BIODEGRADATION OF CELLULOSIC SUBSTRATES

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Biodegradation of Cellulosic Substrates

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13 ABSTRACT

Mechanisms for accelerated biodegradation of cellulosic substrates have been examined, especially analyses and optimization of pre-treatment of the fiber prior to microbial digestion. Methodology has been developed for enumeration of cellulolytic microorganisms and quantitative evaluation of cellulose transformation in the environment. Enzymatic activities of specific microorganisms have been studied, along with effect of specific pollutants on in situ processes, particularly cellulose turnover. PCBs present in cellulosic wastes have been analyzed and schematics proposed for their removal. Overall data suggest that selected physical/biodegradative techniques can be developed to accelerate rates of cellulose decomposition in the aqueous environment.

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FINAL REPORT

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Biodegradation and Shipboard Disposal of Cellulosic Substrates in the Marine Environment

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OBJECTIVES

The major objectives of this research program have been two-fold: (a) To analyze available information on the "state of knowledge" on cellulose assimilation in the marine environment, and (b) to evaluate rates of decomposition of cellulosic wastes and measures to accelerate the biodegradation processes involved.

PROGRAM DEVELOPMENT

The research effort has been concerned with utilization of single and combined biodegradative techniques to ascertain whether rates of cellulose decomposition can be accelerated by chemical/physical pretreatment of the substrate and the impact or "fate" of this material in the sea. Without proper pretreatment, such material may be effectively lost from transformation processes. While, autochthonous (indigenous) cellulose in the estuarine environment appears to be maintained in a steady-state flux, allochthonous materials and their unregulated introduction may upset the homeostatic condition and alter the energy flow of the ecosystem. Included in the study have been evaluation of levels of polychlorinated biphenyls (PCBs) in certain

cellulosics (i.e., Kraft paper, cardboard and highly processed substrates) and mechanisms to degrade such compounds in combination with microbial processes. Correlated investigations have involved analyses of mutualistic microbial associations in the degradative process, effect of combinations of microorganisms on rates of cellulose transformation (leading to formation of microbial bioprotein) and impact of oil intrusion on biodegradative activities of estuarine microorganisms. Reduction of cellulase activity on substrates modified by hydrocarbon intrusion has been considered in the total biodegradation process, especially within hydrocarbon-rich surface films. Overall information obtained has facilitated a better understanding of the kinetics of marine cellulose breakdown, including types and rates of microbial colonization of treated and untreated cellulose, rates of cellulose transformation as affected by prior-treatment processes, particle size vs. decomposition rate, and ultimate fate of the organically-enriched fiber. Evidence suggests that selected physical and biodegradative techniques can be effectively used in combination to accelerate rates of cellulose decomposition in the aqueous environment.

SUMMARY OF RESEARCH ACCOMPLISHED

Major areas of research accomplishments are summarized below. Experimental details and discussion of data are presented in the various reports and publications developing from this research.

Isolation of Isolates and Enumeration of Cellulolytic Activity

A double-layer agar system has been developed for isolation of celluiolytic bacteria. Methodology for isolation of such organisms, and enumeration of cellulase production, has been examined. The procedure involved a double-layer agar system, with a basal portion consisting of a

mineral salt/yeast extract agar and an upper portion of an alkali-treated cellulose-agar overlay. Alkali pre-conditioning of ball-milled cellulose (1% NaOH for 10 min @ 121 C/15 lbs) for incorporation into an agar overlay, increased rate of clearing zone formation as much four-fold over that of conventional cellulose agar preparations. Diagnostic cleared zones were formed in the modified cellulose-agar overlay in 5 days at 30 C. Low colony density is necessary; colonies of certain cellulolytic species may develop without evident clearing of the cellulose agar.

Tests indicate that the most effective cellulosic substrate for detection of cellulolytic microorganisms is a ball-milled cellulose powder subjected to alkali hydrolysis (1% NaOH for 10 min at 121 C/15 lbs). Controlled alkali hydrolysis is necessary to cleave long cellulose chains and swell the fibers, making the substrate more susceptible to microbial attack. Studies using gravimetric determinations, have been made of bacterial rates of digestion of treated and untreated celluloses. Correlated work has included evaluation of C_1 and C_x cellulase activity, especially that elaborated by specific fungi.

Cellulose Treatment

Pre-treatment of cellulosic wastes prior to biological attack has been examined. Pretreated cellulose samples were solubilized in cadoxen (a cellulose solvent) and analyzed for molecular weight and degree of polymerization (DP). Ball-milling or UV-nitrite irradiation lowered the DP to a greater extent than did alkali treatment or UV irradiation without nitrite. The controlled alkali hydrolysis, via chain clearing and fiber swelling, makes the cellulose substrate more susceptible to microbial attack. DP values were examined with respect to bacterial degradation rates for various

cellulose samples to determine if DP reduction occurred concurrently with accelerated cellulose decomposition. In effecting increase in cellulolytic rates, DP is of secondary importance compared with degree of swelling or accessibility of the cellulose fiber to bacterial enzymes. DP reduction is an integral part of cellulose decomposition, for low DP, highly crystalline substrates, have lower digestion rates than higher DP, less crystalline celluloses.

Cellulose treatments that appeared most promising include an alkali treatment of pre-shredded cellulose as well as nitrite photochemical treatment (a combination of sodium nitrite solution and ultraviolet irradiation). This latter process may be relevant to potential shipboard usage in that nitrite levels probably are already high in detergent-loaded waste waters. A reduction of the degradation time of cellulose by 25% using the nitrite-photo-chemical treatment is indicated. In view of the presence of polychlorinated biphenyl (PCB) compounds in brown paper towels, as well as in other highly processed cellulosics; the effect of pre-treatment on such materials has been examined (see subsequent section for PCB work).

Laboratory and In Situ Studies of Cellulose Decomposition

Laboratory and in situ studies were conducted to determine if appropriate pretreatment of cellulosic substrates could decrease recycling time in the environment. A combination of nitrite-photochemical, alkali, and bacteria seeding pretreatments increased the rate of cellulose solubilization by a factor of 6.5. An in situ rate of 3.8 mg solubilized/24 hrs/gram substrate was determined for decomposition of untreated, purified cellulose (filter paper). A specially-designed nylon bag method was used for quantitative in situ studies. Cellulose recycling rates were compared with those calculated for the chitin polymer in the same estuarine environment.

Decomposition rates reached a maximal at the sediment water interface. Introduced purified cellulose substrate was degraded, in situ, 2.7 times slower than that for the indigenous plant substrate, Spartina, indicating that processed cellulose wastes are even more recalcitrant than are natural cellulosics. Such wastes may therefore be particularly susceptible to sequestered elimination from recycling processes. In situ degradation of cellulose can be significantly accelerated by pre-seeding of the substrate with appropriate cellulolytic organisms. Effect of environmental factors on cellulose turnover have been examined.

These in situ studies have involved monitoring of degradation rates in inshore areas, using treated and untreated cellulosic substrates in control and "seeded" hydrocarbon plots. These field studies have been concerned with cometabolism of cellulosic processes, especially those in areas of hydrocarbon intrusion. In the absence of rapid biodegradation, physical coating of microbial substrates with oil will block active sites for enzymatic attack on the cellulosic particle. Oil-coated or oil-saturated detritus poses real problems in acceptability and transformation by the detritivore population, especially in view of the complex balance existing between the meiofauna and its associated microbial biota. Field and laboratory studies indicate a reduction in cellulolytic bacterial populations and in cellulase activity in the presence of crude oil.

Associated Microbial Studies

In conjunction with analyses of enzymatic activities of cellulolytic fungi, literature has been compiled on fungal protein production via biodegradation of various carbohydrate/cellulosic substrates. Nutritive values of microbially-modified substrates are directly related to studies of organically-enriched detritus and the ability of this material to sustain

representative species of marine herbivores. Fungal growth has been evaluated on a range of cellulosics (on a gravimetric basis of grams protein/L) using fungi with diverse carbohydrase activities. For instance, fungal protein (Myrothecium verrucaria) yields from inoculated ball-milled newspaper as great as 3.3 g/L are noted. The species, Pestalotiopsis westerdijkii, produces approximately 1.0 g/L on a wood pulp substrate. These data are more relevant to actual extant biodegradation processes than are measurements of Cx activity alone.

Various taxa of yeasts, notably those of Pichia and Trichosporon, have been further examined for optimal β-glucosidase activity. Specific activity (based on milligrams of microbial protein produced) as great as 0.6 enzyme units/mg protein are noted. Utilization of cellulose breakdown products, i.e., cellobiose, via active β-glucosidase systems have a prime function in favorably affecting rates of cellulose biodegradation and associated microbial protein formation. In other yeast investigations, studies have been made of species of Trichosporon (T. pullulans and T. cutaneum), reported to have cellulolytic activity in addition to their being active β-glucosidase producers. Four strains of T. cutaneum and one of T. pullulans were examined for Cx/C_1 activity on CMC and fibrous cellulose powder. Inspite of vigorous degradation of cellulosic matrices (both native and partially degraded), little enzyme activity, using DNS methods, could be detected, even over long incubation periods. Use of ultrasonic and surfactant treatments of fungal-attacked cellulose gave little indication of surface-bound enzyme(s).

Evaluation of Cellulosic Contaminants

Studies have concentrated on evaluation of toxic contaminants present in a variety of cellulosic waste substrates. Thus, in addition to the refractory

nature of processed cellulosic materials, a further problem exists with respect to toxic contaminants associated with cellulose wastes. Research in developing the technology of increased cellulose utilization requires recognition and proper handling of the hazards of concomitant compounds. Electron-capture gas chromatographic studies indicated over a 90% reduction of PCB residues by appropriate UV irradiation. Under inoculated conditions, approximately 18% of the total PCB concentration (previously dispersed in the liquid phase) was concentrated into the solid phase. Implications of this concentration mechanism in production (and possible use) of cellulosic/microbial biomass have been examined. PCBs in untreated cellulosic substrates were shown to remain stable throughout conventional cellulose decomposition processes and to be concentrated in the microbial-cellulose biomass during biodegradation. Methodology has been developed to determine the fate of these PCB compounds in microbially-degraded cellulose. An extraction procedure has been developed wherein PCB levels in the cellulose substrate and the microbial cells can be quantitatively established.

Evaluation of Hydrocarbon Levels on Microbial Processes and Cellulolytic Activities

Bacterial populations from an oil spill site and from oil-treated and non-treated detrital-based ecosystems showed several differences in proportions of microbial metabolic types. Sites exposed to oil had larger hydrocarbonoclastic bacterial populations than sites without oil. Biomass of cellulolytic bacteria was greatly reduced in oiled areas. A logarithmic fraction ($\log_{10}$ of the number of hydrocarbonoclasts - $\log_{10}$ of the number of cellulolytic organisms) was suggested as a reliable indicator of oil pollution. No consistent fluctuation in the fungal populations of the study areas was noted with oil intrusion.

Selected microbial parameters were monitored in sediments from a pristine and an oil field site. Although numbers of hydrocarbonoclastic bacteria and fungi were significantly greater in the oil field, the values did not show a strong correlation with levels of hydrocarbons. However, a high correlation was noted between ratios of hydrocarbonoclastic and total aerobic heterotrophic bacteria (HB:TB) and levels of hydrocarbons as well as the relative concentration of hydrocarbons (ratio of hydrocarbons to chloroform extractables). Data suggest that this ratio (HB:TB) is a more valid microbial indicator of hydrocarbon abundance than other factors examined. Significant differences in the ratio of pigmented to total colony forming units (cfu), the ratio of different to total cfu, and the diversity index were noted between the natural and oil field marsh. It was suggested that the presence of hydrocarbons alters the relative abundance of the most predominant aerobic heterotrophic bacteria.

Investigations of the interaction of proteolytic organisms and crude oil demonstrated that neither toxicity nor enzyme inhibition occurred. Since a large number of organisms were both proteolytic and hydrocarbonoclastic, crude oil intrusion may increase populations of proteolytic organisms in the environment. None of the cellulolytic organisms tested were inhibited by crude oil. One isolate had reduced growth rate in cellulose medium with crude oil present. The reduction of cellulolytic biomass in areas with oil exposure is probably due to the interaction of several factors. Selection and enrichment processes would seem to be significant factors, since only a small percentage of oil utilizers are also able to metabolize cellulose.

Several applications of microbial cultures to oil medium, "sequential seeding," over a 3-day period induced 32% weight loss. Introduction of a similar quantity of microbial cells in only one application produced only a

25% reduction. Incorporation of butylamine into crude oil at 0.5% levels induced slightly greater oil degradation in most cases. The addition of glucose or carboxymethylcellulose appeared to have no deleterious effect on oil degradation and may bring about more rapid metabolism of crude oil by some organisms.

It was concluded that crude oil does induce a change in overall activity of the estuarine microbial population. The significance of this modification, principally the reduction of cellulolytic organisms, was not determined, but a reduction in cellulolytic activity could seriously affect the energy transfer and regeneration of any oil-affected area.

Proposed Degradation Schematics

Degradation schematics have been proposed, suggesting incorporation of UV irradiation treatment into waste cellulose-SCP systems (or any recycling processes) where bioconcentration of PCB residues may present a significant potential danger. The UV/nitrite treatment may be especially revelant to shipboard usage where normal detergent-loaded waste discharge is already high in nitrites. Work is needed to determine the exact nature of PCB incorporation into the bacterial biomass, although it is assumed that the compound is accumulated in cellular lipid fractions. Rates of cellulose decomposition, in situ, are significantly increased by chemical/physical/microbial pretreatment of the substrate. Detrimental impact of such material on the environment is lessened by pretreatment to reduce levels of associated PCB residues, especially since it has been shown that PCB compounds are present in commercial cellulose products at levels approximately maximum recommended (5 ppm) limits.

MAJOR ACCOMPLISHMENTS

1) Development of methodology for enumeration of cellulolytic bacteria and characterization of cellulose breakdown.

2) Analyses and optimization of pre-treatment of cellulosics wherein the substrate is more susceptable to microbial degradative processes.

3) Laboratory and field rates of cellulose biotransformation have been determined, along with analyses of such rates in pristine and hydrocarbon-polluted detrital-based estuarine systems.

4) Analyses of cellulolytic activities of specific molds and yeasts with particular emphasis on β-glucosidase systems and the role of the latter in cellulose breakdown.

5) Levels of PCB contaminants in various cellulosics have been established with analyses of concentration and removal methodology correlated with cellulose biodegradation.

6) Effect of hydrocarbon levels on microbial processes has been examined with special attention given to rates of cellulose turnover under environmental conditions. Work has permitted establishment of a microbial diversity index to define such stressed environments.

7) Schematics for controlled removal of treated cellulose have been examined and may warrant evaluation in terms of shipboard units.

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