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Containment in industrial biotechnology within wastewater treatment plants

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Both physical and biological containment are considered to be essential parts in the risk analysis of industrial Good Industrial Large-Scale Practice (GILSP) processes using genetically modified organisms (GMOs). Biological containment of industrial microorganisms has become a more important issue since the introduction of recombinant DNA techniques. In the event of an accidental discharge in the production plant, a large amount of organisms could be released into the wastewater treatment (WWT) system. This WWT system should therefore be considered as a part of the containment. This study demonstrates both a hydrodynamic and a microbiological model for the containment aspects of industrial WWT plants. The models are verified by measurements using industrial hosts of GILSP GMOs at full scale. Both models describe the full-scale equipment accurately. The results are supplemented with microcosm studies on survival of GMOs in defined niches. It is shown that WWT plants can be considered as useful additional parts of the containment of microorganisms, in case of an accidental discharge. The effect of drainage of an enormous amount of microorganisms (several tons) through the WWT plant into the environment is shown to be comparable to the direct drainage of a small-scale fermenter. Microcosm experiments correlate well with the survival rates in the WWT and therefore can be of use to predict the behaviour of GMOs in this environment.
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Introduction

Safety is an important aspect of life and has an impact on almost everything we do. This is not important just in normal life, but especially in industry, this issue plays a central role. If neglected, the safety issue can lead to considerable damage, not only to people's lives and to the environment, but also to a company, or even total branch of industry.

Although industry in general was aware of this safety issue, it has been addressed specifically since the late 1970s when recombinant DNA (rec-DNA) techniques were introduced. Both the scientific community and the biotechnological industry have recognized this issue and acted accordingly by implementing containment measures. At first, the issue only occurred on the laboratory scale. However, since the techniques have proven to be successful on the production scale, the safety issues have also been addressed on larger scales.

Most industrial large-scale processes using genetically modified organisms (GMOs) are Good Industrial Large-Scale Practice (GILSP) processes. The safety of these processes is based both on physical and biological containment. Physical containment, e.g., equipment, building, *etc.*, will ensure that living organisms are kept within a defined physical boundary.

As small releases of GMOs are allowed, organisms used in fermentations under GILSP conditions have to fulfill the demands related to biological containment. Biological containment ensures that the chance of survival, the dissemination of the GMOs and the transfer of the newly introduced DNA to other organisms are limited. In GILSP processes, biological containment properties are important to ensure that the GMOs do not cause damage to the environment if they escape in small numbers, due to aeration, sampling, *etc.*, or when they escape as a result of an accidental discharge. The ecological niches of escaped GMOs will meet are air, sewage or WWT facilities, surface water and soil.

In these systems, physical and biological containment are combined. The organisms are exposed in a semiclosed system to a hostile environment, in which they are faced with very strong competition from the organisms that are endogenous to a WWT plant.

Only a few papers have been published on this subject. Kane [9], Bogosian and Kane [1], Bogosian *et al* [2,3] and Heitkamp *et al* [8] were the first to show that rec-DNA *Escherichia coli* strains did not survive in river water or in sewage sludge environment in laboratory microcosm situations. In addition, Bogosian *et al* [3] demonstrated that no gene transfer had occurred from the rec-DNA *E. coli* bovine somatotropin-producing strain to the endogenous microorganisms. Duque *et al* [4] demonstrated that gene transfer did occur in soil, but this concerned a *Pseudomonas* strain engineered to biodegrade polluted environments. From these studies, it is concluded that only when rec-DNA organisms do have a competitive advantage over the microflora will they survive and may transfer genetic material to this endogenous microflora.

This article describes results on the survival of industrial microorganisms in both microcosm experiments and in a simulated accidental discharge at the Delft production site of DSM (formerly Gist brocades) using non-GMO strains, which serve as a parent for industrial GMOs. The survival of these microorganisms was measured in full-scale WWT plant. A model was constructed for describing both the hydrodynamics of the equipment and the killing of the organisms. These models can be used to make an accurate

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estimation of the fraction of organisms that can pass through the WWT plant alive and which reach the settler tank.

Materials and methods

Microorganisms

Two different microorganisms were used: a spontaneous rifampicin-resistant derivative of *Bacillus subtilis* 1S53 (ATTC 33234), and *Kluyveromyces lactis* GAL2B, a derivative of *K. lactis* CBS 487.94. Both strains are parental strains for rec-DNA production strains.

Microcosm survival studies

Microcosm survival studies were carried out on environmental samples from soil and river water from the immediate vicinity of the Delft production site and wastewater from the inlet of the wastewater treatment (WWT) system. Environmental sample sterilization was done with a gamma-irradiation dose of 25,000 Gy (2.5 Mrad). In these experiments, 1 g/ml samples of the respective environment were inoculated with the microorganism to be tested; in sterilized environmental samples, inoculation was at approximately 10^4 g^{-1}; in the natural environmental samples, a starting level of approximately 10^8 g^{-1} was aimed for. Incubations were at 8°C and 25°C, respectively. Survival of the test microorganisms was measured as the number of colony-forming units (cfu) remaining in the environmental samples as a function of time. Control incubations were done by measuring the survival of *Pseudomonas fluorescens* in sterile samples or the endogenous microorganisms in the natural samples, respectively.

WWT installation

A schematic picture is given in Figure 1. The wastewater from the entire production site (approximately 230 m^3/h) enters the WWT at the pumping station. The wastewater acidifies in the buffertank (1245 m^3). From the buffertank, the water is treated in two parallel "streets," each consisting of two parallel anaerobic upflow reactors (285 m^3) followed by one aerobic airlift reactor (350 m^3). Sixty percent of the water is treated in "street 1" and the rest in "street 2." The solids are removed by sedimentation in the settler. The effluent is drained off to the city sewer and the sediment is concentrated.

The mean residence time in the WWT is 10–20 h. Due to mixing, the actual residence time of an organism is distributed around this mean residence time. The influence of the distribution of this residence time on the survival of the organisms is taken into account.

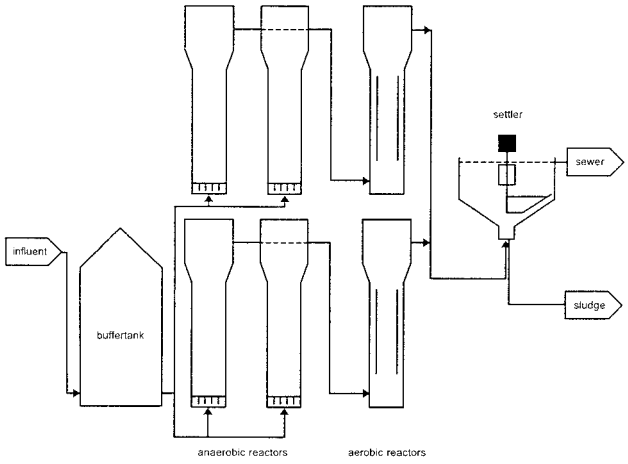

Figure 1 Schematic overview of the WWT system of DSM in Delft (formerly Gist brocades). The top reactors in the figure represent street 1 and the bottom three reactors represent street 2.

Experiments

The two types of organisms were drained to the WWT separately in different experiments. The organisms were produced in the pilot plant fermenters. The fermentation broth was drained off to the WWT as quickly as possible, immediately followed by a large amount of lithium chloride tracer solution, and a large amount of water to decrease the time to reach the WWT.

In these experiments, a total of 3.3×10^{16} cfu of *B. subtilis* and 1.95×10^{17} cfu of *K. lactis* were drained from the pilot plant to the WWT. In both experiments, 7 kg of lithium chloride was discharged to the WWT. Since this drainage was done very quickly (in a few minutes), this can be considered as one flow containing microorganisms and lithium chloride.

Samples

To determine the lithium and microorganism concentration profiles in different parts of the WWT, samples were taken from the liquid entering and leaving the buffertank and aerobic reactors.

Analytical methods

The *B. subtilis* cfu were determined on nutrient agar plates containing 50 ppm of rifampicin. The *K. lactis* cfu were determined on malt–agar plates containing 50 ppm of penicillin, 50 ppm of streptomycin and 50 ppm of rifampicin (to prevent growth of prokaryotes) and 100 ppm of cycloheximide (to prevent growth of eukaryotes other than *K. lactis*, which is resistant to this drug). This analysis is therefore selective for *K. lactis*. The concentration of lithium was determined by atomic emission spectroscopy.

Calculation methods

For the simulation and curve fitting of the data, SAS using FORTRAN routines was used.

Results

Microcosm survival studies

In the so-called microcosm survival studies, small samples (1–2 ml) of a relevant environment are inoculated with the microorganisms to be studied, and their survival rate and competitiveness are observed during time. In these experiments, a number of parameters can be varied in order to create different environmental conditions. For both bacteria tested, an identical set of experiments was designed. In all experiments, the influent of the WWT, as obtained from the buffertank (Figure 1), was used as liquid medium. Variations in the environmental conditions were the incubation temperature (8°C and 25°C) and whether the WWT influent was sterilized before inoculation with the test microorganisms, or taken untreated. Growth in sterilized influent is taken as an indication of the absence of lethal components in the influent. The results in the nonsterilized environment give an indication of the competitiveness in the simulated environment. Studying the

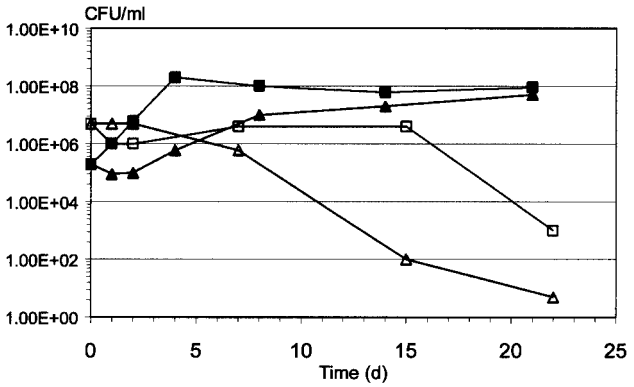

Figure 2 Survival of *K.lactis* in microcosm wastewater. Variations are sterilized (closed symbols) *versus* untreated (open symbols) wastewater; incubations at 8°C (triangles) or 25°C (squares).

survival of microorganisms at two different temperatures is considered to yield more representative results than at a single (e.g., the year's average) temperature, whereas using both sterile and native environments allows one to observe intrinsic survivability of the microorganism as well as the competitiveness with endogenous microbes with respect to available nutrients.

Microcosm survival experiments with *B. subtilis* indicated that within a few days, in both sterile and nonsterile environments (soil, river water, wastewater), the titre of viable *Bacillus* dropped to below the detection limit. This is in full agreement with other observations on rec-DNA *Bacillus* strains [10,11]. Under identical conditions, a *P. fluorescens* control strain was able to grow (sterilized environments) or maintain (nonsterile environment) its titre at appreciable levels (not shown).

K. lactis appears to be more capable of coping with the different environments: in sterilized wastewater at either 8°C or 25°C, the strain grew at approximately the same rate to titres of 10^8 ml^{-1}, as illustrated for wastewater in Figure 2. In nonsterile environments, a slow reduction in surviving yeast cells was observed, the decrease being faster at 25°C than at 8°C (Figure 2). This confirms observations by Gellissen *et al* [7] on rec-DNA yeast strains.

In long-term experiment in wastewater, the decrease was confirmed, with titres falling below the detection limit within 40 days. In other environments, similar trends were observed: growth to stable titres in sterile conditions, slow reduction in competition with the endogenous microorganisms in the natural environment.

Formulation of the hydrodynamic WWT model: In order to obtain a model that adequately describes the different reactors of the WWT, a basic set of equations was set up describing a single continuously stirred tank reactor (CSTR), two CSTRs in series and two CSTRs in parallel, respectively. Subsequently, the best model for each reactor separately was determined by the concentration profiles of the tracer (lithium) during the actual drainage experiment.

The concentration profile in a reactor described by one CSTR is:

$$\frac{dC}{dt} = \frac{F}{V}(C_i - C)$$

where C=concentration of Li (in the outlet) (mg/l); F=flow through the reactor (m^3/h); V=volume of the reactor (m^3) and C_i=concentration of Li in the incoming liquid (mg/l).

The concentration profile in the outlet of a reactor simulated by two CSTRs in series is described by two differential equations:

$$\frac{dC_1}{dt} = \frac{F}{0.4V}(C_i - C_1)$$

and

$$\frac{dC}{dt} = \frac{F}{0.6V}(C_1 - C)$$

where C_1=concentration of Li in the first reactor (mg/l).

The concentration profile in the outlet of a reactor simulated by two CSTRs in parallel is described by the following equations:

$$\frac{dC_1}{dt} = \frac{F_1}{V_1}(C_i - C_1)$$

$$\frac{dC_2}{dt} = \frac{F_2}{V_2}(C_i - C_2)$$

$$C = \frac{(C_1F_1 + C_2F_2)}{F}$$

$$F = F_1 + F_2$$

$$V = V_1 + V_2$$

where C_1=concentration of Li in the first reactor (mg/l); C_2=concentration of Li in the second reactor (mg/l); V_1=volume of reactor 1 (m^3); V_2=volume of reactor 2 (m^3); F_1=flow through reactor 1 (m^3/h); F_2=flow through reactor 2 (m^3/h).

As an example, the modelling of the lithium profile in the effluent of the buffertank is shown (Figure 3).

Formulation of the microbiological WWT model: The microorganisms that are discharged from the pilot plant to the WWT can grow, survive or die in the WWT. To incorporate this

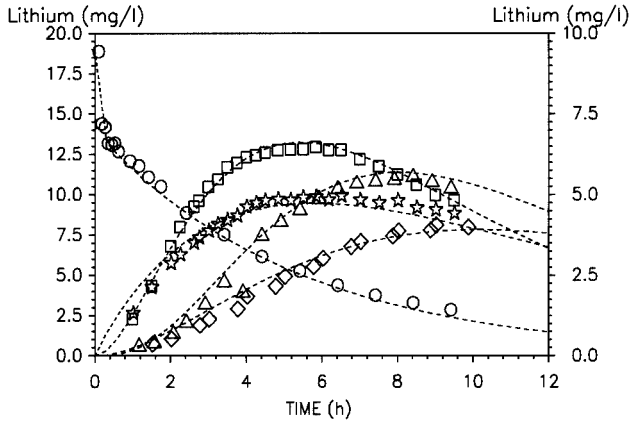

Figure 3 Modelling of lithium profiles in the WWT. (○) Buffertank. (□) Anaerobic reactor street 1. (△) Aerobic reactor street 1. (*) Anaerobic reactor street 2. (◇) Aerobic reactor street 2.

behaviour, the hydrodynamic equations are extended with an extra term for changes in survival:

$$\frac{dC_m}{dt} = -k_d C_m$$

where k_d=specific death rate (h^{-1}). The concentration C_m in the differential equations is number of cfu per millilitre (cfu/ml). k_d is determined by parameter estimation; a positive k_d indicates cell death, a negative k_d growing cells; if k_d=0, the organisms survive.

Hydrodynamic observations: Influent of the buffertank Observations start at the influent of the buffertank. Figure 4 indicates that the organisms and the lithium reached the buffertank at approximately 0.5 h after drainage from the pilot plant. The total amount of lithium and microorganisms entered the buffertank within approximately 12 min. The model does not take into account the "region" between the pilot plant fermentor and the WWT buffertank because the flows and thus the residence times within the sewer system are highly variable. Consequently, the model of the WWT proper will start at the buffertank with a pulse of lithium or organisms and the time scale is changed to hours after entering the buffertank (=hours after drainage -0.5).

Buffertank The concentration profile in the effluent of the buffertank appears to be described best by two parallel CSTRs. One vessel is calculated to be very small (0.01% of the total volume) with a relatively high flow (0.6% of the total). This can be considered a short circuit in the reactor because the residence time is approximately 60 times less than in the other vessel.

WWT In a similar fashion, the appropriate model for the other reactor was determined. For all reactors, the lithium concentration profiles fit with the model for a single CSTR. In Figure 3, it can be seen that this hydrodynamic model describes the lithium concentration profiles very well. This model is taken as the basis for the description of the microorganism concentration profiles.

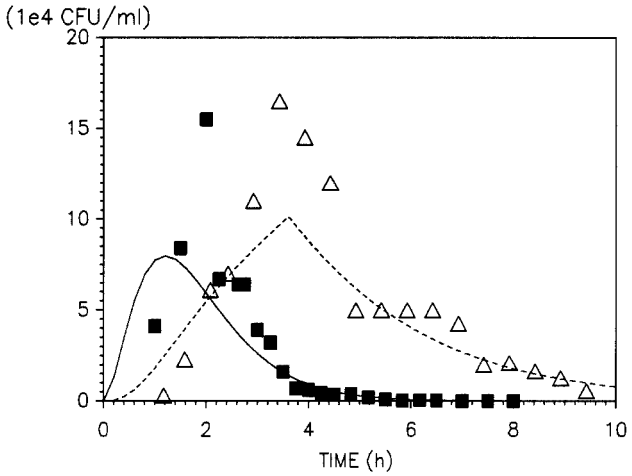

Figure 5 *K. lactis* concentration profile in the anaerobic (■) and aerobic (△) reactor of street 1. Units are in 10^4 cfu/ml.

Microbiological observations: B. subtilis Fitting the observed concentration profiles for *B. subtilis* in the buffertank and anaerobic reactors (not shown) with the model results in a k_d value (death rate) of 3.8 h^{-1}. For the aerobic reactors, the concentration profiles predict a specific death rate of at least 2.1 h^{-1}.

K. lactis In the *K. lactis* experiment (Figures 4–6), the concentration profiles match the model for each of the reactors well if the k_d (death rate) in the buffertank and the anaerobic reactors is about 1–1.4 h^{-1}. The concentration profile in the aerobic reactors appears to reflect survival at approximately the same cfu level.

Containment levels From the models applied to the WWT and taking into account the measured death rates, the number of microorganisms that pass through the complete system and actually might reach the environment can be calculated. According to these

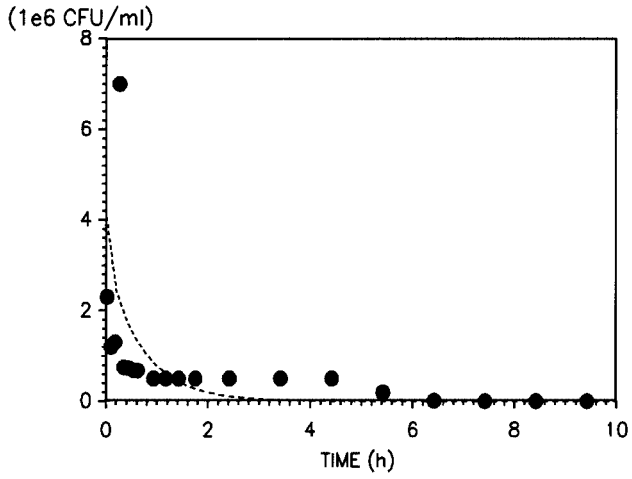

Figure 4 *K. lactis* concentration profile in the buffer tank. Units are in 10^6 cfu/ml.

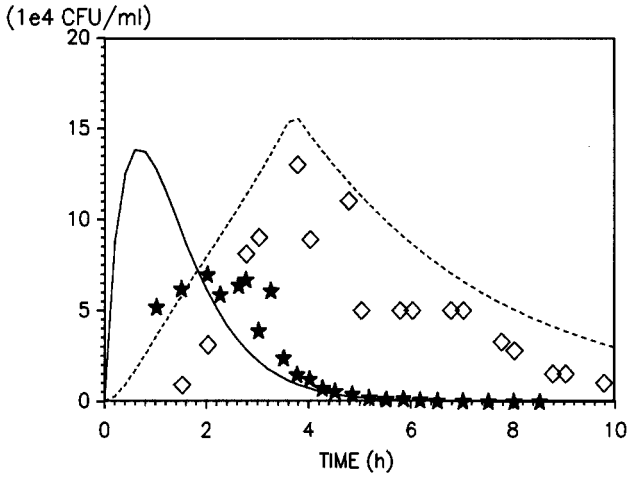

Figure 6 *K. lactis* concentration profile in the anaerobic (*) and aerobic (◇) reactor of street 2. Units are in 10^4 cfu/ml.

calculations, approximately 10^{12} cfu of *B. subtilis* and 10^{14} cfu of *K. lactis* will in fact pass the WWT. These numbers are a reduction by a factor of 10^4 and 10^3, respectively, of the microorganisms drained from the pilot plant to the WWT.

Discussion

Based on the data of a tracer compound and the recovery of living microorganisms, combined with a model describing the dominant hydrodynamic effects, the containment level of the WWT system of the Delft production site of DSM was established.

It was concluded that in case of an accidental discharge with 10 m^3 of *B. subtilis* fermentation broth, only the number of organisms equivalent to 1 l of broth would reach the final reactor (settler) of the system. For *K. lactis*, a volume equivalent to 10 l is expected in the settler.

Taking into account the hydrodynamics of the system, the reduction of viable *B. subtilis* and *K. lactis* cells in the WWT is described quite well by the microbiological model. The observed death rates reflect trends seen in independently conducted microcosm experiments in water from the WWT. Although a quantitative comparison between the two types of analysis is not possible, the microcosm experiments indicated that the viability of *B. subtilis* decreases very rapidly in the wastewater environment, whereas *K. lactis* cells appear more robust. This latter observation is in agreement with Fujimura *et al* [6] who have described similar results with the yeast *Saccharomyces cerevisiae*.

In this study, the use of lithium as a tracer was of paramount importance to determine the correct hydrodynamic model for the different reactors in the WWT, resulting in a description of residence time, distribution and mixing.

The model for the buffertank indicates that a short circuit is present in this vessel. This is based on the observation that the concentration profile of lithium in this vessel is best described by two parallel ideally mixed vessels of which one is small with a very short residence time. Perhaps the performance and containment function of the WWT can be improved by solving the short circuit in this reactor.

The settler, the final vessel of the WWT, was not included in the model because the residence time (of the sediment) in this vessel is several days. However, because of the anaerobic conditions in this reactor, and the observed survival rates of the microorganisms under anaerobic conditions, it is expected that the amount of organisms that actually reach the environment will be even smaller.

Unlike many other published survival studies, e.g., on recombinant *E. coli* K12 strains [11,12], the experiments in this paper were performed with non-recDNA strains but having the properties of actual rec-DNA strains used in large-scale production. Overall, it is concluded that the WWT can be considered an extension of the containment of microorganisms in fermentation production plants. In the event of a large accidental discharge, a reduction to a laboratory-scale volume is reached in the settler. In general, the results are in agreement with studies in laboratory set-ups of differently designed wastewater systems [1,5].

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