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Use of continuous-flow UV-induced mutation technique to enhance chlorinated organic biodegradation

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SUMMARY

In this study, a continuous-flow UV-induced mutation (CUM) device and the CUM device coupled to a selector (CUMS) reactor were fabricated and tested for their ability to enhance the probability of obtaining populations capable of chlorinated organic biodegradation. A mixed culture of bacteria were used as the starting strain for both the CUM and CUMS processes. Populations were obtained from the CUM and CUMS systems capable of 4-chlorobenzoic acid, 2,4-dichlorobenzoic acid and chlorendic acid biodegradation. Non-UV irradiated population served as controls for the experiments and did not demonstrate chlorinated organic biodegradation over the test duration.

INTRODUCTION

The period required for a capable enzyme system to evolve typically is called the acclimation or induction period. Mutation is an inherent process operative in the acclimation period [2]. Natural mutation processes can be accelerated significantly by induced mutation and bacterial strains capable of improving biological degradation to controlling filamentous growth have been studied by Spraker [4], Thibault et al. [6], Zitrides [9], and Straley [5].

The purpose of this paper is to present the findings of an experimental study conducted to assess whether a continuous-flow UV mutation scheme, described previously by Kai and Weber [3], is capable of shortening acclimation times required for chlorinated organic biodegradation. 4-Chlorobenzoic acid (4-CBA), 2,4-dichlorobenzoic acid (2,4-DCBA), and chlorendic (HET) acid (1,4,5,6,7,7-hexachlorobicyclo[2,2,1]-hept-5-ene-2,3-dicarboxylic acid; CAS Number 115-28-6) were used as target chemicals. Those compounds were chosen because chlorinated benzoic acids have been used as model compounds to study chlorinated organic biodegradation and chlorendic acid has been shown to be highly resistant to microbial attack [7,8].

The experimental investigation was conducted in two phases. In a previous study [3], the continuous-flow UV mutation (CUM) system used in this research was shown to be an effective method for producing streptomycin resistant mutants. To demonstrate the utility of this mutation device for enhancing chlorinated organic biodegradation, shaker flask biodegradation studies were conducted in Phase I using inoculums produced with the CUM device. In the second phase, the continuous-flow UV mutation system was coupled to a reactor. This coupled system (CUMS) was examined for its potential in establishing a population capable of 4-CBA biodegradation.

MATERIALS AND METHODS

The experimental systems used in this investigation were housed in the Environmental Engineering Research Laboratory of the State University of New York at Buffalo. The equipment and procedures associated with system operation and testing are delineated in this section.

UV mutation devices

CUM system. The CUM device used in Phase I of this investigation is illustrated in Fig. 1. The ultraviolet lamp (Spectronic Models 11SC-1 or 11SC-2) was housed in the tubular mutation chamber placed between the cultivation and stabilization reactor. The average lamp intensities were 4500 $\mu W/cm^2$ and 2000 $\mu W/cm^2$ of 254 nm radiation for Model 11SC-1 and 11SC-2 at 2 cm, respectively. The cultivation reactor (R-1), mutation chamber and stabili-

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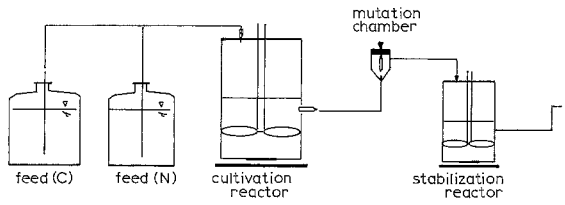

Fig. 1. Continuous-flow UV mutation (CUM) system.

zation reactor (R-2) had the operating volumes of 1420 ml, 5 ml and 1230 ml, respectively. All reactors were constructed of plexiglass. Positive displacement pumps were used to achieve the delivery of the flows between the reactors and the mutation chamber through vinyl tubing. Humidified air was delivered to each reactor to provide the necessary oxygen for bacterial culture growth. Reactor mixing was accomplished by magnetic stirrers in the cultivation reactor and stabilization reactor and with humidified air in mutation chamber. Reactor off-gases were vented to an exhaust fumehood. All bioreactors and ancillary equipment used in the experimental investigation were housed in a dimmed light environment with a yellow lamp serving as the main light source. The ambient room temperature was maintained between 20–22 °C.

CUMS system. The CUMS system was comprised of the CUM apparatus operated in series with a selector the reactor (R-3) as shown in Fig. 2. Although not part of the CUMS system, a fourth reactor (R-4) was coupled to system to serve as a control. Reactor R-4 received non-UV-irradiated culture from R-1. The target compound

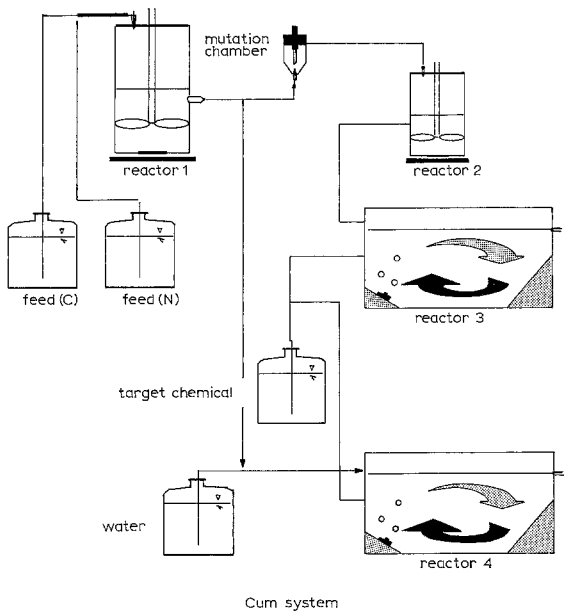

Fig. 2. Continuous-flow UV-induced mutation and biodegrading-population selection system.

was delivered by positive displacement pump to reactors R-3 and R-4. Reactors R-3 and R-4 were constructed of plexiglass and had operating volumes of 9550 ml. Mixing of both reactors was achieved by delivery of humidified air.

Methods

Operation of CUM. The CUM system was used to produce mutants for shaker flask investigations. The microbial culture for mutation was cultivated in the starter strain cultivation reactor (R-1) as shown in Fig. 1. After quasi-steady state in R-1 of the CUM device was achieved, the following traits were borne by the cultivated population: (1) multiple strains, (2) exponential phase growth, (3) viable cell count of approximately 10^8 colony forming units (cfu)/ml of culture fluid, and (4) an aqueous phase total organic carbon (TOC) concentration of around 200 mg/l. Multiple strains in the reactor were desired because the evolution of a new pathway for biodegradation of complex organics is greatly enhanced by the greater genetic pool represented by multiple strains. Exponential growth phase conditions were desired because fast growing bacteria are more susceptible to mutation than under other growth conditions. A viable cell count of 10^8 cfu/ml was favored to ensure adequate amount of bacteria for the mutation experiment. The residual aqueous phase TOC concentration was needed for mutant stabilization in R-2.

The cultural suspension in R-1 was filtered with a double layer of wiper paper (Kimberly-Clark) every day, the reactor was washed, to remove the flocculent growth. The tubings and fittings connected from the feed to R-1 were replaced with the clean ones periodically to prevent significant bacterial growth in the feed lines. Other tubing, fittings and flow rates for the reactors were checked and adjusted periodically.

Parameters pertinent to the volume, feed rate, and detention time for CUM operation are summarized in Table 1. The feed solution composition for R-1 was identical to that used in earlier studies by Kai and Weber [3].

Operation of CUMS. The CUMS system was constructed and operated for continuous bacterial mutation and selection. Mixed culture from R-1 was irradiated in the UV chamber under complete mix conditions. Bacteria from the mutation chamber were recovered in R-2. Substrate not utilized in R-1 was removed in R-2 during the stabilization process. Biodegradation efficacy of the mutants obtained from R-2 was tested in R-3 which received the target compound.

As stated earlier, R-4 was established to serve as a control for the experimental system. For these studies, 4-CBA was delivered to R-3 and R-4 at such a rate as to maintain an influent concentration of 100 mg/l. R-4 was

TABLE 1

Reactor parameters in CUM and CUMS devices

Reactors	Volume ml	Flow rate ml/min	Detention time h
CUM device			
R-1	1420	9	2.63
R-2	1230	5.17	3.97
UV chamber	5	5.17	0.016
CUMS device			
R-1	1420	9	2.63
R-2	1230	5.17	3.97
R-3	9550	5.74	27.7
R-4	9550	5.74	27.7
UV chamber	5	5.17	0.016

fed a comparable amount of bacteria as R-3 using R-1 as the inoculating source. Because the concentration of bacteria in R-1 was greater than in R-2, the flow rate from R-1 to R-4 was reduced so that the flow of viable bacteria into R-4 would be similar to that of R-3. To maintain comparable hydraulic detention times in R-3 and R-4, the flow rate from R-1 to R-4 was augmented with distilled water. Flow rates and corresponding hydraulic detention times for R-3 and R-4 are shown in Table 1.

Starter culture. Activated sludge from three sources was utilized as the inoculum for R-1 in different experimental phases. In Phase I, the starter culture for testing the CUM system was obtained from a laboratory operating reactor fed 2,4-dichlorophenoxyacetate. For testing the CUMS system, starter cultures from the activated sludge basins at the Lackawanna, NY Publicly Owned Treatment Works (POTW) and Amherst, NY POTW were employed.

Operation of shaker studies. Shaker flask studies were conducted to demonstrate the utility of the CUM device in acquiring the strain with enhanced chlorinated organic biodegradation. Inocula of 100 ml for the shaker flask studies were taken from R-2, which contained a bacterial concentration of approximately 10^8 cfu/ml. In addition to the inoculum, each flask contained 100 ml of basic medium supplemented with target chlorinated organic. Initial concentrations of 4-CBA, 2,4-DCBA, and chlorendic acid were 200 mg/l, 200 mg/l and 210 mg/l, respectively. After inoculation, samples were withdrawn from the flasks periodically as a function of time for sample analysis. For comparison, controls containing an equal amount of non-irradiated inoculum from R-1 were used.

Analysis. Target compounds used in this experiment were dissolved as a stock solution of 1 g/l in a phosphate buffer, pH 7.2. Parameters commonly used for characterization of wastewater were measured in accordance with the standard methods [1]. These parameters included: pH, Section 423 (Accumet pH Meter 910), Total Organic Carbon, Section 505 B (Dohrmann Carbon Analyzer, DC-80), Chemical Oxygen Demand, Section 508 B.

TABLE 2

Composition of basic medium

Constituent	Amount
$(NH_4)_2SO_4$	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaSO_4 \cdot 2H_2O$	0.01 g
Conc. H_2SO_4	5×10^{-3} ml
$Fe_2(SO_4)_2 \cdot nH_2O$	4.3×10^{-3} g
$MnCl_2 \cdot 4H_2O$	1×10^{-3} g
$ZnSO_4$	4.05×10^{-4} g
$CuSO_4 \cdot 5H_2O$	4.05×10^{-4} g
$CoCl_2 \cdot 6H_2O$	1.2×10^{-4} g
Na_2MoO_4	1.05×10^{-4} g
H_3BO_4	4×10^{-5} g
KH_2PO_4/Na_2HPO_4 Buffer (pH 7.2)	0.04 M
EDTA (disodium salt)	5×10^{-6} M
Distilled water to 1 l	

Two chloride analyzer models were used in this work. An Orion Research Expandable Ion Analyzer EA 940 was used in Phase I, while an Orion Ion Analyzer 407A was used in Phase II.

UV absorbance was obtained with Bausch and Lomb Spectronic 2000 spectrophotometer. Wavelengths of 250, 240 and 216 nm were chosen for measuring 4-CBA, 2,4-DCBA, and chlorendic acid, respectively. In this investigation, UV absorbance is expressed as 'relative UV absorbance' which was calculated as a ratio between initial and time dependent UV absorbance. Under these conditions, if the UV absorbance decreased the relative UV absorbance values were less than 1. Values for relative UV absorbance were greater than 1 when UV absorbance increased with time.

A high performance liquid chromatography (Shimadzu, with a SPD-6AV UV-VIS Spectrophotometric Detector and a Beckman Ultrasphere ODS 5U column) was used in Phase 2 to measure 4-CBA. Chlorendic acid was measured with a Gas Chromatograph (Hewlett-Packard 5840) with a flame ionization detector and a Hewlett-Packard 530U column.

RESULTS AND DISCUSSION

Phase I

Shaker flask biodegradation tests were used to assess the utility of the continuous-flow mutation (CUM) device

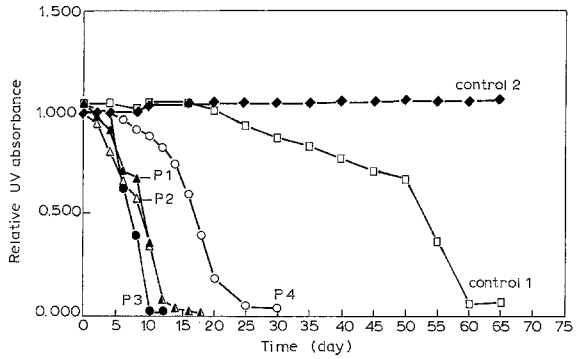

Fig. 3. Biodegradation of 4-CBA with chlorinated organic degrading starter culture.

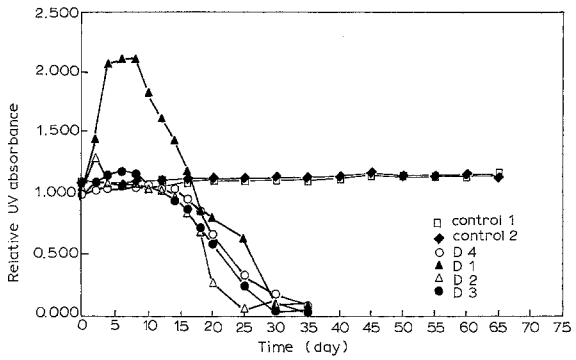

Fig. 4. Biodegradation of 2,4-DCBA with chlorinated organic degrading starter culture.

for enhancement of 4-CBA, 2,4-DCBA and chlorendic acid biodegradation. Non-irradiated populations taken from R-1 were used as controls for the populations obtained from R-2.

4-CBA. The 4-CBA biodegradation test concentration profiles, as measured by UV absorbance, for the four mutant replicates, designated as P1, P2, P3 and P4, and two control (non-mutated populations) replicates, designated as Control 1 and Control 2, are presented in Fig. 3. As shown, mutated cultures were able to affect biodegradation of 4-CBA in a significantly shorter period of time as compared to non-mutated cultures. In general, there was good consistency between the four replicates and 4-CBA biodegradation was completed in less than 25 days for each of the mutant replicates. There was, however, some inconsistency in the control data obtained for this experiment. For one control flask (Control 1), the non-mutated population initiated 4-CBA biodegradation at about 25 days. A slower rate of biodegradation was observed for this control relative to the rate observed for the mutated populations. No biodegradation of 4-CBA was observed in the second control (Control 2) during the 65-day test period.

TABLE 3

COD and chloride release measurements for 4-CBA biodegradation studies

Sample	COD, mg/l		Chloride, mg/l	
	Initial	Final	Initial	Final
P1	302	25	0.35	43.4
P2	302	20	0.35	43.6
P3	302	31	0.35	42.5
P4	302	37	0.35	40.9
Control 1	286	28	0.35	43.2
Control 2	286	288	0.35	0.44

As additional evidence of the enhanced 4-CBA biodegradation, initial and final shaker flask COD and chloride concentration measurements were made for each population as shown in Table 3. Those populations which demonstrated biodegradation of 4-CBA based on the relative UV absorbance data also affected COD removal and chloride release.

2,4-DCBA. The 2,4-DCBA biodegradation concentration profiles obtained in shaker flask experiments for the mutant replicates, designated as D1, D2, D3, and D4, and two control (non-mutated populations) replicates, designated as Control 1 and Control 2, are presented in Fig. 4. As shown, mutated cultures were able to effect biodegradation of 2,4-DCBA in a significantly shorter period of time as compared to non-mutated cultures. There was good consistency between the four mutant replicates and the two control replicates. It is interesting to note that during the degradation process of replicate the UV absorbance values increased over initial values for some of the replicates. This suggests that intermediates may have been formed by the UV irradiated population which had a greater UV absorbance at 240 nm than did the parent compound 2,4-DCBA.

Chlorendic acid. Inspection of chlorendic acid biodegradation results presented in Fig. 5 reveals that the mutated cultures brought about a reaction not achieved by the non-mutated controls. Evidence of this reaction is in the form of increased relative absorbance measurements. In three of the mutated replicates, the reaction was initiated after approximately 4 days of reaction time. Increased relative absorbance was observed for the fourth mutated replicate on day 8. No increase in relative absorbance was noted for either of the controls.

To further assess the likelihood of chlorendic acid biodegradation resulting from the mutated populations, the concentrations of chlorendic acid, TOC, and chloride in two replicates were measured after 20 days of reaction time. During the 20-day test period, chlorendic acid con-

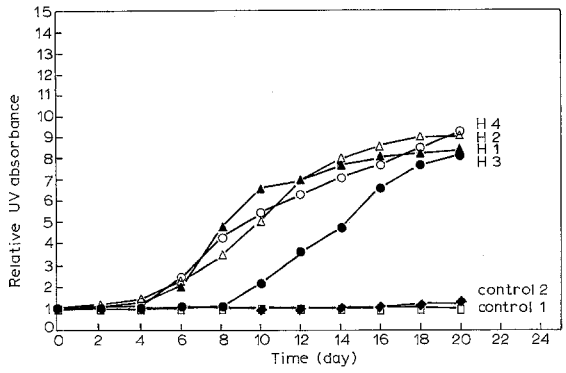

Fig. 5. Biodegradation of chlorendic acid with chlorinated organic degrading starter culture.

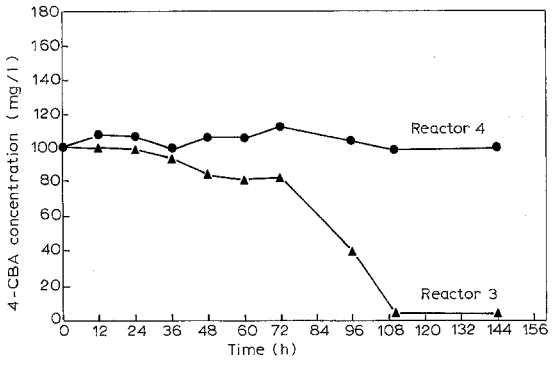

Fig. 6. Relationship between time and 4-CBA concentration change in reactors 3 and 4, with POTW-L bacterial source.

centration dropped from 210 mg/l to 166 and 145 mg/l for populations H1 and H2, respectively, which represents a 21 and 31% decrease in chlorendic acid. Total organic carbon concentration also dropped during the same period from an initial concentration of 58 mg/l to 45 and 43 mg/l for H1 and H2, respectively, which represents removal percentages of approximately 22 and 26%. Additional evidence of chlorendic acid biodegradation can be seen from the chloride release data. Chloride was released in all test flasks containing mutant cultures and averaged 7.34 mg/l Cl^-. This constitutes an approximate chloride release efficiency of 6% [(7.34/210) × 0.556)].

Given the limited data available for chlorendic acid, TOC removal and chloride release, it is difficult to determine the fate of chlorendic acid with any degree of confidence. Although the chlorendic acid and TOC removal percentages are similar, leading one to postulate that about 25% removal of parent chlorendic acid was transformed, the chloride data do not support this hypothesis. However, based on the data presented above it is clear that the mutated cultures affected some transformation of chlorendic acid while the controls did not.

Phase II

From the results of Phase I, the CUM system tested was shown to shorten the acclimation times required for obtaining populations capable of chlorinated organic biodegradation. In the case of chlorendic acid, which had been shown to be highly persistent to biodegradation in previous studies, use of the CUM system may aid in obtaining a population capable of compound biotransformation.

While the CUM system tested in Phase I was successful in facilitating the procurement of a capable population, the full potential as a continuous flow device was not tested. In Phase 2, rather than taking individual inocula from R-2 for shaker flask studies as was done in Phase I, the population from the CUM was passed through the R-3 selector which received the target compound. To test this CUMS system, a series of experiments were conducted in Phase II using 4-CBA as the target substrate.

In the first series of experiments, the CUMS system was assessed for obtaining a 4-CBA degrading population using the two different inocula. The benefits of R-3 were assessed by comparing results of the CUMS system to the control reactor R-4. After these investigations, the necessity for R-2 in the CUMS system was assessed. The results of these tests are presented here.

CUMS system testing. In the first CUMS trial, the system was tested using an inoculum from the Lackawanna, NY POTW (POTW-L) as a starter culture. During the experiment, the concentrations of 4-CBA and chloride were measured as a function of time for both the reactor receiving the mutants (R-3) and the control (R-4). These data are presented in Figs. 6 and 7 for 4-CBA and chloride, respectively. As shown in Fig. 6, after 36 h of CUMS run time, the R-3 population appeared to initiate biodegradation of 4-CBA. At the time of 4-CBA biodegradation initiation, culture fluid containing a total bacterial count of 1×10^{12} cfu had been passed through the UV chamber and subsequently screened. Over the next

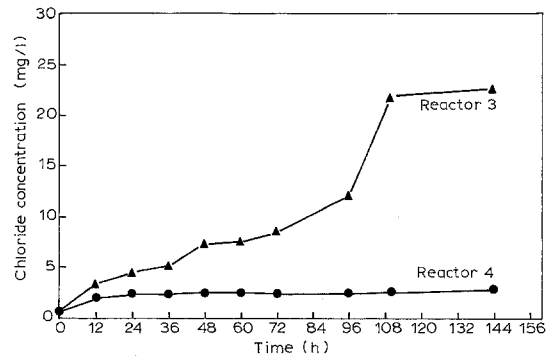

Fig. 7. Relationship between time and chloride concentration change in reactors 3 and 4, with POTW-L bacterial source.

36 h of system operation, about 20% conversion of 4-CBA was achieved by the R-3 population. After 72 h of operation, a significant increase in 4-CBA degradation was achieved, with 4-CBA levels reduced from near 100 mg/l to approximately 4 mg/l. No degradation of 4-CA was observed in R-4 during the experiment.

The two tiers of 4-CBA degradation observed in this first experiment, which may be the result of two separate mutation events, also are evident in the chloride release data. As shown in Fig. 7, chloride production in R-3 as compared to R-4, was first observed after 36 h of operation and increased significantly after 72 hours of CUMS operation. After approximately 108 h of operation, the chloride concentration in R-3 approached the stoichiometric equivalent for 100 mg/l of 4-CBA. Thus, in addition to near complete removal of 4-CBA observed in Fig. 5, dechlorination of 4-CBA was accomplished by the population in R-3. No dechlorination was observed for the non-UV-irradiated population cultured in R-4.

For comparative purposes, a second CUMS system run was conducted with a microbial inoculum from the Amherst, NY POTW (POTW-A). The chloride release data derived from this experiment are shown in Fig. 8. Based on these data, 4-CBA biodegradation was initiated by the R-3 population after about 20 h of CUMS operation while no chloride release was achieved by the control population in R-4. Initiation of 4-CBA biodegradation was achieved after approximately 6×10^{11} cfu had been passed through the UV chamber and screened in R-3. Based on stoichiometry, about 50% of the 100 mg/l 4-CBA feed solution to R-3 was dechlorinated. No 4-CBA analyses were conducted for this experiment.

Function of reactor 2. Stabilization of mutated organism after exposure to ultraviolet light was the primary function of R-2. This function was conducted in a separate reactor to prevent target compound inhibition of the unstable UV irradiated population. To test whether R-2 could be

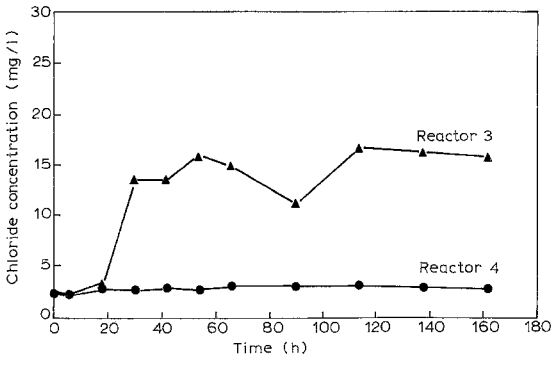

Fig. 8. Chloride concentration change in reactors R-3 and R-4 of CUMS system inoculated with POTW-A microbial population.

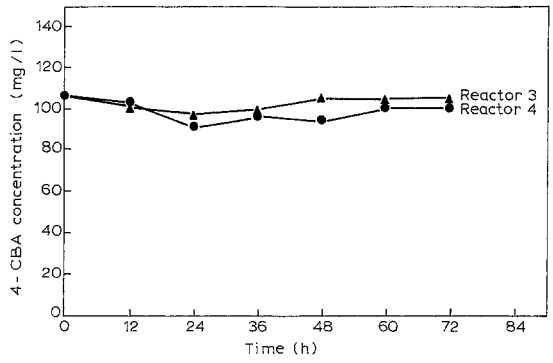

Fig. 9. 4-CBA concentration as a function of time in reactor R-3 of CUMS system at control reactor R-4 when operated without reactor R-2.

removed from the CUMS system without adverse impact on obtaining a capable organism, CUMS studies were conducted with and without R-2 in the reactor train.

During the initial part of the study, R-2 was deleted from the CUMS system. The CUMS system was run in this manner for 72 h which previously had been adequate for obtaining an organism capable of 4-CBA biodegradation. Experimental data consisting of 4-CBA concentration and chloride concentration from this initial phase are plotted in Figs. 9 and 10, respectively. From inspection of the data, there was no change in either 4-CBA or chloride concentration occurring in R-3 during the initial 72 h of operation.

After 72 h of operation, R-2 was placed back in the CUMS system, which was then operated for another 144 h. The data collected in this phase of testing were reported earlier (Figs. 6 and 7) and are shown again here with the earlier 72 h of CUMS operation without R-2. The concentration of 4-CBA and chloride in reactors R-3 and R-4 operated with and without R-2 are presented in Figs. 11 and 12, respectively. Briefly stated, when R-2 was placed back in operation, a population was obtained in

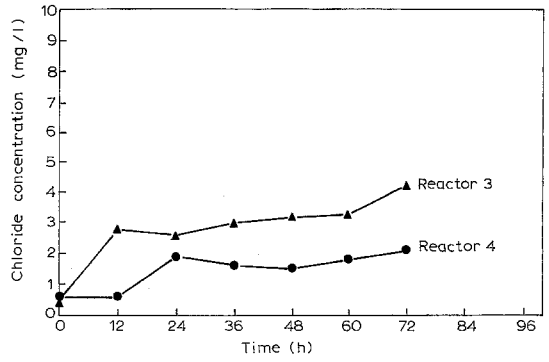

Fig. 10. Chloride concentration as a function of time in reactors R-3 and R-4.

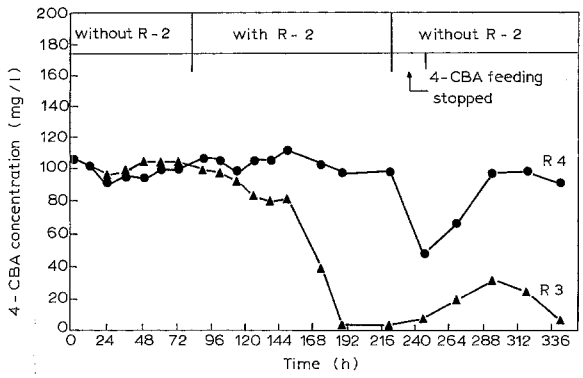

Fig. 11. 4-CBA concentration as a function of time in R-3 of CUMS system and control reactor R-4 with and without the presence of reactor R-2 in CUMS system.

R-3 that was capable of 4-CBA degradation. This fact is highlighted by a decrease in 4-CBA concentration in R-3 as shown in Fig. 11, which occurred between hours 72 and 216 with concurrent increases in chloride concentration as shown in Fig. 12. After 144 h of operation with R-2 in place, near complete biodegradation of 4-CBA was achieved in R-3 as compared to no 4-CBA removed or chloride release in the control R-4 reactor.

Once the utility of R-2 had been confirmed for obtaining a capable population, it was removed again to determine if it was necessary for maintaining a 4-CBA degrading population in R-3. It was hypothesized that R-2 would be necessary if the dilution rate of R-3 was greater than the 4-CBA degrader growth rate. Under these conditions, R-2 would be necessary as a continual reinoculum to prevent population washout. Concurrent with the second removal of R-2 from the CUMS system, 4-CBA feeding to R-3 was stopped for approximately 20 h (0.75 hydraulic resident times) to accelerate population washout.

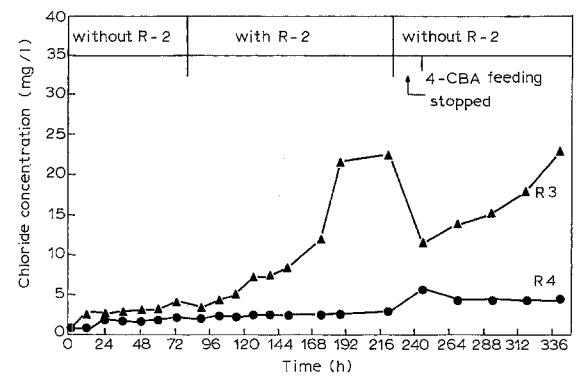

Fig. 12. Chloride concentration as a function of time in R-3 of CUMS system and control reactor R-4 with and without the presence of reactor R-2 in CUMS system.

Upon reintroduction of 4-CBA to R-3 on hour 246, the concentration of 4-CBA initially increased and then decreased after hour 288. Initial increases in 4-CBA concentration resulted from prior 4-CBA degrader population washout. Because of this population loss, the mass flux of 4-CBA to R-3 was greater than the biodegradation capacity between hours 246 and 292. At hour 292, the biodegradative capacity of the 4-CBA degraders appeared equal to the influent 4-CBA flux. With continued growth of 4-CBA degraders, the 4-CBA concentration in R-3 decreased to previous levels after 292 h. The experiment was terminated after 340 h of operation.

After R-2 was removed from the CUMS system, an initial dilution of 4-CBA occurred in R-4 from hours 216 to 246 which resulted from a stoppage in the 4-CBA feed. Once 4-CBA feeding was reintroduced, the 4-CBA in R-4 concentration increased to influent levels between hours 246 and 292. At no time during the test was 4-CBA degradation observed in R-4.

While based on the results presented here, it is hypothesized that R-2 enhances system operation; whether R-2 is vital for obtaining a capable population can not be ascertained from this preliminary study. Clearly, for the compounds studied, the CUMS system is more efficient with the R-2 than without.

In summary, populations capable of 4-CBA, 2,4-DCBA, and chlorendic acid degradation were obtained from the continuous-flow UV mutation device tested in this investigation. Near complete degradation was observed for 4-CBA and 2,4-DCBA, while partial degradation or transformation of chlorendic acid was observed.

The feasibility of the CUMS system was demonstrated in this work using 4-CBA as a model target compound. Desired mutants were produced and selected efficiently from the CUMS system. 4-CBA degrading populations were obtained after passing culture fluid containing 6×10^{11} cfu for the POTW-A inoculum and 1×10^{12} cfu for the POTW-L microbial source. A potential application of the CUMS system may be as a support facility for a wastewater biotreatment system receiving recalcitrant compounds.

The necessity of reactor R-2 in the CUMS system was assessed also. Based on the initial data collected, it appears that the R-2 is important to the successful operation of the CUMS. The maintenance of a selective pressure in R-3 was found to be important in maintaining a high concentration of 4-CBA degraders.

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