EFFECT OF INOCULATION ON THE BIODEGRADATION OF BUTTERFAT-DETERTENT MIXTURES IN FIXED-FILM SAND COLUMNS

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Abstract

The purpose of this work was to investigate the feasibility of biodegradation of butterfat using an aerobic sand column system. Two bench-scale sand columns were inoculated with lipolytic microorganisms to colonize the sand with an active biofilm, and two uninoculated columns were used as controls. Butterfat was fed daily to the columns in artificial wastewaters that were supplemented with a detergent to provide emulsification. Sand columns exhibited physical infiltration, trapping butterfat in the sand, and resulted in an apparent high degree of COD and BODs removal from the effluent. Accumulation of biomass on the sand grains was demonstrated by measurement of ATP. Biomass accumulated in the columns over the 259-day experiment. At the end of the experiment, the amount of biomass was comparable in inoculated and uninoculated columns. Uninoculated columns were shown to lag behind inoculated columns by about 100 days in COD removal. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Key words: Sand column, lipolytic microorganisms, COD, BODs, ATP.

INTRODUCTION

Animal fats, vegetable oils, and detergents from restaurants (especially quick-service restaurants), dairy farms, and food processing plants have led to an increase in oil and grease content in wastewater. Oil and grease in waste streams can cause problems such as blockages in drainage systems and accumulation on interior pipe surfaces in treatment plants. Traditionally, greasy and oily wastewaters from dairy and other food processing industries have not been the subject of specialized treatment. Specific engineering designs are available to physically separate grease and oil from wastewaters, but none are available to promote their removal via biodegradation with lipolytic biofilms in sand columns. Other specialized processes developed for high BODs (5-day biochemical oxygen demand) waste streams include anaerobic reactors to treat slaughterhouse wastewater (Sayed et al., 1987; Toldra et al., 1987), and upflow anaerobic sludge blanket reactors (Gutierrez et al., 1991) or moving bed biofilm reactors (Rusten et al., 1992) to treat dairy waste streams. Although the COD (chemical oxygen demand) load in these systems is greatly reduced, the fate of the lipid fraction in these treatment systems remains unclear.

Biofilms as fixed-film reactors are employed for a variety of applications for wastewater treatment and for remediation of soil and water. Packed bed reactors, rotating biological contactors, and biological fluidized reactors are some typical examples of fixed-film bioreactors used in wastewater treatment (Bryers and Characklis, 1990). Many different media, characterized by high specific surface areas, are suitable for these systems as long as the solid phase is chemically and biologically inert to support microbial colonization and biofilm formation. A biofilm is a matrix of microbial cells and cellular products attached to a surface. The formation of a biofilm is a complex process of sorption, desorption, attachment, detachment, cell growth, nutrient assimilation, product formation, entrapment, and decay of dead cells (Peyton and Characklis, 1995). In early stages of biofilm formation, microorganisms attach to surfaces that provide an interface to compete for nutrients, followed by growth to an established biofilm community.
...water treatment processes, its low cost, and high with and without initial inoculation of lipolytic filter medium. This study employed a lipolytic fixed-gradation of butterfat in biofilms with sand as the underlying biological mechanisms greatly vary (Van Loosdrecht and Heijnen, 1993).

The goal of this study was to evaluate the biodegradation of butterfat in biofilms with sand as the filter medium. This study employed a lipolytic fixed-film system to degrade fat in the presence of a supplemental detergent formulation. Lipolytic microorganisms are ubiquitous in nature and distributed among many genera of prokaryotic and eukaryotic microorganisms. Lipases may be extracellular or intracellular (Ota, 1977), and those that act on the fatty acid chains of triglycerides are active at the lipid–water interface. Enhanced biodegradation of lipids is usually achieved in the presence of surfactants because, due to their amphipathic nature, they emulsify or solubilize lipids in aqueous solutions and thereby increase the interfacial area. The specific objectives of this study were to (i) evaluate the effects of inoculation on the performance of fixed-film sand filters in the removal of COD and BOD₅, and (ii) estimate biomass accumulation with and without initial inoculation of lipolytic microorganisms. Butterfat was chosen as the substrate in the present work because of its high content in dairy farm and dairy industry discharges. Sand was selected as the filter medium for this work by virtue of the suitability of sand filters in wastewater treatment processes, its low cost, and high availability.

**METHODS**

**Microorganisms and culture media**

Lipolytic microorganisms were enriched from a soil sample collected at a land application site used for dairy farm wastewater disposal, a soil sample from a river bank, and a sample of spoiled pork. For enrichment purposes, a liquid medium was used that contained 0.1% w/v butterfat, 1% w/v tryptone, and 0.5% w/v yeast extract (BTYA medium). About 1 g samples of soil were added to shake flasks containing 100 ml of the enrichment medium. The flasks were incubated at 22±2°C on a shaker at 150 rev/min. The enrichment cultures were subcultured twice in BTY medium before plating on a solid medium that contained 1% w/v tryptone, 0.5% w/v yeast extract, and 1.2% w/v agar (BTYA medium). For spoiled pork, isolation was directly performed by streaking samples on BTYA medium. Lipolytic colonies on BTYA medium could be visualized due to formation of clear zones around the colonies capable of degrading butterfat. A total of 13 lipolytic bacterial cultures were isolated in this study. The isolates were stored at -80°C in glycerol.

**Sand columns**

For inoculation of the sand columns, lipolytic stock cultures from storage were inoculated in 5 ml aliquots of tryptone-yeast extract medium (TY medium) that contained 1% w/v tryptone and 0.5% w/v yeast extract. After three subcultures (24 h each), these cultures were transferred (0.1% v/v) into 5-fold diluted TY medium. The diluted, inoculated medium (90 ml) was introduced as a seed into the sand columns previously sterilized twice by autoclaving (121°C for 30 min each). The inoculated columns were incubated for 2 weeks at 22±2°C before initiating a daily application of artificial wastewater.

The columns were constructed of glass (inside diameter of 3.8 cm) and filled with sand to a height of 27.5 cm (450 g sand and 90 ml void volume per column). The sand was sieved and the particle size fraction of 0.85–1.7 mm in diameter was used for the columns. Two sand columns were inoculated with the lipolytic test cultures and the other two were uninoculated and used as controls. The sand columns were covered with sterile cotton plugs and operated next to each other at 22±2°C.

**Column operation and sampling**

On a daily basis, 50 ml aliquots of sterile artificial wastewater were fed into each column from the top and drained (by gravity) immediately from the bottom. The commercially available dish-washing detergent, Ivory (Procter and Gamble, Cincinnati), was used as the supplement of the artificial wastewater to facilitate the dispersion of butterfat in the aqueous phase (Table 1). The ratio of butterfat to detergent was standardized to 1:0.4 in order to completely dissolve the butterfat in the aqueous phase. The artificial wastewater formulation contained 0.1% (w/v) butterfat. The artificial wastewater was supplemented with sterile mineral solutions that contained (per liter) 8.5 mg KH₂PO₄, 21.75 mg K₂HPO₄, 33.4 mg Na₂HPO₄·7H₂O, 1.6 mg

Table 1. Mean removal efficiencies of COD and BOD₅ in the sand columns at the end of the column experiments (259 days)

<table>
<thead>
<tr>
<th>Column no.</th>
<th>Influent</th>
<th>% COD removal*</th>
<th>% BOD₅ removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2 (inoculated)</td>
<td>0.04% (w/v) Ivory+0.1% (w/v) butterfat</td>
<td>45.8±16.1 a</td>
<td>49.6±19.6 a</td>
</tr>
<tr>
<td>3 and 4 (uninoculated)</td>
<td>0.04% (w/v) Ivory+0.1% (w/v) butterfat</td>
<td>37.5±10.6 b</td>
<td>40.0±17.5 b</td>
</tr>
</tbody>
</table>

* Each set is based on the mean of 38 data points ± standard deviation. The values with different letter designations are significantly (P<0.05) different from each other within each column.
A is the average influent COD (or BODs) and B is the average effluent COD (or BODs) of each sample expressed as mg O\textsubscript{2} l\textsuperscript{-1}. The removal efficiencies (E) of COD and BOD\textsubscript{s} removal was calculated as $E = \frac{(A - B) \times 100}{A}$, where $A$ is the average influent COD (or BOD\textsubscript{s}) and $B$ is the effluent COD (or BOD\textsubscript{s}) of each sample expressed as mg $\text{O}_2$ l$^{-1}$.

Adenosine triphosphate (ATP), a universal component in living cells, was used as a measure of biomass in this work. Sand samples from the upper, middle, and bottom layers of the columns were retrieved from each sand column after the first month of operation and at the end of the experiment. ATP was extracted as described by Tseng et al. (1996) except that all the samples were centrifuged at 8000 g for 20 min at 4°C. ATP concentrations were measured with the luciferin-luciferase bioluminescence system using ATP bioluminescent assay kit (Adenosine 5’-Triphosphate Bioluminescent Assay kit, Sigma Chemical Co., St Louis) and a photometer (Monolight 2010, Analytical Luminescence Laboratory, Ann Arbor).

The data were analyzed by ANOVA using the Minitab software (release 8.2, Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Effect of inoculation on the performance of sand columns

The performance of the sand columns was evaluated on the basis of changes in the effluent COD and BOD\textsubscript{s} over time. Removal efficiencies over time of the sand columns are shown in Fig. 1.

During the first 3 days of the experiment, the removal efficiencies for the inoculated sand columns were negative, i.e. the effluent COD and BOD\textsubscript{s} were higher than the respective influent values. These transient increases were attributed to wash-out of loosely adherent inoculum bacteria and residual diluted TY medium as a carry-over from the inoculation.

Within 2 weeks of operation, COD and BOD\textsubscript{s} removal efficiencies of inoculated sand columns were improved and higher than the uninoculated columns. The differential of the removal efficiencies between inoculated and uninoculated columns continued to increase during the following few weeks. The first month of operation of inoculated sand columns can be regarded as a lag period of biofilm formation. This may also be responsible for the initially low removal efficiencies.

The COD and BOD\textsubscript{s} removal efficiencies in all sand columns continued to increase during the next 3 months. For inoculated and uninoculated sand columns, the rates of increase in COD removal efficiencies were similar (Fig. 1) on the basis of the respective slopes of the fitted lines. However, the removal efficiencies in inoculated columns were higher than in the uninoculated columns. In the beginning of this period, COD removal efficiencies of inoculated columns reached about 40%. It can be estimated from the regression lines in Fig. 1 that the COD removal efficiencies of uninoculated columns reached this 40% level about 100 days later. After another 1 month (i.e., after 5 months of operation), the COD removal efficiencies of uninoculated columns were comparable to those in the inoculated columns. After 7 months of operation, the effluent COD and BOD\textsubscript{s} values remained at a more or less steady level with only minor differences between the inoculated and uninoculated sand columns.

The statistical analyses (ANOVA) of the COD and BOD\textsubscript{s} results for the entire time course (259 days) are shown in Table 1. The inoculated sand columns had significantly ($P < 0.05$) higher COD and BOD\textsubscript{s} removal efficiencies than the uninoculated columns over the entire time course.

Biomass accumulation in sand columns

The ATP concentrations upon termination of the experiment (259 days) were in the range of 1–5 µg ATP g$^{-1}$ dry weight sand (Fig. 2). After 259 days of operation, the ATP concentrations of the four columns significantly ($P < 0.05$) increased from an average of 0.15 µg ATP g$^{-1}$ dry weight sand at 30 days to 2.25 µg ATP g$^{-1}$ dry weight sand (Table 2). This suggests that the feed solution formulation was utilized by microorganisms, leading to biomass accumulation and biofilm formation in the columns. In addition to butterfat and Ivory, no other organic carbon source was available in the daily feed solution.

After the first month of the operation, inoculated sand columns showed significantly ($P < 0.05$) higher ATP levels than the uninoculated columns (Table 3). The presence of ATP in the uninoculated columns indicated contamination. The daily feed solution was sterilized but not routinely monitored for contamination. Airborne contamination resulting from exposure to air to load the feed solution into each column may also account for
bacterial entry into the uninoculated columns. Subsequent experiments revealed the presence of spore-forming bacteria in comparable columns that were not initially inoculated.

The ATP concentrations in inoculated and uninoculated sand columns after 259 days of operation were not significantly ($P > 0.05$) different. Results of statistical analysis of ATP concentrations in upper, middle, and bottom sand layers of all the sand columns after 30 days and 259 days of operation are shown in Fig. 2. After the first month of operation, no significant ($P > 0.05$) difference was observed in ATP concentrations among upper, middle, and bottom layers of all sand columns (Fig. 2). After 37 weeks of operation, the highest level of ATP was found in the upper layer of inoculated columns (Fig. 2) and this was a statistically significant ($P < 0.05$) difference. The relative ranking order of ATP concentrations in the inoculated columns (259 days) was upper > middle > bottom.

CONCLUSIONS

This study examined the use of sand columns to remove COD and BOD$_5$ from butterfat- and deter-
Butterfat degradation in sand columns

Over the 37-week period, columns inoculated with lipolytic bacteria performed better than did uninoculated sand columns in terms of COD and BOD₅ removal efficiencies from effluents. The bioluminescence assay for ATP measurement was suitable for biomass estimation in samples of sand columns. ATP concentrations increased with time, confirming that COD and BOD₅ removal was associated with growth of biomass in the sand columns. ATP has been previously used to evaluate biomass accumulation in slow sand filtration systems for drinking water treatment (Mackay et al., 1996), but not for sand filter media used in wastewater treatment. Several conversion factors from ATP to cellular-C (on a weight basis) have been reported in the literature; these fall in the range of 120 for soils and 250-286 for aquatic environments (Atlas and Bartha, 1993). Validation of a conversion factor applicable to sand filtration systems was not within the scope of the present work. The fixed-film systems with sand as filter medium reached 40% COD and BOD₅ removal efficiencies as early as 4 weeks after operation. Inoculation accelerated the time course to reach a steady removal efficiency. In treatment practice, however, feed solutions and sand would not be sterile and inoculation may not be warranted. In an effort to optimize the biodegradation activity in sand filtration systems, further studies are also needed to characterize biofilm formation and stability of fixed-film microorganisms and to evaluate the beneficial use of detergents and surfactants.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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**Table 2. Concentration of ATP in the sand columns measured after 30 days and 259 days of operation**

<table>
<thead>
<tr>
<th>Length of operation</th>
<th>ATP (µg g⁻¹ dry weight sand)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days</td>
<td>2.25 ± 1.12 a</td>
</tr>
<tr>
<td>259 days</td>
<td>0.15 ± 0.07 b</td>
</tr>
</tbody>
</table>

¹Each set is based on the mean of four columns (four duplicate samples for each column) ± standard error. The values with different letter designations are significantly (P<0.05) different from each other within each column.

**Table 3. Concentration of ATP in the sand columns measured after 30 days and 259 days of operation**

<table>
<thead>
<tr>
<th>Column no.</th>
<th>ATP (µg g⁻¹ dry weight sand)¹</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>30 days</td>
</tr>
<tr>
<td></td>
<td>259 days</td>
</tr>
<tr>
<td>1 and 2 (inoculated)</td>
<td>0.20 ± 0.08 a</td>
</tr>
<tr>
<td>3 and 4 (uninoculated)</td>
<td>0.10 ± 0.01 b</td>
</tr>
</tbody>
</table>

¹Each set is based on the mean of two columns (four duplicate samples for each column) ± standard error. The values with different letter designations are significantly (P<0.05) different from each other within each column.


