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14. ABSTRACT Simulated greywater or blackwater was fed to laboratory scale bioreactors that were operated as continuous flow systems with or without 100% biomass recycle. The responses of the microbial community were evaluated. The complex microbial communities found in biomass recycle reactors formed a robust and resilient system for the rapid biodegradation of complex organic wastes found in greywater and blackwater. The shipboard treatment of wastewater requires a rapid and reliable reduction in organic matter. Microbial degradation can be useful if operation is rapid, reliable, and with minimal sludge production. Our studies addressed microbial physiological state at near-zero growth rates, the resiliency of the microbial system to shock treatment, and the dynamics of microbial community structure; all of these are central to determining the success of this approach for biotechnological remediation.
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FINAL REPORT

Grant#: N00014-94-1-0318

PRINCIPAL INVESTIGATORS: Dr. A. Konopka and Dr. C.H. Nakatsu

INSTITUTION: Purdue University

GRANT TITLE: Biotechnological remediation of shipboard waste effluents.

AWARD PERIOD: 15 January, 1994 - 30 December, 2001

OBJECTIVE: Apply biotechnological principles to optimize organic matter reduction in bioreactors treating greywater effluents on Navy ships.

APPROACH: Simulated greywater or blackwater was fed to laboratory scale bioreactors that were operated as continuous flow systems with or without 100% biomass recycle. The responses of the microbial community are evaluated, with particular emphasis on the kinetics of organic biodegradation, and analysis of the physiological state of the microbial population existing under conditions of very low substrate flux per unit biomass. Functional characteristics of the microbial community in initiating the degradation of complex, polymeric organic materials have been determined by assaying the activities of hydrolytic ectoenzymes. The stability of the microbial community has been assessed by employing nucleic acid biomarkers of community composition. The laboratory scale reactor has been used to determine performance reliability to the environmental perturbations likely to occur on shipboard. These include inputs of toxic materials and temporal changes in substrate supply.

ACCOMPLISHMENTS: The bioreactor is a continuous-flow system in which 100% of biomass is retained by an ultrafiltration membrane (nominal 100,000 MW exclusion). This design has desirable characteristics from the perspectives of both engineering (low "sludge" output) and biotechnology (high biocatalyst concentration, and operation at a short hydraulic residence time). However, it raises unanswered questions about the physiological state and stability of the microbes.

There is a shift in physiological state of the microbial population when forced to grow at very slow rates. This shift results in a very low growth yield. Whereas conventional aerobic wastewater treatment processes produce yields of 0.3 g bacterial biomass per g COD, our systems operate in the range of 0.02-0.05 g bacteria per g COD.

The system is capable of degrading simulated greywater at temperatures from 15 to 70° C. Microbial diversity in the system decreased as temperature was increased. We have also found that the physiological characteristics of the system are affected by temperature. Although the microbial community present at moderately thermophilic temperatures is capable of metabolic activity over a temperature range of 25-30° C, the populations at higher temperatures exhibit a higher maintenance energy requirement, and therefore are more susceptible to interruptions in substrate supply than mesophilic reactors.

Microbial communities capable of degrading biopolymers and surfactants typically found in graywater were selected in bioreactors operated at 30, 44, 53, and 62° C. The effect of temperature upon microbial activity and community composition was determined. The microbial community in each reactor was optimally adapted to the operating temperature. Distinct consortia were present at each temperature. Community complexity was inversely related to temperature.

Under starvation conditions, microbes in the 62° C system lost membrane integrity 30 to 100-fold faster than microbes at lower temperatures.

Whereas previous work had shown that the biomass recycle reactor's microbial community was physiologically stable over extended periods of time, molecular analyses of community structure have indicated substantial changes in population structure with time. When assayed by denaturing gradient gel electrophoresis of PCR-amplified fragments of the 16S rRNA gene, microbial communities are relatively stable over a few day period, but then diverge such that similarity coefficients are less than 0.25.

The capacity of a starved bioreactor community to reinitiate biodegradation was determined. Starvation over 32 days caused a 10-fold decrease in cells, but  $> 10^8$  cells remained. All populations were capable of resuming exponential growth at approximately the same rate ( $0.3 - 0.4 \text{ h}^{-1}$ ). The length of time it took for this rate of exponential growth to occur was correlated to the length of the starvation period. From a process perspective, there was little difference in performance between samples starved between 0 and 32 days. Residual substrate concentrations would appear to be  $< 5\%$  of the initial values within 30 h of inoculation. Lyophilizing an active bioreactor community was not an effective strategy for storage and use in re-starting the system, as there was an extensive lag period. Cells quick-frozen in the presence of 10% glycerol were as active as an unstarved bioreactor population.

The degradation of complex particulate waste materials in blackwater has been simulated by the use of yeast cells and spinach homogenate. Both materials can be degraded at hydraulic residence times near 2 hours. The microbial communities contain a suite of hydrolytic ectoenzymes that initiate catabolism of particulate biopolymers. The activity of these enzymes is regulated; the level of induction/derepression differs for different enzymes. A defined community of 17 strains was used to test competitive interactions in the community. The communities collapsed to  $< 10$  members; many of the successful strains could degrade more than one polymer.

The relationship between structure and physiology of mixed microbial communities grown under substrate-limited conditions was investigated using continuous-flow bioreactors with 100% biomass recycle. Community structure was analyzed by denaturing gradient gel electrophoresis (DGGE) of the PCR and RT-PCR amplified V3 region of 16S rDNA and 16S rRNA templates, respectively. Starvation of reactor biomass demonstrated the link between community structure and metabolic activity assessed by RT-PCR of 16S rRNA and DGGE. In biomass recycle reactors in which substrate limitation becomes progressively more stringent with time, the specific content of community RNA declined by more than 10-fold and closely followed the decline in specific growth rate. In contrast the DNA content was variable and did not follow the same trend. Cluster analysis of the presence or absence of individual bands indicated that the fingerprints generated by the two templates were different. It also indicated that community response was first observed in the rRNA fraction. However, both the rDNA and rRNA fingerprints provided a picture of temporal population dynamics. Dice similarity coefficients gave a quantitative measure of the differences and changes between the communities. A limited screen of bacterial isolates using standard plating techniques recovered a third of the phylotypes detected by DGGE. An isolate that corresponded to the brightest band in the rRNA profile was readily cultivated; whereas, no isolates were obtained that corresponded to any of the bright bands in the rDNA profile. Nucleotide sequence analysis of the almost complete

16S rRNA genes of these isolates indicates that  $\beta$  and  $\gamma$  Proteobacteria and high and low GC Gram positive bacteria are present in these systems.

Experiments with reconstituted communities on a synthetic greywater medium have shown that the system can support a microbial diversity of 5-8 microbes. If a larger number of microbes are introduced into the system, there is a reduction in diversity over the first ten days. There is no tendency for generalist microbes (capable of using multiple polymeric substrates) to dominate in the system over specialists (use only one polymeric substrate).

**CONCLUSIONS:** The complex microbial communities found in biomass recycle reactors form a robust and resilient system for the rapid biodegradation of complex organic wastes found in greywater and blackwater.

**SIGNIFICANCE:** The shipboard treatment of wastewater requires a rapid and reliable reduction in organic matter. Microbial degradation can be useful if operation is rapid, reliable, and with minimal sludge production. Our studies address microbial physiological state at near-zero growth rates, which is central to determining the success of this approach for biotechnological remediation.

**PUBLICATIONS AND ABSTRACTS:** (for total period of grant)

Konopka, A., T. Zakharova, L. Oliver, D. Camp and R. F. Turco. 1996. Biodegradation of organic wastes containing surfactants in a biomass recycle reactor. *Appl. Environm. Microbiol* 62: 3292-3297.

Konopka, A. and Zakahrova, T. (1997) Physiological state at very low growth rate. *Abstr. Annu. Meeting Amer. Soc. Microbiol.*

Konopka, A., L. Oliver, and R.F. Turco. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial Ecology* 35: 103-115

Konopka, A., T. Zakharova, L. Oliver, and R. F. Turco. 1997. Microbial biodegradation of graywater in a continuous-flow reactor. *J. Industr. Microbiol. Biotechnol.* 18:235-240.

Konopka, A., T. Zakharova, E. Paseuth, L. Oliver, and R. F. Turco. 1998. Physiological state of a microbial community in a biomass recycle reactor. *J. Industr. Microbiol. Biotechnol* 20: 23-237.

Konopka, A., L. Oliver, and R.F. Turco. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial Ecology* 35: 103-115.

Konopka, A., T. Bercot and C. Nakatsu. 1999. Bacterioplankton community diversity in a series of thermally stratified lakes. *Microbial Ecology* 38: 126-135.

Konopka, A. T. Zakharova, and T. M. LaPara. 1999. Bacterial function and community structure in reactors treating biopolymers and surfactants at mesophilic and thermophilic temperatures. *J. Industrial Microbiology and Biotechnology* 23: 127-132.

Konopka, A. 1999. Theoretical analysis of the starvation response under substrate pulses. *Microbial Ecology* 38: 321-329.

- Konopka, A. 2000. Microbial physiological state at low growth rate in natural and engineered ecosystems. *Current Opinion Microbiol* 3: 244-247.
- LaPara, T., A. Konopka and J.E. Alleman. 2000. Energy spilling by thermophilic aerobes in potassium-limited continuous culture. *Water Research* 34: 2723-2726.
- LaPara, T. M., A. Konopka, C. H. Nakatsu, and J. E. Alleman. 2000. Thermophilic aerobic wastewater treatment in continuous-flow bioreactors. *J. Environ. Eng.* 126: 739-744.
- LaPara, T. M., A. Konopka, C. H. Nakatsu, and J. E. Alleman. 2000. Effects of elevated temperatures on bacterial community structure and function during aerobic biological wastewater treatment. *J. Ind. Microbiol. Biotech.* 24: 140-145.
- Oliver, L., Zakharova, T., Konopka, A., and Turco, R.F. 2000. Sodium hypochlorite perturbation of a graywater treatment system. *J. Ind. Microbiol. Biotech.* 24: 191-197.
- Morgan, CA, Nakatsu CH, Zakharova TP, Carrero-Colon M, Lapara TM and Konopka A. 2000. Survival of the fittest? Generalists vs. specialists. Annual Meeting of Canadian Society for Microbiology.
- Nakatsu CH, Morgan, CA, Zakharova TP, and Konopka A. 2000. Perturbation of a microbial biomass recycle community. Annual Meeting of Canadian Society for Microbiology.
- LaPara, T. M., A. Konopka, C. H. Nakatsu, and J. E. Alleman. 2001. Thermophilic aerobic treatment of a synthetic wastewater in a membrane-coupled bioreactor. *J. Ind. Microbiol. Biotech.* 26: 203-209..
- M. Carrero-Colon, C.A. Morgan, A. Konopka, T. Zakharova, and C.H. Nakatsu. Laboratory scale bioreactors: a model system to study microbial community structure and function. *Abstr. Annu. Meeting Amer. Soc. Microbiol.* (2001)
- Konopka, A., M. Carrero-Colon, C.A. Morgan, T. Zakharova, and C.H. Nakatsu. Microbial community structure and function in a biomass-recycle bioreactor. *Abstr. Internat. Soc. Microb. Ecol.* (2001)
- Morgan, C.A., A. Hudson, A. Konopka and C. H. Nakatsu. 2002. Analysis of microbial activity in biomass recycle reactors using based on denaturing gradient gel electrophoresis of 16S rDNA and 16S rRNA. *Can. J. Microbiol.* 48: 333-341.
- Konopka, A. and Zakharova, T. 2002. Analysis of bacterial ecotoenzymes by polyacrylamide gel zymograms. *J. Microbiol. Meth.* 51: 273-282.
- Konopka, A., Zakharova, T. and Nakatsu, C.N. 2002. Effect of starvation length upon lags in substrate degradation in a biomass recycle reactor. *J. Industr. Microbiol. Biotech* 29(5): 286-291.
- LaPara TM, Zakharova T, Nakatsu CH, and Konopka, A. 2002. Functional and structural adaptations of bacterial communities growing on particulate substrates under stringent nutrient limitation *Microbial Ecol* 44 (4): 317-326.