or biology of macromolecular metabolism, because they do not account for mass transfer limitations to the cell surface or the complexity of extracellular hydrolysis and transport across the cell membrane. This inaccuracy is conspicuous when negative values for the kinetic parameters μ_{max} and K_s are obtained in wastewater systems (Saunders and Dick, 1981). The contributions of mass transfer and hydrolysis/transport to overall macromolecular metabolism need to be better understood and incorporated into models that assess the performance of wastewater treatment systems.

This study is part of a larger project that investigates two important biological questions of macromolecule metabolism. First, does hydrolysis occur in close proximity to the cell or are the hydrolytic enzymes released into solution (extracellular enzymes)? Second, assuming hydrolysis occurs in close proximity to the cell, are hydrolyzed fragments directly assimilated or are they released back into bulk solution? A teleological perspective would favor cell-associated hydrolysis of macromolecular substrates. Hydrolytic enzymes released into bulk solution are themselves soluble proteins that can become microbial substrates. After initial hydrolysis, hydrolytic fragments would be kept in the pericellular environment and hydrolysis continued until the fragments were small enough to be transported into the cytoplasm. Such a scenario would minimize energy expenditures by producing extracellular enzymes that directly benefit only the cell that produces them.

In natural systems most studies indicate macromolecule hydrolysis is cell-associated, although some studies in eutrophic environments demonstrate a dominance of cell-free hydrolysis (Hoppe, 1983; Somville and Billen, 1983; Chróst, 1989). Unlike the Gram-positive bacteria that predominate in soil and have been shown to naturally secrete enzymes into the surrounding environment, Gram-negative bacteria predominate in aquatic bacterial communities and the frequency of enzyme secretion is much lower (Burns, 1982; Chróst, 1991).

Engineered systems similarly demonstrate that hydrolytic enzymes are primarily cell-associated. In a bench-scale activated sludge system metabolizing soluble starch, Banerji *et al.* (1967, 1968) found that starch was degraded primarily in contact with cells rather than in cell-free solution based on the significantly higher rate of starch disappearance in cell-containing solution. Boczar *et al.* (1992) used commercially available colorimetric enzyme assays (API-ZYM and LRA-ZYM Esterase test system, Analytab Products, Plainview, NY) to test for the activity of enzymes in cell-free and cell-containing preparations of municipal activated sludge. No measurable enzyme activity was observed in any cell-free samples, but there was significant hydrolytic activity in all cell-containing samples. Other experiments in this laboratory employing the reactor configurations used in the present study and highly sensitive fluorometric assays show that polysaccharide and protein hydrolytic activity is cell-associated in suspended culture and biofilm-associated in fixed-film reactors (Confer, 1996; Confer and Logan, 1997a).

The purpose of this study is to determine whether small (< 1000 amu) and/or intermediate (1000–10,000 amu) size fragments are released during bacterial degradation of soluble large-molecular-weight (> 10,000 amu) model polysaccharides. The fate of hydrolytic fragments of macromolecules were monitored during degradation experiments with membrane ultrafiltration to determine the size distribution of molecules in solution. This study continues the investigations begun by Haldane and Logan (1994) and broadens the scope of their axenic suspended culture experiments with dextran (70,000 amu) to include experiments by mixed cultures, biofilms and a second model substrate, dextrin (86,000 amu). Both dextran and dextrin are soluble glucose polymers, but they have different structures due to different linkage and branching patterns.

MATERIALS AND METHODS

Inocula

Both pure and mixed cultures were used in experiments. Mixed cultures were obtained from the biotower influent of the Roger Road trickling filter wastewater treatment plant in Tucson, Arizona. Because the Roger Road plant recycles secondary clarifier solids, this inoculum contained bacteria from both the primary clarifier effluent and the biotower biofilm. A dextran-degrading isolate was obtained from Roger Road biotower influent as described by Haldane and Logan (1994). This Gram-negative isolate was identified using the Biolog GN MicroPlate (Biolog, Heyward, CA) as belonging to the group CDC II-I (Center for Disease Control, Atlanta, GA). CDC II-I is closely related to sphingobacteria and is in the Cytophagia group. Cytophagia are noted macromolecular metabolizers which possess the ability to degrade cellulose, a β-linked glucose polymer.

The liquid media for all experiments was a mineral salt buffer (MSB). It contains, per liter of ultrapure water (Millipore, Bedford, MA), 0.5 g KH_2PO_4, 1.266 g $K_2HPO_4 \cdot 3H_2O$, 0.3 g NH_4Cl, 0.2 g $MgSO_4$, 0.04 mg $FeCl_3$ and 0.02 mg vitamin B_{12} (Baillod and Boyle, 1969). NH_4Cl was substituted for $NaNO_3$ used by Bailloid and Boyle since nitrate was found to interfere with the anthrone test used to quantify saccharides. All experiments were performed at room temperature (22 ± 1°C).

Suspended culture reactors

Suspended cell experiments used either 2.5-liter fermentors (Virtis Omniculture, Gardiner, NY) containing 1 liter of liquid, mixed at 250 rpm and aerated at 2.5 liters min^{-1}, or 1.0-liter fermenters (Applicon, Foster City, CA) containing 0.5 liters of liquid, mixed at 250 rpm and aerated at 1.25 liters min^{-1}. Suspended culture reactors were operated in both batch and continuous culture configurations. Continuous culture reactors were fed using Masterflex peristaltic pumps to supply media through a drip tube and to withdraw fluid using a suction tube to maintain

a constant liquid volume. All continuous culture reactors were inoculated and operated in batch mode until culture absorbance (600 nm) was approximately 0.10, at which time continuous culture operation was begun. Reactors were run in excess of three detention times, at which point the reactor was assumed to be at steady state (Grady and Lim, 1980) based on previous dextran culture experiments (Haldane and Logan, 1994).

Biofilm reactors

Fixed-film reactors were employed as described in Confer and Logan (1997b) except that dextran (140 mg liter^{-1}) was used as substrate. These reactors consist of biofilms grown on the inside of 4-liter polypropylene bottles filled with 400 ml of Roger Road biotower influent (changed four times per week) and rolled horizontally. Before performing experiments reactors were adapted to dextran by adding 150 mg liter^{-1} dextran to the biotower influent and feeding daily for 1 week.

Model polysaccharides

Dextran, a soluble storage polysaccharide produced by bacteria and yeasts was used as the primary model polysaccharide. Unlike α(1-4) backbone linkages of glucose units found in starch, dextrans are characterized by a predominance (>95%) of α(1-6) backbone linkages and varying proportions of α(1-2), α(1-3) and α(1-4) linkages typically at branch points (Lehninger, 1975; Janson, 1972). Dextran was chosen because of its high solubility and because it was posible to select a defined molecule size from dextrans having a variety of molecular weights. In contrast to more highly branched polysaccharides such as dextrin (see below), dextran contains more than 95% of only one type of glucose linkage. Thus, the number of enzymes necessary for the degradation of dextran should be small compared to other polysaccharides.

The dextran chosen for this study had an average molecular weight of 70,000 (produced by *Leuconostoc mesenteroides*; Sigma, St Louis, MO). Previous experiments in this laboratory have shown that 70,000-amu dextran (as received) has a significant amount of material less than 10,000 amu and that it is difficult to remove this fraction by ultrafiltration of concentrated solutions (50 g liter^{-1}) due to gel layer formation at the ultrafiltration membrane. Because of the large volumes of media required for these experiments and the necessity to sterile filter rather than autoclave all dextran solutions, it was impractical to remove the intermediate-molecular-weight dextran. Dextran was added to autoclaved media reservoirs from sterile-filtered, concentrated stock solutions. The molecular weight distribution of carbohydrates in media prepared from concentrated stock solutions was typically 0% less than 1000 amu, 20–25% between 1000 and 10,000 amu and 75–80% between 10,000 amu and 0.2 μm.

To show that results obtained in these experiments were not unique to dextran and could be generalized to other polysaccharides, a continuous culture experiment was performed with a wastewater inoculum using dextrin, a relatively soluble polysaccharide prepared by the partial hydrolysis of starch. The dextrin used is a patented corn starch product that was kindly provided by Dr Robert N. Ammeraal (American Maize-Products Co., Hammond, IN). This bacterial α-amylase hydrolysate of waxy corn starch has been fractionated using strong base ion-exchange resins and contained molecules in a more narrow molecular weight range (average 86,000 amu) than most commercially available dextrins. It has an α(1-4) backbone and is highly branched, with the branch points being α(1-6) linkages. The presence of two types of linkages increases the likelihood that hydrolytic fragments will be released into solution, since two different enzymes are required to break α(1-4) and α(1-6) linkages.

Sample handling, separation and determination of molecular weight distribution

All sample handling, centrifugation, vacuum filtration and molecular weight separation methods used in these experiments were identical to those used previously (Confer and Logan, 1997b), except that polysaccharide hydrolytic fragments were assigned to three, not two, size fractions. Size distributions were determined by parallel processing of samples through 1000 and 10,000 amu cut-off ultrafiltration membranes (Amicon YM1 and YM10 Beverly, MA). Material was assigned by subtraction to three discrete fractions, small (<1000 amu; YM1 permeate), intermediate (1000–10,000 amu; YM10–YM1) and large (10,000 amu to 0.2 μm; 0.2 μm filtrate–YM10).

Controls

Parallel experiments in separate sterile reactors containing MSB and substrate were conducted to ensure that any change in substrate molecular weight profile in experimental reactors was the result of bacterial metabolic processes. These reactors were operated under identical experimental conditions for more than 45 h, a period longer than those used in the batch or CSTR reactors containing cultures. Initial and final molecular weight separations all showed stable molecular weight profiles for both dextran and dextrin. Because a single CSTR media reservoir bottle might be in use for approximately 10 days, molecular weight distribution of the media (under quiescent conditions) was monitored for 2 weeks. Again, initial and final molecular weight separations showed stable molecular weight profiles for both dextran and dextrin.

Analytical techniques

Polysaccharide concentrations in samples were measured by the anthrone method as modified by Gaudy (1962). Samples (1 ml) were added to an anthrone solution (3 ml) (Sigma; 2 g liter^{-1} anthrone dissolved in concentrated sulfuric acid) in a test-tube, mixed and boiled for 15 min in a water-bath. Polysaccharides are hydrolyzed by the acid, and the resulting monosaccharides react with the anthrone to form a colored complex which absorbs light at 620 nm. Experimental samples were compared to glucose standards. In the present study, the detection limit for this technique was 1 mg liter^{-1}. More consistent results were obtained when the reaction tubes were cooled in the dark at 4°C for 4 h before spectrophotometric readings were performed.

RESULTS

Dextran degradation in batch suspended culture

Small-molecular-weight hydrolytic fragments (<1000 amu) accumulated during dextran degradation in axenic batch suspended culture. This size fraction increased from a concentration below the detection limit (<1%) to 34% of total carbohydrates at the first analysis (14 h), when 27% of the dextran initially present had been metabolized (Fig. 1). The percentage of substrate present as small-molecular-weight carbohydrates continued to increase as substrate was consumed, with small-molecular-weight material comprising 75 and 83% of total carbohydrates at the 20-h and 24-h sampling times, respectively. Because there was a substantial percentage of intermediate-molecular-weight carbohydrates present in uninoculated media, measurement of the intermediate size fraction is a less sensitive indicator of the dynamics of the metabolic processes occurring than the measurement of either small- or large-mol-

ecular-weight fractions. The relatively smaller change in intermediate size fraction as compared to either small- or large-molecular-weight fractions at 14-h and 16-h measurements most likely indicates that, as intermediate-molecular-weight carbohydrates are hydrolyzed to small-molecular-weight fragments, large-molecular-weight carbohydrates are hydrolyzed to intermediate-molecular-weight fragments that replenish the intermediate-molecular-weight pool. The high percentage of small-molecular-weight material that accumulates in solution suggests that metabolism of this fraction, rather than the hydrolysis of large and intermediate size carbohydrates, is rate-limiting in this pure culture system. If this small-molecular-weight fraction were not rate-limiting, it would not have accumulated in solution. The accumulation in solution of dextran hydrolytic fragments is similar to the trend reported by Haldane and Logan (1994), although a higher concentration of small-molecular-weight fragments (< 1000 amu) was observed to accumulate in solution in the present study than previously reported.

Dextran degradation in continuous suspended culture

Continuous suspended culture dextran degradation experiments were done to see if the accumulation of hydolytic fragments observed in batch culture could be reproduced under continuous culture conditions for both pure and mixed cultures. In continuous culture at steady state, small-molecular-weight carbohydrates accumulated in pure culture reactors at all detention times when a measureable concentration of substrate remained (Fig. 2a), a finding that is consistent with the batch suspended culture data. The percentage of small-molecular-weight carbohydrates present in reactors at steady state increased with detention time, while the percentage of large-molecular-weight carbohydrates decreased. Small-molecular-weight carbohydrates composed less than 1% of total carbohydrates in the feed solution but increased to 54% at a 16-h detention time, when

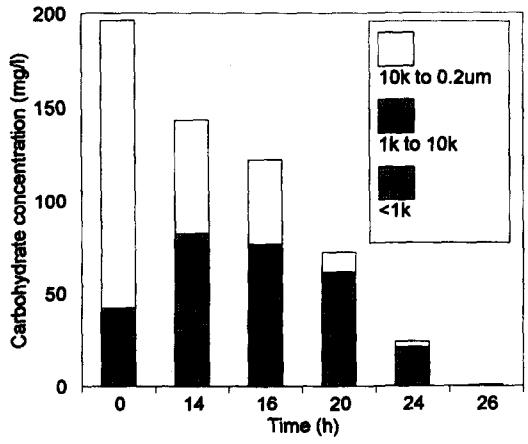

Fig. 1. Molecular weight distributions during dextran degradation by a pure culture in batch suspended culture.

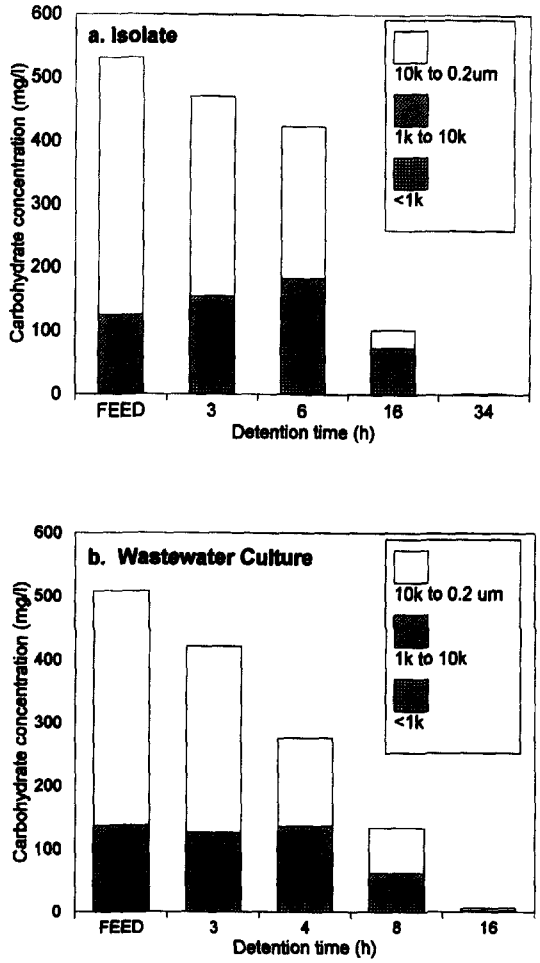

Fig. 2. Molecular weight distributions during dextran degradation by (a) pure and (b) wastewater cultures in CSTRs at steady state.

total substrate concentration was reduced to 19% of the original concentration. Large-molecular-weight carbohydrates were 75% of total carbohydrates in the feed solution but only 26% at a 16-h detection time. Similar to the results observed in batch suspended culture experiments, there was less change in intermediate-molecular-weight carbohydrates as compared to either small- or large-molecular-weight material.

High concentrations of small-molecular-weight carbohydrates also accumulated in chemostats using mixed cultures developed from a wastewater inoculum (Fig. 2b), but the amount was less than that observed for the corresponding pure culture reactors. In the wastewater inoculated reactor containing 25% of the feed substrate concentration (8-h detention time), only 17% of carbohydrates were found in the small-molecular-weight fraction. In the pure culture reactor containing a similar amount of substrate (16-h detention time; 20% of the feed substrate concentration), 54% of carbohydrates were small molecular weight. Except for the shortest detention

time, which was near washout, intermediate-molecular-weight dextran was always a greater percentage of total dextran in wastewater inoculated reactors than in pure culture reactors. This shift to a higher percentage of intermediate-molecular-weight material in mixed cultures than in pure cultures probably reflects the greater efficiency of the diverse wastewater consortium to assimilate small-molecular-weight dextran fragments. The greater overall efficiency of the wastewater consortium is also evident in significantly faster kinetics. The pure culture required almost twice the detention time of the wastewater consortium to reach 80% substrate depletion.

Dextran degradation in rotating biofilm reactors

Fixed-film dextran degradation experiments were done in batch mode using rolled biofilm reactors. To perform this experiment, six reactors were filled with 400 ml of MSB containing 140 mg liter^{-1} of dextran, placed on the roller and rolled at 1.5 rpm. Molecular weight distributions obtained by sacrificing the contents of each reactor at different times showed that small-molecular-weight polysaccharide fragments accumulated in the bulk solution during biofilm degradation of dextran (Fig. 3). Hydrolysis proceeded rapidly after contact of substrate with dextran-adapted biofilms. The dextran feed solution used in the rolling biofilm reactors contained no detectable small-molecular-weight dextran, but, within 0.1 h, 25% of dextran in solution was less than 1000 amu. This molecular weight transformation occurred even though less than 10% of total substrate was assimilated into the biofilm. This accumulation of hydrolytic fragments is consistent with results from both batch and continuous suspended culture experiments. This is evidence that there can be a change in molecular weight distribution without substrate uptake and that hydrolysis and uptake are not tightly coupled.

The rapid hydrolysis of dextran observed in this experiment is similar to results obtained by Haldane and Logan (1994) in batch suspended culture. In their experiments they inoculated dextran-containing batch reactors with washed exponential and stationary phase cultures. Molecular weight distributions done on samples taken within 5 min of inoculation indicated that intermediate-size carbohydrates increased from 5.8% (no microbes) to 8.5% with the exponential phase inocula and to 13.4% with the stationary phase inocula.

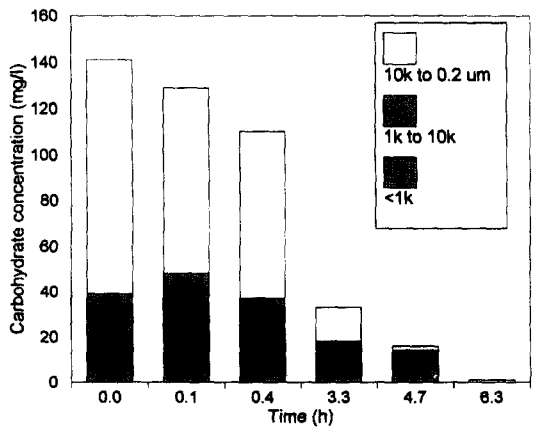

Fig. 3. Molecular weight distributions during dextran degradation in batch mode biofilm reactors.

The present authors wondered whether the release of hydrolytic fragments during dextran degradation could be due to oxygen limitation, but they were unable to mechanically aerate the rolling biofilm reactors to remove oxygen concentration gradients above the biofilm. However, oxygen concentration remained high in solution, and aeration would probably not have altered biofilm oxygen concentration. Using the same technique to monitor the bulk dissolved oxygen concentration as was used in as in protein degradation experiments (Confer and Logan, 1996b), it was found that solution oxygen concentrations remained in excess of 7.6 mg liter^{-1} during dextran degradation, suggesting that biofilms were not oxygen limited. However, since oxygen concentration was not measured within the biofilm, it cannot be certain that the biofilm itself was not oxygen-limited.

Dextrin degradation in suspended continuous culture

To see if the release of polysaccharide hydrolytic fragments during dextran degradation was representative of other carbohydrates, continuous culture dextrin degradation experiments were performed with a wastewater inoculum. As in the dextran experiments, feed solutions contained no small-molecular-weight carbohydrates, and intermediate-molecular-weight material comprised approximately one-third of total carbohydrates. Consistent with the dextran degradation experiments, continuous suspended culture dextrin reactors at steady state contained small-molecular-weight carbohydrates in solution at all detention times (Fig. 4). At various detention times, small-molecular-weight carbohydrates accounted for 20–40% of total carbohydrates. These data indicate that release of polysaccharide fragments from cells after partial hydrolysis is not unique to dextran but also occurs with starch preparations such as dextrin that are considered to be more representative of non-cellulosic polysaccharides in wastewater treatment systems.

Although dextrin is more highly branched polysaccharide than dextran, dextrin is an easier substrate for mixed microbial assemblages to metabolize than dextran (Fig. 5). Total carbohydrate concentration in dextrin degradation reactors was lower and a higher percentage of total carbohydrates were hydrolyzed to intermediate- or small-molecular-weight fragments than was observed in dextran reactors at similar hydraulic detention times. This suggests that hydrolysis of the predominant α(1-4) linkage in dextrin is a

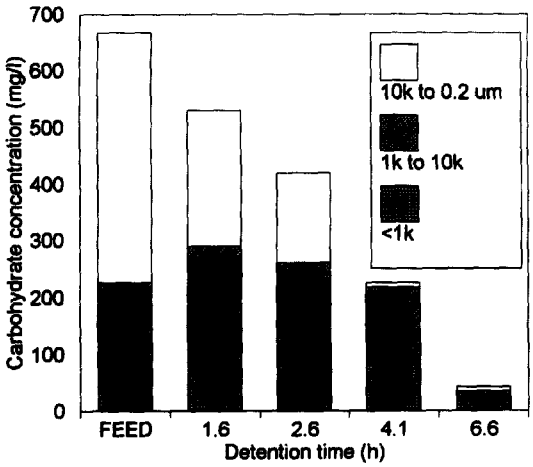

Fig. 4. Molecular weight distributions during dextrin degradation by suspended wastewater cultures in CSTRs at steady state.

more rapid than the predominant α(1-6) linkage in dextran in these wastewater inoculated reactors. Even though overall substrate degradation was faster for dextrin than dextran, a significantly higher proportion of small-molecular-weight material accumulated in solution during dextrin degradation than during dextran degradation. These small-molecular-weight oligosaccharides that accumulate during dextrin degradation are possibly the more branched oligosaccharides that remain after the linear portions of the molecule are metabolized.

DISCUSSION

Because polysaccharide hydrolytic enzymes are primarily cell-associated (Banerji *et al.*, 1967; Chróst, 1989, 1991; Boczar *et al.*, 1992; Confer and Logan, 1997a), the experiments reported here demonstrate that hydrolytic fragments that accumulate in bulk solution during polysaccharide degradation are released into solution by cells. Hydrolytic fragments were measured in solution for all combinations of reactor configurations, inocula and substrates investigated, indicating that the release of hydrolytic fragments is a fundamental aspect of polysaccharide degradation. This is in agreement with other macromolecular degradation studies both for polysaccharides and protein. Haldane and Logan (1994) studied molecular weight distribution during the degradation of dextran (70,000 amu) by a wastewater isolate in both batch and continuous culture suspended cell reactors. In both reactor configurations there was accumulation of partially hydrolyzed polysaccharide fragments. Although large-molecular-weight material (>10,000 amu) comprised more than 80% of total polysaccharides in feed solutions, small- or intermediate-molecular-weight compounds comprised more than 80% of total polysaccharides in late exponential growth phase in the batch reactor. Similarly, a continuous culture reactor at steady state contained over 50% intermediate- and small-molecular-weight carbohydrates. The results of the present study extend the applicability of the pure suspended culture work with dextran done by Haldane and Logan (1994) to mixed culture systems, fixed-film systems and to a second substrate, dextrin.

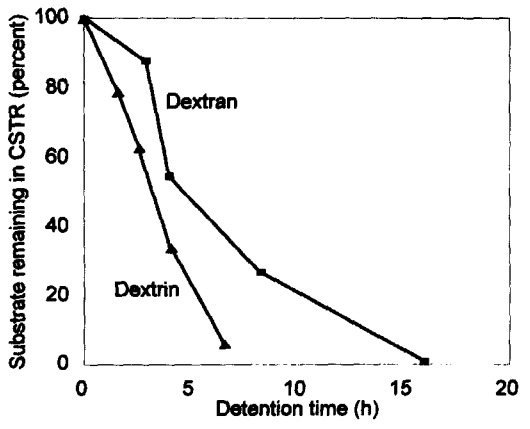

Fig. 5. Steady state concentrations of dextran (■) and dextrin (▲) in wastewater inoculated CSTRs at various detention times.

Two other studies used mixed cultures metabolizing soluble starch to study the mechanism of polysaccharide degradation. Banerji *et al.* (1968) used a bench-scale activated sludge system and found that carbohydrate intermediates with molecular weights smaller than the original starch accumulated in solution during degradation. In an annular biofilm reactor, Larsen and Harremoës (1994) also reported an accumulation of small-molecular-weight polysaccharides. Although Larsen and Harremoës attributed this accumulation to bulk liquid hydrolysis, they did not measure biofilm hydrolysis rates, which may have been more important than bulk liquid hydrolysis.

General model for macromolecule degradation

When the present and previous studies are considered in conjunction with other studies demonstrating that enzymes for polysaccharide hydrolysis are cell-associated (Banerji *et al.*, 1967; Chróst, 1989, 1991; Boczar *et al.*, 1992; Confer and Logan, 1997a), the accumulation of dextran and dextrin hydrolytic fragments in solution confirm that the macromolecule hydrolytic model presented for protein hydrolysis (Confer and Logan, 1997b) also applies to polysaccharides. In this model, macromolecules diffuse or are transported to cells where they are hydrolyzed. The hydrolytic fragments are then released and may return to the bulk solution. This process is repeated until the hydrolytic fragments are small enough (<1000 amu) to be assimilated into cells. This model is consistent with all of the following observations: macromolecule hydrolysis is primarily cell-associated; hydrolytic fragments smaller than the parent molecule accumulate in solution; and these hydrolytic fragments are metabolized. Alternatives to

this model that theorize that hydrolysis occurs by dissolved enzymes because hydrolytic fragments are found in solution (Larsen and Harremoës, 1994) are not consistent with studies that demonstrate that macromolecule hydrolysis is primarily cell-associated. Models that predict all hydrolysis and metabolism is cell-associated cannot explain the observations that hydrolytic fragments smaller than the parent substrate accumulate in solution and that these hydrolytic fragments are metabolized.

One reason that it may be necessary for cells to release hydrolytic fragments into bulk solution is that several different cell-associated enzymes may be required to completely hydrolyze macromolecules. It may not be possible to hold a macromolecule at the surface of the cell while these different enzymes hydrolyze the macromolecule. There are exo-glucanases that hydrolyze at non-reducing terminals and endo-glucanases that attack interior bonds. Dextranases have been identified that are able to hydrolyze polysaccharides comprised of linear α-1,6 linkages but unable to hydrolyze α-1,2, α-1,3 and α-1,4 linkages (Walker, 1978) or even α-1,6 linkages adjacent to branch points (Walker and Pulkownik, 1974). Similarly, various protein hydrolytic enzymes exist and exhibit specificity for different peptide bonds. Amino- and carboxy-exo-peptidases hydrolyze terminal amino acids, while endopeptidases hydrolyze interior amino acid linkages. For these different hydrolytic enzymes to function, the substrate and cell-bound enzymes need to contact each other in the correct orientation. There must exist either a complex handoff system to convey hydrolytic fragments from one enzyme to the next, or the cell must release the partially hydrolyzed substrate back into the bulk solution (or aggregate, biofilm matrix) and rely upon random re-collision to provide the correct orientation for subsequent hydrolytic events. A handoff system would need to be quite sophisticated and versatile to be able to direct the hydrolysis of all the different linkages which might be present. There are presently no studies that indicate the existence of such a system.

Implications for wastewater treatment system modeling

The importance of the finding that hydrolytic products are released into solution during macromolecule degradation for wastewater treatment reactors will be a function of the concentration and types of macromolecules in the wastewater. The major identifiable component of domestic wastewaters in the U.S. is thought to be proteinaceous material with carbohydrates and lipids comprising the smaller mass fractions (Metcalf and Eddy, 1991). In the previous study on degradation of a model protein (bovine serum albumin), it was shown for a wastewater-derived consortium that relatively small concentrations of hydrolyzed proteins, relative to the polysaccharides examined here, accumulated during the degradation of the macromolecules (Confer and Logan, 1997b). Since domestic wastewaters typically have relatively more protein than polysaccharide material, it is hypothesized that little dissolved material will accumulate in solution during wastewater treatment due to the hydrolysis of macromolecules in domestic wastewaters. There are special conditions, however, where the presence of intermediate-sized compounds could produce significant concentrations of intermediate-sized compounds. A wastewater containing a high proportion of polysaccharides, such as certain food-processing wastewaters, for example, might produce a relatively large concentration of intermediate (1000–10,000 amu) molecules. The production of smaller compounds during the degradation of a macromolecule must be considered as a separate issue from the synthesis and excretion of proteins or polysaccharides by bacteria for specific functions such as cell attachment. Polymers used for attachment should primarily remain attached to the cell (as a capsule or fibers) and therefore contribute to the particulate and not dissolved organic material.

The release of macromolecules into solution during macromolecule hydrolysis has important implications for models used to predict biological oxygen demand (BOD) removal in engineered reactors, since both the size and composition of the daughter molecules (those molecules produced by hydrolysis that are released into solution) will be different from those of the parent molecules. In one modeling approach, such as that used to model BOD removal in activated sludge plants, it is assumed that overall substrate degradation of DOM in wastewater is limited by microbial kinetics (Metcalf and Eddy, 1991). In other models, such as those used to describe substrate removal by biofilms in trickling filters (Logan *et al.*, 1987a, b), it is assumed that the removal of DOM is primarily limited by diffusion through the liquid film to cells in the biofilm and that all molecules that diffuse into the biofilm are degraded before they can diffuse back out into the bulk wastewater. Macromolecule hydrolysis can alter the composition of the substrate by creating a series of smaller molecules of different sizes with a different overall composition of monomer linkages. Thus, while a system can start out with only one type of molecule, the system can quickly accumulate many different molecules, all with different enzymatic degradation kinetics and diffusivities. This change in the composition of the substrate material will affect the values of the overall enzyme kinetic coefficients used in kinetic models and will alter the average molecule diffusivities necessary for calculating chemical fluxes in other types of models.

The production of molecules with different sizes and degradation kinetics may explain why consistent kinetic parameters (V_{max}, K_s) are difficult to determine in systems with large concentrations of macromolecules (Saunders and Dick, 1981). Substrate degradation models based on Michaelis–Menten kinetics

are formulated to describe a system that contains one enzyme and one substrate. When similar kinetic models are used to describe complex wastewater treatment systems, it is assumed that the complex system behaves as a single-enzyme/single-substrate system. For this assumption to be correct, the substrate size and composition must be invariable over time, so that all necessary degradation enzymes in the system operate simultaneously, resulting in a total enzyme activity that can be expressed as the superposition of individual enzyme activities. During macromolecule degradation, however, the character of the substrate itself changes and the required enzymes may work sequentially rather than simultaneously. Consider for example, a culture metabolizing 100 mg liter^{-1} of glucose and a similar culture metabolizing 100 mg liter^{-1} of dextrin. In the glucose metabolizing culture, when 90% of substrate has been consumed, the remaining substrate is still glucose. The same enzymes that were able to metabolize the first 10 mg liter^{-1} of substrate are capable of metabolizing the last 10 mg liter^{-1}. In the dextrin metabolizing culture, however, as hydrolysis of the original polysaccharide molecules proceeds, the remaining substrate is different both in structure and degradability than the original, unhydrolyzed dextrin. When 90% of the substrate has been consumed, the remaining polysaccharides will likely be smaller, more highly branched and may have hydrolysis and uptake kinetics that are faster or slower than those of the original molecules. If different enzymes, with different kinetic characteristics, are used to hydrolyze the remaining linkages, the resulting kinetic parameters V_{max} and K_s would also change.

The rate that molecules are degraded by biofilms is proportional to the chemical flux into the biofilm, which is in turn a function of both the diffusivities and microbial degradation kinetics of the organic compounds in the wastewater (Rittmann and McCarty, 1981; Logan *et al.*, 1987a, b). Small molecules with large diffusion coefficients diffuse to cells much faster than large molecules with small diffusion coefficients. Molecular diffusivities needed to calibrate biofilm models can be ascertained from molecular weight distributions of biodegradable DOM in the wastewater (Logan, 1994; Confer *et al.*, 1995). In experiments reported here for polysaccharides and previously for proteins (Confer and Logan, 1997b), it was found that molecular weight may not be conserved during degradation, indicating that the average diffusivities of the DOM will change during biodegradation of the parent compounds. This could result in the overall DOM removal rate in a biofilm reactor, such as a trickling filter, being a time-dependent function of not only the concentration but also the composition of the substrate pool.

Changes in the sizes and composition of the macromolecules during degradation demonstrates that changes in the size distribution of the DOM produced by macromolecule hydrolysis has the potential to affect both the microbial kinetics of suspended cultures and substrate fluxes into biofilms for fixed films. Identifying the coupled interactions between substrate diffusivity and biodegradability that results from macromolecule hydrolysis should allow a more complete understanding of the removal rates of DOM in wastewater treatment reactors. Mechanistic models may need to account for the changes in compound sizes and degradability for wastewaters when a large fraction of the DOM pool is significantly altered in composition due to macromolecule hydrolysis. In the case of domestic wastewater treatment in a plastic media trickling filter, it has been possible to predict overall wastewater treatment plant performance in terms of the initial molecular size distribution of the soluble BOD (Logan *et al.*, 1987a, b). For wastewaters composed of a high concentration of polysaccharides, however, such an assumption may not be justified, and more complex models may need to be developed that allow for hydrolytic by-products to diffuse out of the biofilm to rejoin the bulk wastewater. Thus, a more detailed description of the composition of wastewater during its degradation by microorganisms may make it possible to derive new models to better predict wastewater treatment plant performance.

CONCLUSIONS

A 70,000-amu polysaccharide contains more than 400 monosaccharides and, depending on the complement of enzymes present in the bacterial system, must undergo no fewer than 70 (and perhaps several hundred) hydrolytic reactions before the resulting fragments are small enough (<1000 amu) to be assimilated by bacterial cells. Because macromolecule hydrolysis is uncoupled from uptake, hydrolytic fragments can accumulate in the bulk solution. This causes a change in overall substrate molecular weight which can affect mechanistic treatment models. The released hydrolytic fragments can be different in degradability and require different hydrolytic enzymes than the original molecule, and this can affect kinetic-based treatment models. Consideration of this macromolecule degradation mechanism may lead to more accurate wastewater treatment models capable of better predicting the performance of bioreactors used to treat complex wastewaters.

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