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Streptomycin-resistant mutant production in a continuous-flow UV mutation device

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SUMMARY

A continuous-flow UV-induced mutation device which incorporates starting strain cultivation, UV irradiation and mutant reproduction was conceptualized and tested in this study using streptomycin resistance as an indicator of mutant production. For the experimental conditions employed and populations used, the mutation frequency for streptomycin resistance ranged from 10^{-4} to 10^{-5} cfu/ml. These mutation frequencies are comparable with conventional batch UV mutation methods and represent a gain of 3 orders of magnitude over the spontaneous mutation frequency.

INTRODUCTION

Induced mutation has been applied in industrial microbiology for many years. To date, the most dramatic practical success of mutagenesis has been in the pharmaceutical industry with specific interest on antibiotic production. For example, the initial penicillin isolated from nature yielded only about 1–10 μg/ml of culture fluid of penicillin. By 1982, the yield obtained under laboratory conditions had been increased to approximately 20 000 μg/ml [8] as a result of strain mutation and selection. Accordingly, mutation is often suggested as a feasible method for obtaining new and/or improved strains [1,11,12]. Ultraviolet light (UV) induced mutagenicity appears to be especially pertinent to strain improvement. Ultraviolet light produces various mutagenic effects and is considered a priority mutagen [5,9]. Thoma [11] reported some selected examples of induced mutation in a variety of microorganisms used for production of antibiotics of enzymes. Twenty-eight chemical mutagens, UV and γ irradiation were used over a 15-year period. Among the 14 produced strains with superior productivity after the mutagenic treatment, 10 were obtained by UV irradiation treatment alone or UV plus chemical mutagen treatment.

From a statistical standpoint, as the number of mutation events increase, the probability of an individual organism acquiring the desired trait also increases. Thus, it is desirable to develop processes which are capable of maximizing desirable mutant production. Today, most conventional UV-induced mutation work has been done using batch processes [2,4]. However, from the standpoint of satisfying of maximizing mutant production, it is hypothesized that a continuous flow mutation process is more desirable. The purpose of this research was to conceptualize and develop a continuous-flow UV mutation technique for evolving mutant organisms capable of degrading recalcitrant chemicals. The focus of this paper is the preliminary testing of this device using streptomycin resistance as an indicator of mutant formation.

CONTINUOUS FLOW MUTANT PRODUCTION DEVICE

Based on the calculations presented by Kai [7], a large number of mutants is necessary to statistically account for all possible mutations induced by more than one dimer within one gene. To satisfy this requirement, a continuous flow UV mutation device was conceptualized. This device, consisting of a starter strain cultivation reactor, UV light mutation chamber, mutant stabilization reactor, and selector reactor is shown in Fig. 1.

Briefly stated, the purpose of the cultivation reactor is to provide a culture under exponential growth to increase the probability for DNA damage from UV irradiation in the UV mutation chamber. In addition, the culture flowing into the UV mutation chamber should be well dispersed to minimize shielding. Once irradiated, phenotype stabili-

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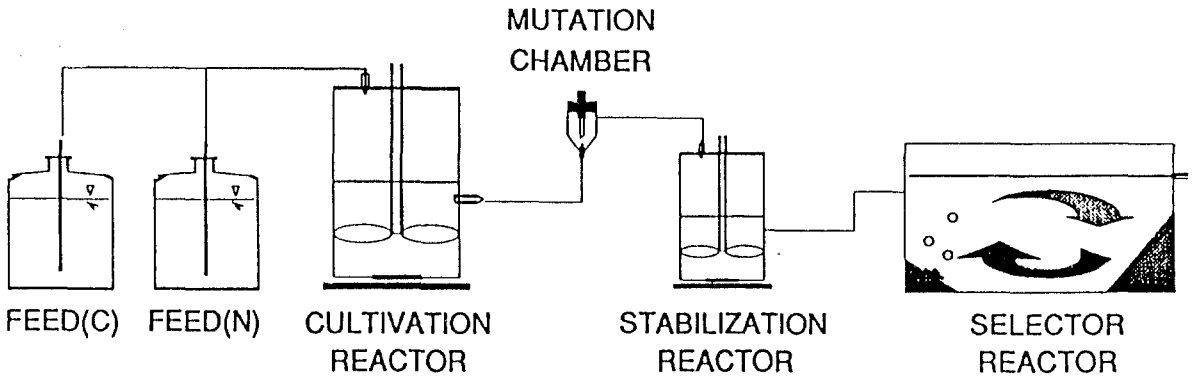

Fig. 1. Continuous-flow UV-induced mutation and biodegrading-population selection device.

zation is achieved in the stabilization reactor. Residual soluble feed stock not consumed in the cultivation reactor is used for this purpose or additional organic carbon can be added directly to the reactor. Selection of desired mutants is achieved in the selector reactor by adding the target substrate.

A continuous flow configuration for the process was selected because: (1) continuous flow reactors are well suited for handling the large volumetric flow requirements deemed necessary for evolving a xenobiotic degrading organism; (2) strict process control can be maintained; (3) a consistent population at a specific growth phase can be obtained from continuous flow processes operated under steady-state conditions; and (4) the operation of the selector reactor under continuous flow conditions provides the most effective vehicle for screening the wide array of mutants produced by the device.

MATERIALS AND METHODS

To study the mutant production capabilities of the device, study was limited to the cultivation reactor and mutation chamber. Starter cultures were mutated and directly tested for their resistance to the antibiotic streptomycin under a number of device variables.

UV mutation device. The cultivation reactor was constructed of plexiglass with an operating volume of 1420 ml. Contents in the cultivation reactor were completely mixed by magnetic stirrer. Aeration was achieved with humidified air. The tubular mutation reactor had an internal diameter of 1 cm and a total operating volume of 5 ml. Delivery of flows between the cultivation reactor and the UV chamber was accomplished with the use of positive displacement pumps through vinyl tubing. Reactor off-gases were vented to an exhaust fume-hood. After attainment of steady-state operation, a bacterial concentration about 10^8 cfu/ml was achieved in the cultivation reactor. These bacteria were highly dispersed, which facilitated optimal UV light penetration in the UV irradiation chamber.

The ultraviolet lamps were housed in the tubular mutation chamber. Clearence between the UV lamps and the mutation chamber averaged 0.25 cm. Two models of UV lamps from Spectronics Corporation, Model 11SC-1 and Model 11SC-2, were used. The average lamp intensities were 4500 μW/cm^2 and 2000 μW/cm^2 of 254 nm radiation for Models 11SC-1 and 11SC-2 at 2 cm, respectively. The irradiation dosage administered by each lamp was dictated by UV chamber hydraulic detention time.

Culture media employed. The composition of feed solution for the cultivation reactor was a basic medium (Table 1) supplemented with 1.25 g/l fructose.

TABLE 1

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}
$ZnSO_4$	4.05×10^{-4} g
$CuSO_4 \cdot 5H_2O$	4.05×10^{-4}
$CoCl_2 \cdot 6H_2O$	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	

Initial inoculum for cultivation reactor. Various bacterial strains were isolated from activated sludge populations and grown as pure cultures on a Soybean-Casein digest agar (BBL) plate. Strains showing growth on a streptomycin assay agar (Antibiotic Medium 5, Difco) plate with 250 mg/l concentration of streptomycin (Sigma) were not used.

Measurement of streptomycin resistance. Resistance to streptomycin (sulfate) was used to measure mutation rate and frequency. Rubin [10] standardized the procedure with the 'multiple agar layer technique'. In this work, Rubin's multiple agar layer technique was used, with some minor modifications. Aliquots of irradiated culture were pipetted and spread to the streptomycin assay agar plate. A thin soft agar (7 g/l Difco Antibiotic Medium 5, pH 8) was plated on the plate 1 h later to cover the culture. Before pouring, the temperature of the agar was kept at 42–45 °C. After a 5-h period of incubation at 21 °C, the plate was covered with a third agar layer containing dissolved streptomycin. The Petri dishes were then refrigerated overnight at 4 °C and finally incubated at 21 °C again for 5 days. The visible colonies in the second agar layer were counted and taken to be streptomycin resistant mutants. At least three concentrations of bacterial cultures and three plates for each concentration were made.

RESULTS AND DISCUSSION

To study the prototype continuous flow mutation device, studies were conducted to assess survivor number, mutant production, and mutation frequency as a function of UV irradiation dose. In Phase I, experiments were conducted at an average UV intensity of 4500 $\mu W/cm^2$ at 2 cm and a bacterial inoculum from a laboratory chemostat fed 2,4-dichlorophenoxyacetic acid. Phase II experiments were conducted at a UV intensity of 2000 $\mu W/cm^2$ and a bacterial inoculum obtained from the Amherst, New York POTW. The results derived from these studies are presented in this section.

Phase I

The efficacy of UV irradiation in the mutation device was investigated using bacterial concentrations ranging from 5.7×10^6 cfu/ml to 2.5×10^8 cfu/ml. These concentrations were achieved by diluting cultivation reactor effluent. As shown in Table 2, the surviving bacterial number decreased as function of increasing irradiation time for each initial bacterial concentration studied. Further illumination of this trend is illustrated in Fig. 2. Plotted on semi-log scale, it would appear that the decrease in survival can be modelled using a first order rate expression.

First order decay rates for each bacterial concentration were determined for the purpose of assessing irradiation efficacy. Decay coefficients, as a function of

TABLE 2

Effect of irradiation time and bacterial survival and mutant production

Bacterial concentration (cfu/ml)	Irradiation time (s)	Surviving bacteria (cfu/ml)	Percent survivors	Number of mutants (cfu/ml)
2.5×10^8	0	2.47×10^8		
	5	1.20×10^8	48.6	3485
	10	4.17×10^7	16.9	5003
	20	3.39×10^7	13.7	4775
	30	1.19×10^7	4.8	2713
	50	7.24×10^5	0.3	150
3.4×10^7	0	3.40×10^7		
	5	2.09×10^7	61.5	1194
	10	1.28×10^7	37.6	1829
	20	7.30×10^6	21.5	1668
	30	2.00×10^6	5.9	456
	50	3.80×10^5	1.1	84
5.7×10^6	0	5.68×10^6		
	5	3.31×10^6	58.3	208
	10	2.04×10^6	35.9	326
	20	1.59×10^6	28.0	362
	30	1.20×10^6	21.1	274
	50	1.66×10^5	2.9	72

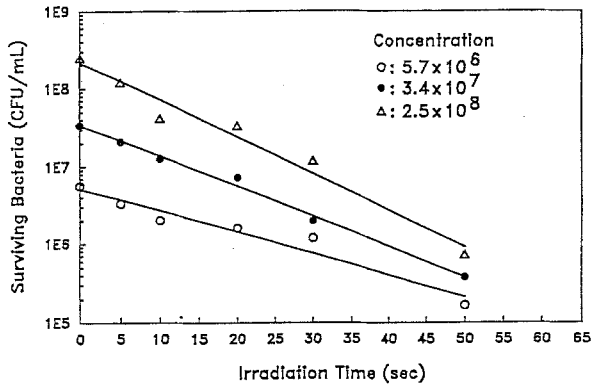

Fig. 2. Surviving bacterial numbers as a function of bacterial concentration and irradiation dosage.

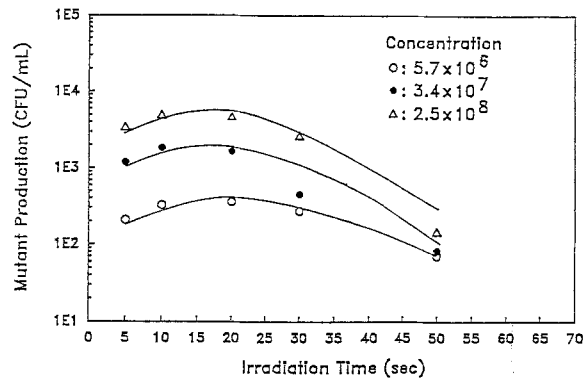

Fig. 4. Streptomycin resistant mutants produced as a function of bacterial concentration and irradiation dosage.

initial bacterial concentration, were obtained from the slopes of $-\ln (C/C_0)$ vs. time presented in Fig. 3. Decay coefficients obtained in this manner were 0.069, 0.092, and 0.111 s^{-1} for initial bacterial concentrations of 5.7×10^6, 3.4×10^7, and 2.5×10^8 cfu/ml, respectively. Increases in decay coefficient as a function of increasing bacterial concentration suggests that the irradiation efficiency was greater at a higher culture concentration. Typically, the opposite trend is observed [3]. It is possible that the higher survival ratios measured at the lower bacterial concentration were caused by competing UV adsorption by constituents in culture media used in diluting to the lower bacteria concentration.

In Fig. 4, the relationship between mutant production and UV exposure is illustrated for each initial bacterial concentration. The streptomycin mutant production increased with initial increases in irradiation time and arrived at its maximum after approximately 20 s of UV exposure which corresponded to a relative irradiation dosage of 14–28% survival. Mutant production decreased after about 20 s exposure period.

Mutation frequencies (no. of mutants/no. of survivors), calculated as a function of bacterial concentration and irradiation time, are shown in Fig. 5. The relationship between induced mutation frequency and UV exposure obtained in these experiments are comparable to those reported in the literature by Drake and Blatz [3], and Jagger [6]. The mutation frequency increased with the increases in irradiation time for the first 20 min. After approximately 20–25 s of UV exposure the mutation frequency became asymptomatic at approximately 3.5×10^{-4}. This value is approximately 3 orders of magnitude greater than the spontaneous mutation frequency for streptomycin resistance which was measured to be 1.1×10^{-7} for a bacterial culture concentration of 2.1×10^8 cfu/ml. Bacterial concentration did not appear to play a significant role in determining mutation frequency.

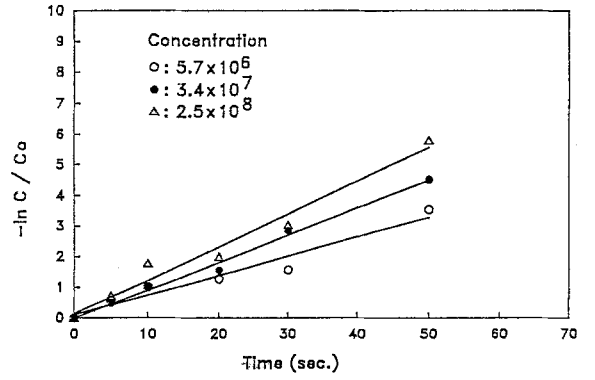

Fig. 3. Three bacterial decay curves in the mutation system with model 11SC-1 UV lamp.

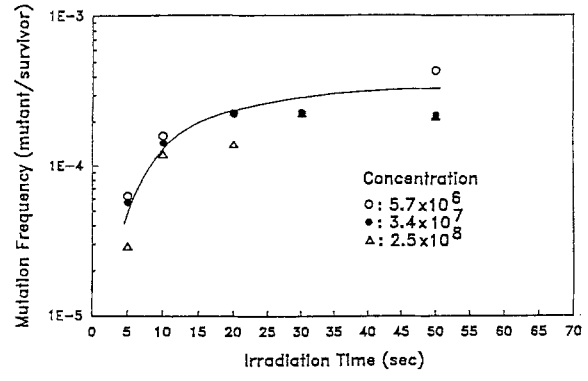

Fig. 5. Mutation frequency as a function of irradiation time for mutation system with model 11SC-1 UV lamp.

Phase II

Survivor ratios calculated from Phase II experiments conducted with a UV lamp intensity of 2000 $\mu W/cm^2$ as a function of irradiation time are presented in Fig. 6. For comparison, data obtained from the Phase I investigation at 10^8 cfu/ml concentration also are presented. As shown in Fig. 6, both curves decreased as irradiation time increased. The slopes for the two decay curves are similar and were calculated to be $4.0 \times 10^{-3}\ s^{-1}$ for the 4500 $\mu W/cm^2$ lamp and $4.3 \times 10^{-3}\ s^{-1}$ for the 2000 $\mu W/cm^2$ lamp, respectively.

Although the two slopes observed for survivor rates are similar, the survivor rate at a given UV dose was not. For the lower intensity lamp used in Phase II, population survival was nearly 100% under short irradiation times. After approximately 15–20 s of irradiation with the 4500 $\mu W/cm^2$ lamp, the survivor ratio decreased with a rate similar to that measured for the 2000 $\mu W/cm^2$ lamp. Thus, at any given UV exposure period, the lower intensity lamp yielded a higher survivor ratio. The nonlinear rate of deactivation found for Phase II experiment is typical of UV as a bacterial disinfectant, while the shape of the curve from the Phase I data is more typical of conventional UV mutation investigations. Dissimilarity in the two responses may be a result of bacterial flocculation. At lower UV lamp dosages, the amount of irradiation may have been insufficient to penetrate the bacterial suspension, resulting in a greater number of viable organisms after UV irradiation.

Mutation frequencies as a function of irradiation time are presented in Fig. 7 for Phase II. Mutation frequency was found to increase with increasing irradiation time until an apparent maximum was reached at approximately 60 s when using a UV lamp with 4500 $\mu W/cm^2$. For comparative purposes, the data obtained with a UV lamp having 2000 $\mu W/cm^2$ during Phase I also are presented in Fig. 7. A maximum mutation frequency of

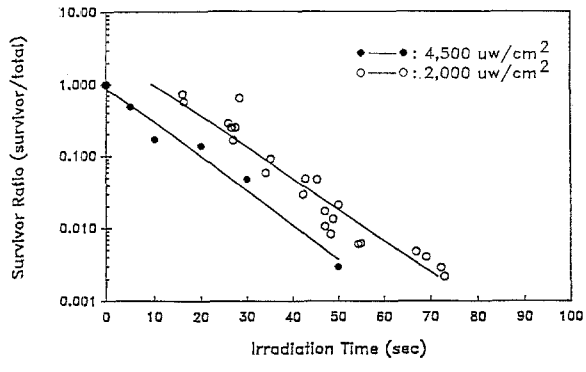

Fig. 6. Comparison of survivor ratios with model 11SC-1/2 UV lamps.

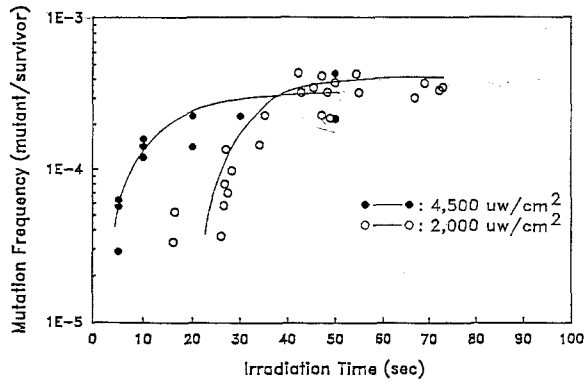

Fig. 7. Comparison of mutation frequencies with model 11SC-1/2 lamps.

4.0×10^{-4} was obtained for a higher intensity lamp as compared to 3.5×10^{-4} under lower lamp intensity. Comparing these to the spontaneous mutation frequency of 1.1×10^{-7}, the mutation frequency was increased by factors of 3600 and 3200 with the 4500 $\mu W/cm^2$ and 2000 $\mu W/cm^2$ lamps, respectively. Based on the data collected during these preliminary experiments, it is difficult to discern whether the differences between the two lamp sources are significant.

To sum up, in this investigation, a prototype continuous-flow UV-induced mutation system tested has been proven to be a feasible and effective method for mutant production. Because of the short distance between the bacteria suspension irradiated and the UV light source, the collecting and washing cell procedures typically employed were not required in this system. For the experimental conditions employed and populations used, the mutation frequency for streptomycin resistance ranged from 10^{-4} to 10^{-5} cfu/ml. These mutation frequencies are comparable with conventional batch UV mutation methods [13] and represent a gain of 3 orders of magnitude over the spontaneous mutation frequency.

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