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Effects of Ultraviolet Light Irradiation in Biotreatment of Organophosphates

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ABSTRACT

Excess biomass accumulation in reactor biodegradation processes is undesirable: it increases the disposal cost and upsets the operation of biological reactors if not properly controlled. In this study, we investigated the feasibility of using ultraviolet (UV) light irradiation to reduce biomass accumulation and increase the specific biodegradation activity. UV irradiation has been widely used to introduce DNA damage in bacteria. Here we apply this technology to the biodegradation of organophosphates by recombinant *Escherichia coli* strains that contain a *recA* mutation and a cloned organophosphate hydrolase gene. We show that the *recA* negative strains after UV irradiation reduce the growth rate but increase the specific organophosphate hydrolase activity. This increase in specific enzyme activity is not owing to continued protein synthesis from the plasmid after the damage of chromosomal DNA by UV irradiation. Rather, it is likely to be caused by an increase in membrane permeability to the substrate. Kinetic analysis suggests that the membrane transport of paraoxon is the rate-limiting step in its biodegradation.

Index Entries: Organophosphate; ultraviolet; biotreatment.

INTRODUCTION

Biodegradation in biological reactors has been recognized as one of the effective processes for treating industrial and municipal wastes (*1*). Most bioreactors employ active biomass either suspended as free organisms or attached as biofilms on support media. Design of biological reactors is at

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least partially based on the amount of biomass generated and the oxygen required (*2*). Excess biomass is undesirable because of increased oxygen consumption, increased sludge disposal cost, and decreased mass transfer efficiency in biofilm (*3–6*). An optimal design should therefore minimize the microbial biomass in the reactor while maximizing the specific biodegradation activity. Ideally, one would like to use nongrowing cells that are metabolically active for biodegradation. Many approaches, such as cell immobilization (*7,8*), permeabilization (*9*), growth inhibition (*10*), and nutrient limitation (*7*) have been used for restricting cell growth in bioreactors. Unfortunately, permeabilization by solvents and growth inhibition by drugs are both expensive and environmentally unfriendly. Moreover, nutrient-limited, nongrowing cells in most cases have decreased metabolic activity for biodegradation. Cell immobilization on a supported biofilm is perhaps the most promising. Biomass production can also be avoided by use of crude or purified enzymes. However, several disadvantages, such as the cost of enzyme purification, the difficulty of recycling them, and the instability of enzymes, limit the usage of this approach.

In this article, we explore the feasibility of using ultraviolet (UV) light irradiation to reduce biomass accumulation while maintaining a high specific biodegradation activity. This technology parallels the maxicell technology (*11*), which was developed for detecting protein synthesis from plasmid DNA. The maxicell technology enables the preferential degradation of chromosomal DNA by ultraviolet (UV) light without significantly damaging proteins in the cells. The resulting cells cannot grow but are expected to maintain a high enzymatic activity. In biotreatment processes, one does not need to produce truce maxicells. Rather, the retardation of biomass accumulation and the maintenance or improvement of biodegradation activities are the main objectives.

Organophosphate degradation by genetically engineered strains of *Escherichia coli* is used as a model system in this work. Organophosphates have well-documented acute neurotoxicity (*12*) and have been used as pesticides and chemical weapons. Detoxification of these compounds thus requires serious attention (*13–17*). Paraoxon is used as a model organophosphate because of the convenience in detecting its degradation product, *p*-nitrophenol. The degrading enzyme, organophosphate hydrolase (OPH), has been characterized (*18–21*), and its gene has been cloned from *Pseudomonas* and *Flavobacterium* (*22*). The *E. coli* strains used here contain a plasmid encoding a cytoplasmic organophosphate hydrolase and are deficient in the *recA* gene, which is essential for the induction of SOS repair mechanisms for damaged DNA (*23*).

In this article, we report the applicability of UV irradiation to reduce cell growth while maintaining a high specific biodegradation activity. Because the complete conversion to maxicells is unnecessary, UV irradiation can be conducted periodically in the bioreactor without the tedious steps for maxicell preparation. Therefore, the only additional cost to the process is the UV source and periodical power consumption.

Table 1
E. coli Strains Used in This Study

E. coli	Genotypes	Source
HB101	*hsd*20 (*rB*$^-$, *mB*$^-$) *lacY*1 *proA*2 *supE*44 *ara*14 *galK*2 *repL*20 *xyl*-5 *mtl*-1 *recA*13 *mcrB mrr*	Lab collection
MC4100	araD139 Δ(*argF-lac*)*U*169 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR*	Lab collection
JCL1194	HB101/pLR540 *opd*$^+$	This study
JCL1095	MC4100/pLR540 *opd*$^+$	This study
JCL1096	MC4100 Δ*recA*/pLR540 *opd*$^+$	This study

MATERIALS AND METHODS

Chemicals and Microorganisms

Paraoxon was purchased from Sigma (St. Louis, MO). Protein assay reagents and bovine serum albumin were from Bio-Rad (Hercules, CA). All other chemicals used in this article were products of Sigma or Aldrich (Milwaukee, WI). The *E. coli* strains used in this study are listed in Table 1. JCL1095 and JCL1096 are isogenic except that the latter was made *recA* negative by standard P1 transduction. These two strains were used to determine the importance of *recA* mutation in this process. Plasmid pLR540, a gift from Jim Wild (Department of Biochemistry and Biophysics, Texas A&M University), encodes the *opd* gene, and the gene product, OPH, is a soluble cytoplasmic protein. The expression of this gene is driven by a resident *tac* promoter from the vector, which also contains the *bla* gene conferring ampicillin resistance.

The medium for growing bacteria is the TB medium, which consists of the following (per liter): 12 g Bacto-tryptone; 24 g yeast extract; 4 mL glycerol; 2.32 g K_2HPO_4; and 16.44 g KH_2PO_4. In addition, 0.6 g of ampicillin and 1 mL of 1*M* $CoCl_2$ were added into the above solution. All experiments were initiated from overnight cultures, which were used as inoculants to fresh TB media (50 mL) in 250 mL flasks. These cultures were incubated in a circulating air shaker at 30°C and 150 rpm for about 24 h until OD_{550} reached 13 to 15. At this time, cells were harvested for UV treatment.

UV Irradiation

The process for UV irradiation was modified from Sancar et al. (*11*). Because the cell density (measured by OD_{550}) of the harvested cells was very high, shielding effect caused by the high concentration of cells would protect some cells from UV irradiation. To reduce this problem, the high density culture was diluted by potassium phosphate buffer (K_2HPO_4, 2.32

g/L; KH_2PO_4, 16.44 g/L) to about 10^8 cells/mL. Ten milliliters of diluted culture was pipetted onto a petri dish on a shaker, and a prewarmed UV lamp was used to generate irradiation intensity about 5.5 erg/mm^2/s above a rotating shaker for an indicated amount of time. This procedure was conducted in the absence of visible light.

Organophosphate Hydrolase Assay

The enzyme assay essentially followed the procedure published previously (*14*), and was conducted with unbroken cells and with cell extract. The cell extract was prepared by passing the cell suspension through a French Pressure Chamber. The slurry was centrifuged to remove cell debris. The supernatant was used for enzyme and total protein assay. Five different assay substrate (paraoxon) concentrations were used: 0.125 m*M*, 0.25 m*M*, 0.5 m*M*, 1 m*M*, and 2 m*M*. The buffer used was 50 m*M* potassium phosphate buffer (pH 7.4); 0.2 mL of the cell extract or the whole cell suspension was then added to the assay substrates, and one of the products, *p*-nitrophenol, was monitored spectrophotometrically at 400 nm as a function of time. The extinction coefficient of *p*-nitrophenol used was 17,000$M^{-1}cm^{-1}$.

RESULTS

The Specific OPH Activity and Growth Rate

To determine the effect of UV irradiation on cell viability, three strains (JCL1194 *recA*, JCL1095, adn JCL1096 *recA*) were exposed to UV irradiation and the viable cell counts were determined. As expected, *recA* strains were much more sensitive to UV irradiation than the *recA*$^+$ strain. These survival curves give the dosage of irradiation necessary to reduce viability to a given value. UV dosage is perhaps the most important factor in the process. Insufficient irradiation resulted in out-growth of surviving cells. Over-irradiation reduced the activity and stability of the enzymes.

To evaluate the biodegradation activity, strain JCL1194 *recA* was UV-irradiated for various amounts of time. After irradiation, the cells were incubated for 10 h, and samles were taken for OPH assay. Figure 1 shows the cell growth (measured in terms of OD_{550}) and the specific OPH activity at various times. UV irradiation did result in a decreased growth rate, and an increased specific OPH activity. Irradiation for 220 s (1210 erg/mm^2) appears to be the optimal irradiation dosage, as UV dosage higher than 1210 erg/mm^2 did not improve the specific OPH activity (data not shown). These results suggest that UV irradiation in *recA* cells may be applicable in reducing cell growth and increasing the specific OPH activity. However, because of the low growth rate of the UV-treated cells, the total OPH activity is low. Therefore, an optimal design should take into account both the rate of degradation and the cost of biomass disposal. Practical applications of this approach will be discussed shortly.

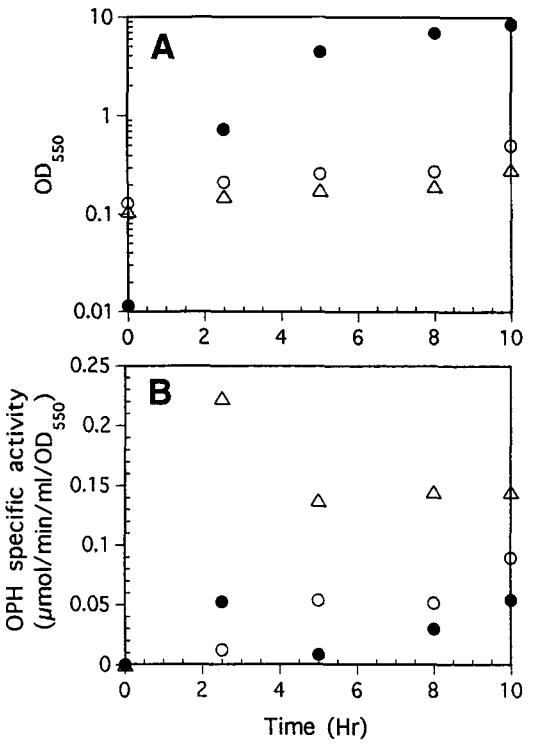

Fig. 1. The growth curves (**A**) and the OPH specific activity (**B**) of strain JCL1194 as functions of time after UV irradiation. The symbols correspond to different UV irradiation dosage (1 s = 5.5 erg/mm^2): closed circles, 0 s; open circles, 180 s; open triangles, 220 s.

To determine whether the increase in specific enzyme activity requires a *recA* mutation, a pair of strains, JCL1095 and JCL1096 *recA*, which were otherwise isogenic, were irradiated with UV for various amounts of time. The *recA* strain (JCL1096) again showed an increased specific OPH activity after UV treatment, but its isogenic *recA*$^+$ strain (JCL1095) failed to show the effect (data not shown). Therefore, the increase in specific OPH activity after UV irradiation is *recA* dependent. The UV effect in *recA*$^+$ strains presumably were repaired by the SOS genes.

Nutrient Limitation

Several methods can be used to reduce cell growth. Among them the most common is nutritional limitation. Here we examine whether nutrient limitation can result in a high specific enzyme activity. Without UV irradiation, JCL1194 was cultured in 100, 10, and 1% concentrations of TB medium. The growth rate and the specific OPH activity were measured. As shown in Fig. 2, the nutrient limitation did effectively reduce the growth of the cell. However, the specific OPH activity was greatly reduced under nutrient limitation. To determine if UV irradiation followed by nutrient limitation can enhance the performance, we irradiated JCL1194 for 220 s

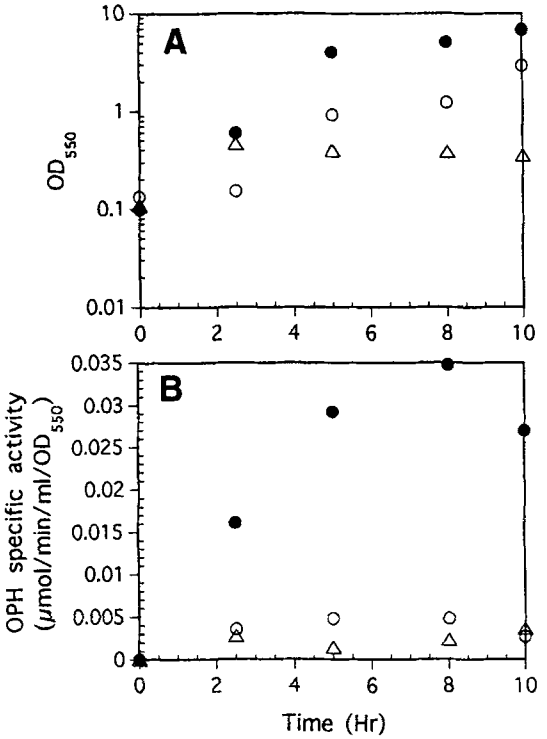

Fig. 2. The growth curves (**A**) and the OPH specific activity (**B**) of strain JCL1194 as functions of time in different nutrient conditions without UV treatment. The symbols are: closed circles, 100% TB medium; open circles, 10% TB medium; open triangles, 1% TB medium.

(1210 erg/mm^2) and incubated the cells in 100, 10, or 1% TB medium. Results (Fig. 3) show that nutrient limitation did not enhance the performance of the UV-treated cells. Therefore, although nutrient limitation is effective in reducing biomass formation in biotreatment reactors, it also reduces the specific enyzme activity.

Kinetics of Organophosphate Degradation in UV-Treated Cells

The increased specific OPH activity in UV-treated cells suggests at least three possibilities. First, the increased enzyme activity was owing to continued OPH enzyme synthesis from the plasmid after the chromosomal DNA in the UV-treated cells was damaged. Second, the UV treatment caused the leakage of proteins out of the cell membrane. Third, the UV irradiation made the cell membrane more permeable to paraoxon and thus increased the apparent specific OPH activity. The second possibility could be tested easily by measuring the OPH activity in cell-free supernatant. No activity was detected, indicating that the OPH enzyme did not leak out of the cells. The first and third models can be distinguished by measuring the specific OPH activity in intact cells and in cell extract.

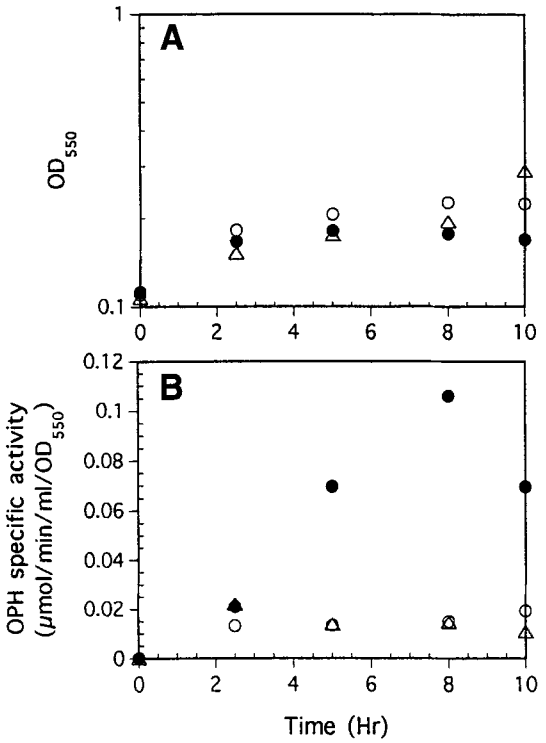

Fig. 3. The growth curves (**A**) and the OPH specific activity (**B**) of strain JCL1194 as functions of time in different conditions. The symbols are: closed circles, 100% TB medium; open circles, 10% TB medium; open triangles, 1% TB medium. The cells were UV-irradiated for 220 s (1210 erg/mm²).

Table 2
Kinetic Parameters of OPH in the Cell Extract and in Unbroken Cells of JCL1194[a]

	Cell extract		Unbroken cells	
Parameters	Control	UV-treated	Control	UV-treated
V_{max} (μmol/min/mg)	0.066 ± 0.012	0.067 ± 0.015	0.033 ± 0.006	0.048 ± 0.008
K_m (m*M*)	0.30 ± 0.05	0.22 ± 0.07	1.2 ± 0.09	0.48 ± 0.08

[a]The parameters were estimated from the Lineweaver-Burke plots of the initial rates of the OPH reaction at various paraoxon concentrations (0.125 m*M*, 0.25 m*M*, 0.5 m*M*, 1 m*M*, and 2 m*M*). The controls are the cells without UV irradiation.

Therefore, we disrupted the UV-treated cells and the control cells (no UV irradiation) and measured the OPH activities in cell extract at various substrate concentrations. By use of the Lineweaver-Burke plot, the apparent V_{max} and the K_m values were determined (Table 2). The intact UV-treated cells showed a significant increase in the apparent V_{max} and a significant decrease in the apparent K_m. These changes were not found in

cell extract. Therefore, the observed increase in the specific OPH activity in unbroken cells was not owing to continued protein synthesis from the plasmid. The increased organophosphate degradation activity in UV-treated cells may be explained by increased membrane permeability to paraoxon after UV treatment.

Kinetic Modelling for Analyzing Membrane Transport

To determine the rate of membrane transport, we modelled the system in terms of three steps: membrane transport of paraoxon, the OPH reaction, and membrane transport of the product, *p*-nitrophenol. The whole process was described as:

$$\text{vol}_e\,(dS_e / dt) = -V_1 W_p \tag{1}$$

$$\text{vol}_i\,(dS_i / dt) = (V_1 - V_2) W_p \tag{2}$$

$$\text{vol}_i\,(dP_i / dt) = (V_2 - V_3) W_p \tag{3}$$

$$\text{vol}_e\,(dP_e / dt) = V_3 W_p \tag{4}$$

where vol_e and vol_i are the extracellular and intracellular volumes, respectively; V_1, V_2, and V_3 are the specific rates of the paraoxon cross-membrane transport, OPH reaction, and *p*-nitrophenol cross-membrane transport, respectively; W_p is the total protein added to the reaction mixture; S and P are the concentrations of paraoxon and *p*-nitrophenol, respectively; subscripts i and e refer to intracellular and extracellular compartments, respectively. Vol_i was calculated based on the size of *E. coli* and the cell concentration, assuming that the cell is spherical and its diameter was 1 m. Vol_e is the volume of total reaction mixture less vol_i. The extracellular and intracellular concentrations of paraoxon and *p*-nitrophenol were assumed to be homogeneous. The mass transfer resistance in the boundary layer outside of a cell was assumed to be small and lumped into V_1.

Two models were tested for the rate of paraoxon transport (V_1).

(i) Simple diffusion:

$$V_1 = K_1(S_e - S_i) \tag{5}$$

where K_1 is a coefficient to be determined.

(ii) Carrier-mediated transport:

$$V_1 = (V_{1max} S_e / K_{m1} + S_e) \tag{6}$$

where V_{1max} and K_{m1} are the apparent $V_{\max}$ and K_m, respectively, for the membrane transport.

The hydrolysis of paraoxon (V_2) catalyzed by organophosphate hydrolase has been shown to follow the Michaelis-Menten kinetics without product inhibition (*14*).

$$V_2 = (V_{2max} S_i / K_{m2} + S_i) \tag{7}$$

where $V_{2\max}$ and K_{m2} were determined above using cell extract (Table 2).

Table 3
Kinetic Parameters in Eq. (6) Estimated from the Reaction Progress Curves of Unbroken Cells of JCL1194[a]

	Control		UV-treated cells	
S_e	V_{1max}	K_{m1}	V_{1max}	K_{m1}
1.67 m*M*	0.037	1.19	0.069	0.46
0.83 m*M*	0.030	1.37	0.050	0.50
0.42 m*M*	0.035	1.24	0.058	0.41
0.21 m*M*	0.035	1.22	0.050	0.54

[a] S_e is the initial extracellular paraoxon concentration. The units for V_{1max} and K_{m1} are μmol/min/mg and m*M*, respectively. The UV-treated cells received 1210 erg/mm² of irradiation. The control cells were without UV irradiation. Other parameters used were V_{2max}, 0.068 μmol/min/mg; K_{m2}, 0.38 m*M*; and K_3, 1000 mL/min/mg.

The transport of *p*-nitrophenol (V_3) was assumed to be very fast and followed the linear nonsaturable kinetics:

$$V_3 = K_3(P_i - P_e) \tag{8}$$

where K_3 is an arbitrarily large number, 1000 (mL/min/mg).

With the above equations, the substrate and product concentrations were solved. The only unknown parameters were those associated with V_1. Therefore, the unknown parameters could be estimated from experimental values of product or substrate concentrations as a function of time. To do so, we measured the time course of product (*p*-nitrophenol) formation at various initial paraoxon concentrations. The resulting data were used in a parameter estimation program (*24*) to estimate either K_1 in Eq. (5) or V_{1max} and K_{m1} in Eq. (6). If Eq. (5) was used, K_1 value was dependent on initial substrate concentration, a result consistent with the model. Therefore, simple diffusion (Eq. 5) was not a good model for paraoxon transport. On the other hand, V_{1max} and K_{m1} in Eq. (6) (*see* Table 3) were reasonably independent of initial substrate concentrations, suggesting that paraoxon transport was carrier mediated. Moreover, V_{1max} and K_{m1} of the UV-treated cells were higher and lower, respectively, than those of the control cells, suggesting that membrane transport was enhanced by UV irradiation.

To determine whether the membrane transport was rate limiting, we calculated the normalized sensitivity of the quasi-steady state rate of product formation ($V_{3,ss}$) with respect to V_{1max} and V_{2max}, based on the parameters estimated from the carrier-mediated transport model. The calculation was carried out by use of a numerical method described by Caracotsios and Stewart (*25*). Results show that the rate of *p*-nitrophenol production (or paraoxon degradation) was limited completely by the membrane transport, since the sensitivities with respect to V_{1max} and V_{2max} were 1 and 10^{-4}, respectively. Therefore, a 1% increase in V_{1max} would increase the quasi-steady state rate by 1%, but a corresponding increase in V_{2max} (or OPH

enzyme concentration) would have almost no effect. This result suggested that the membrane transport was indeed rate-limiting in both the control cells and the UV-treated cells.

DISCUSSION

The above investigation demonstrated that UV treatment can effectively reduce the cell growth and increase the specific OPH activity. In contrast to other treatments for cell growth control, UV irradiation does not reduce the specific biodegradation rate. Therefore, it can be used in processes where excess biomass accumulation upsets the operation. The increase in the specific enzyme activity may be caused by an increase in paraoxon transport across the cell membrane, possibly owing to UV damage in the membrane structure. The decreased apparent K_m of the membrane transport in intact UV-treated cells is consistent with this explanation. The kinetic model also suggests that the paraoxon transport is a carrier mediated process and that it is the limiting step in paraoxon biodegradation. This finding is important because *E. coli* has been used commonly as a host for expressing many biodegradation enzymes. In cases such as paraoxon degradation, the substrate transport across cell membrane may become the rate-limiting step after the enzymes are sufficiently expressed. The UV treatment in *recA* strains may offer a simple way to increase membrane permeability to the substrate, in addition to the biomass reduction. Nutrient limitation, although effectively reducing cell growth, also reduces specific enzyme activity, possibly owing to stress responses. Other means of biomass reduction and membrane permeabilization such as solvent treatment, detergent treatment, or membrane disruption are potentially more expensive than the UV treatment in large scales.

Although the present work investigates the UV-treatment in a small scale, scale-up of this process is straightforward. We envision that the UV irradiation can be carried out through a circulating tube with a UV-transparent window. The cells in the bioreactor are to be circulated through this tube and UV-irradiated. The irradiation process begins when the cell density reaches a pre-set value. Therefore, one can utilize the high specific enzyme activity of UV-treated cells without significantly compromising the cell density. Because of the high cell density and the large liquid volume, only a portion of cells will receive UV irradiation per passage through the window. The cells that do not receive sufficient UV irradiation will soon out-grow the UV-treated cells. Thus, the irradiation process can continue for a period of time when the cell growth rate reduces to a desired value, and recommence when the cell growth rate increases again. Obviously, the dosage of irradiation and the circulation rate must be optimized according to process economy. The irradiation process need not be 100% effective, as long as the growth rate is sufficiently reduced without reducing the total biodegradation activity. The process requires a *recA* mutation, or any other similar mutations that block the DNA repair mechanisms. This requirement

presents some problem if the host bacteria do not have *recA* strains readily available. Fortunately, more and more *recA* mutations in microorganisms other than *E. coli* have been isolated, and an effective method to isolate *recA*-like mutants in gram-negative bacteria has been reported (*26*).

The above process has been discussed in the context of pure cultures, particularly in recombinant strains where biodegrading enzymes are non-native to the host. In mixed cultures with defined population, the UV-treatment approach may still be possible. But it is much more difficult to implement in a consortium of microbes where population components are not easily defined. This approach can also be used in bioreactors with cells attached (or immobilized) on the surface of support particles. The UV irradiation will be able to reduce the growth of the biofilm on the support particles. However, this process is feasible only in bioreactors; it is not suitable for *in situ* bioremediation, as the UV-treated cells will be out-competed by the growing cells.

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