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Enhancement of the Conversion of Toluene by *Pseudomonas putida* F-1 Using Organic Cosolvents

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Abstract

Pseudomonas putida F-1 (ATCC 700007) was used as a model organism in stirred tank reactors to study conversion enhancement of poorly soluble substrates by organic cosolvents. After a literature study, silicone oil was used as a solvent system to enhance the mass transfer rate. To study the benefits of the organic solvent addition, batch experiments were conducted in two side-by-side fermentation vessels (experimental and control) at three different levels of silicone oil (10, 30, and 50%). Results showed that the presence of silicone oil resulted in a 100% increase in the toluene mass transfer compared to the control. Experiments in continuous stirred-tank reactors showed that improved conversion could be obtained at higher agitation rates.

Index Entries: *Pseudomonas putida* F-1; toluene; organic solvents; silicone oil.

Introduction

To promote higher bioconversion of poorly water-soluble components, cosolvents and surfactants are often added to the fermentation broth. The logarithm of the partition coefficient (log P or log P_{ow}) of an organic solvent in a standard octanol-water two-phase system is a useful parameter to predict what solvent would be most suitable for a bioconversion *(1,2)*. The partition coefficients for some common organic solvents are listed in Table 1. The relationship between log P and bioactivity is based on the assumption that the octanol-water system provides a sufficient description of hydrophobic and transport interactions when it is introduced into a biologic system *(3,4)*. In general, organic solvents with a log P value between 1 and 5 are toxic to microorganisms *(5)*.

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Table 1
Log *P* Values for Selected Solvents *(2)*

Solvent	Log *P*	Solvent	Log *P*
Ethanol	–0.24	1-Decanol	4.0
1-Heptanol	2.4	Dodecanol	5.0
Toluene	2.5	Decane	5.6
1-Octanol	2.9	Dodecane	6.6
Hexane	3.5	Oleyl alcohol	7.5
Heptane	4.0	Hexadecane	8.8

In general, Gram-negative bacteria appear to have a higher solvent tolerance than Gram-positive bacteria, and species within a genus sometimes show a range of tolerances *(6–9)*. It has been suggested that the difference in solvent tolerance is caused by the presence of the outer membrane in Gram-negative bacteria containing lipopolysaccharides, which protect the cells against hydrophobic compounds. The most resistant Gram-negative species have been reported in the genus *Pseudomonas (10–12)*. One of the key processes in the adaptation of some *Pseudomonas* strains enabling them to tolerate organic solvents appears to be the isomerization of *cis*- into *trans*-unsaturated fatty acids *(13)*.

Silicone oil has been used for the biological elimination of alkanes from gases using biotrickling filters. One study reported the use of silicone oil with an aqueous medium in a 1:1 ratio being recirculated in a biotrickling filter to remove hexane *(14)*. An 89% elimination efficiency of hexane was achieved. The oil was reused after separation by natural gravity or centrifugation. Column experiments were performed with intermittent replacement of nutrients. In these studies, a control column was not used, making it difficult to positively prove the benefit of the oil addition.

In a study by Budwill and Coleman *(15)*, silicone oil was used as an additive in peat-based biofilters for the removal of hexane. Peat was coated with 20% (v/v) silicone oil and loaded into the biofilter columns. An average 60%, or 16 g/(m^3·h), hexane removal was reported in the column containing silicone oil compared to 24%, or 8.2 g/(m^3·h), in the untreated control. These investigators speculated that the presence of silicone oil increased the mass transfer of hexane from the gas to the liquid phase by increasing the contact of microorganisms with the dissolved gas at the water–silicone oil interface.

A group of French researchers have been investigating the applications of silicone oil as an organic solvent for the degradation of poorly water-soluble xenobiotic compounds such as xylene, butyl acetate, 2,4,6-trichlorophenol, and styrene *(16–19)*. One of their studies reported that microorganisms were able to grow more in a two-phase system (70% medium and 30% silicone oil [v/v]) on xylene and butyl acetate (70%/30% [w/w]) than in a one-phase system *(16)*. The two-phase system resulted in an

increase in optical density at 540 nm for several substrate concentrations. No appreciable growth was noted in the one-phase system.

Other studies performed by the French group have also used a biphasic aqueous-silicone oil system with 20% oil (v/v) in a continuous stirred-tank reactor (CSTR). One of these studies demonstrated a more efficient degradation of 2,4,6-trichlorophenol in the biphasic system when compared to a monophasic aqueous system *(17)*. As the dilution rate was changed from 0.033 to 0.22/h, the volumetric conversion rate increased from 21.3 to 85.8 g/(m^3·h) in the biphasic system compared with an increase from 13.9 to 40.2 g/(m^3·h) in the monophasic system. Ascon-Cabrera and Lebeault *(18)* demonstrated the effect of silicone oil in the degradation of chlorinated and nonchlorinated mixed compounds. They found that the specific growth rate of the microorganisms used in the study was about two times higher in the biphasic system (0.48/h) than in the monophasic system (0.27/h). Statistical analysis showed that the biphasic system was more efficient in the degradation process when compared to the monophasic system. Finally, the French group showed in another study that this type of biphasic system was effective in the degradation of styrene by *Pseudomonas aeruginosa (19)*. Without silicone oil, the microorganisms were unable to oxidize styrene, but in the presence of silicone oil, the lethal dose of styrene in aqueous medium (70 mg/L) was avoided.

The French studies were performed by mixing an aqueous phase and silicone oil laden with organic contaminants, and they were able to demonstrate the benefit of silicone oil as a "reservoir" for the contaminant. By doing this, the toxic levels of some of the compounds in the aqueous phase could be lowered and toxicity avoided. By contrast, our scope was to study the benefit of using silicone oil for enhancement of mass transfer of dilute gaseous organics (not necessarily toxic) to microorganisms in the aqueous phase when conducting batch or continuous fermentations.

Materials and Methods

Microbial Culture and Media

Pseudomonas putida F-1 strain ATCC 700007 was obtained from the American Type Culture Collection (Manassas, VA). Seed cultures were grown aerobically at 30°C in a mineral salts medium consisting of the following ingredients: 0.4 g/L of KH_2PO_4, 0.5 g/L of K_2HPO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.04 g/L of $CaCl_2$, 0.5 g/L of NH_4Cl, 0.5 g/L of KNO_3, and 1 mL of Pfennig trace metals *(20)*. The medium was distributed in 50-mL aliquots in 125-mL serum bottles and sealed with butyl rubber stoppers and aluminum crimps (Wheaton, Millville, NJ). Toluene was added (20 µL) as a liquid to each culture as a carbon source.

Reactor Experiments

Experiments were conducted in 1-L nominal volume, stirred batch reactors (SBRs) and in a CSTR (Virtis, Gardiner, NY). Silicone oil (DC 200,

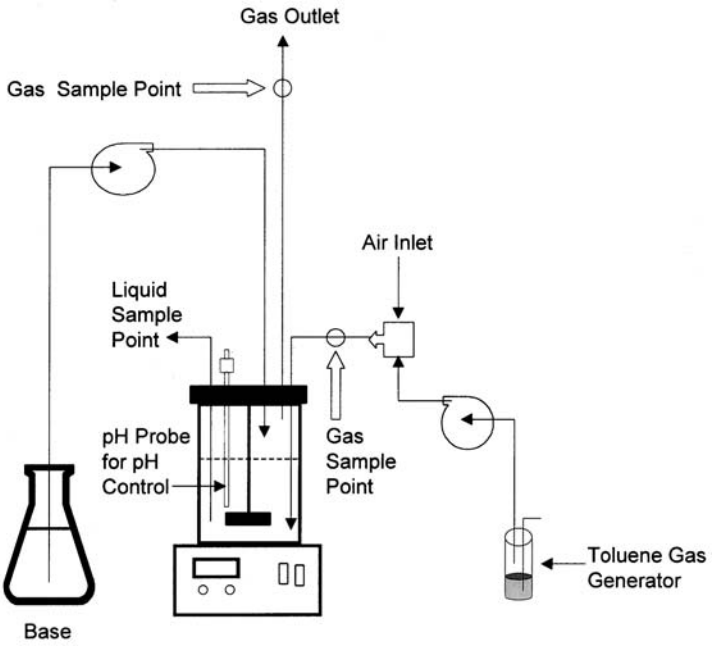

Fig. 1. Schematic of experimental setup for SBRs.

~53 mPa·s, polydimethylsiloxane) (Sigma/Fluka) was selected as an organic solvent to increase the conversion of toluene. A schematic of the batch reactor configuration is shown in Fig. 1. Modifications to the reactor for continuous feed included the addition of two feed pumps (for medium and oil). Both gas and liquid exited the continuous reactor through a tube through the top of the reactor positioned at the interface (Fig. 2).

The batch experiments were conducted in two side-by-side fermentation vessels (experimental and control) with a total liquid volume in each reactor of 1 L. The SBRs were operated at 30°C and an agitation of 300 rpm. The pH was controlled at 7.0. The aeration was set at 1 L/min, and the air was prefiltered using a Gelman Sciences Acrodisk (ACRO 50 APT, 0.2 µm polytetrafluoroethylene). Toluene was pumped as a gas into the inlet air at a flow rate of 10 mL/min, resulting in concentration of approx 35 ppmv (1.4×10^{-6} mol/L). After an initial growth phase without silicone oil, the contents of both reactors were mixed and a portion of the broth was returned to the fermentation vessels. Then, either silicone oil or water was added to a total volume of 1 L. Thus, the same population of viable cells was present in each reactor when measurements began. Silicone oil was tested at three different concentrations: 10, 30, and 50% (v/v).

The continuous experiments were conducted with a total liquid volume in the reactor of 1 L. The CSTR was operated at 30°C and an agitation of 300 rpm. The pH, aeration, and toluene addition was the same as in the SBRs. After an initial growth phase without silicone oil, the oil and medium feed streams were started at a total flow rate of 0.45 mL/min (15% silicone oil).

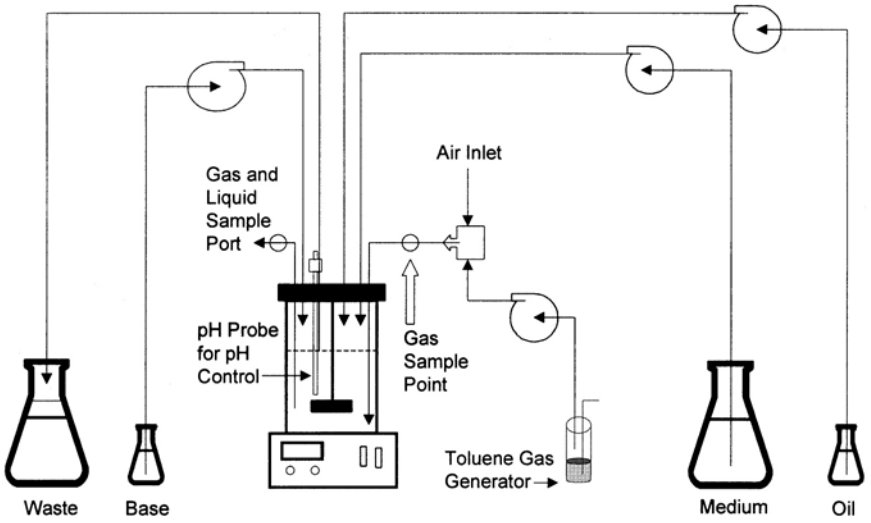

Fig. 2. Schematic of experimental setup for CSTR.

Analytical Techniques

Gas samples were collected from the inlet gas and the reactor headspace in gastight syringes, and 100 µL was injected into a gas chromatograph (Hewlett Packard HP 5890 Series II) equipped with an HP WAT (cross-linked polyethylene glycol) capillary column (30 m × 0.53 mm) with a 1.0-µm film thickness. Temperatures of the column, injection port, and flame ionization detector were 40, 175, and 200°C, respectively. Helium was used as a carrier gas. The calibration was based on 8.66 and 86.6 µg/L of toluene standards in hexane. Liquid samples were collected from the aqueous phase, which was allowed to settle by temporarily turning off the agitation. To measure toluene in the aqueous phase, samples were centrifuged for 5 min at 14,200*g* and 2 µL of the aqueous phase was injected into the gas chromatograph. The column temperature program was initially 35°C followed by ramping to 50°C at 25°C/min with a 2.0-min hold, then followed by ramping to 150°C at 20°C/min with a 0-min hold. Temperatures of the injection port and the flame ionization detector were 245 and 265°C, respectively.

The increase in dry cell weight (DCW) was measured by optical density (OD) at 600 nm after hexane had been used to extract the remaining silicone oil present in each sample. No emulsion was observed in the samples after extraction. The calibration curve was prepared from samples with known cell concentration. The potential interference of hexane in the procedure was determined by comparing OD measurements of hexane-extracted and nonhexane-extracted samples.

Results and Discussion

The addition of silicone oil enhanced the conversion of gaseous toluene for all conditions studied in the batch experiments. In Fig. 3, the conver-

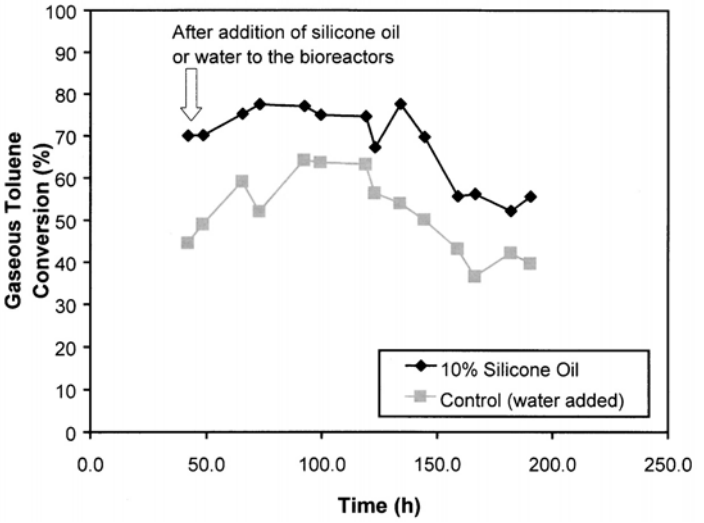

Fig. 3. Toluene conversion in SBRs with or without the addition of 10% (v/v) silicone oil.

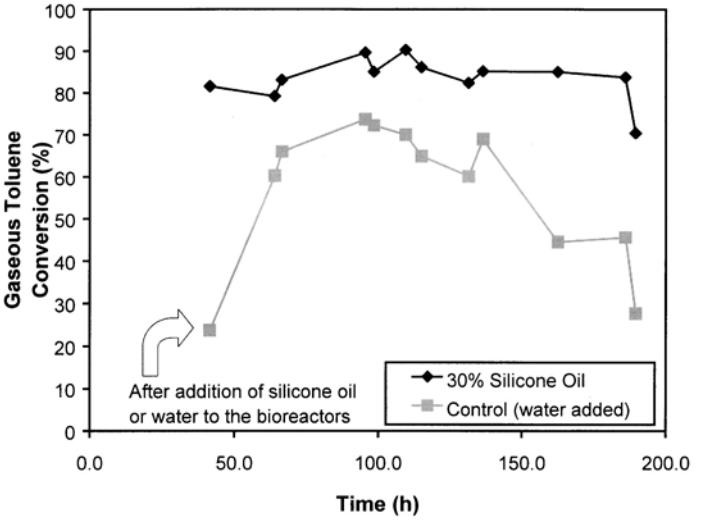

Fig. 4. Toluene conversion in SBRs with or without the addition of 30% (v/v) silicone oil.

sion of toluene has been plotted as a function of fermentation time when 10% (v/v) silicone oil was present in one of the reactors. Data prior to the addition of the oil has not been plotted, and it should be noted that the contents of the reactors were mixed just before the addition of the silicone oil. The toluene conversion was substantially higher when silicone oil was present. At the end of the batch fermentation, the conversion dropped

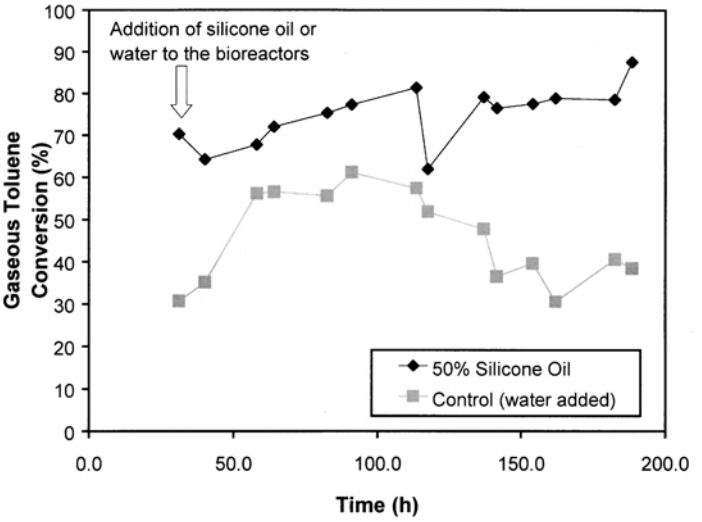

Fig. 5. Toluene conversion in SBRs with or without the addition of 50% (v/v) silicone oil.

in both cases, presumably because of nutrient limitations. A short lag phase was noted in most cases after the mixing/initiation of the experiment. Figures 4 and 5 show results from the addition of 30 and 50% silicone oil. In both cases, the conversion of toluene was higher in the reactor containing silicone oil. Toluene concentration in the aqueous phase remained below detection (data not shown) in these experiments, indicating gas mass transfer limiting conditions.

The cell growth in the study in which 30% silicone oil was used is shown in Fig. 6. The increase in cell concentration is dramatic in the case in which silicone oil was used. This, of course, can be attributed to the higher conversion obtained in this reactor by the apparent improved mass transfer rate of the toluene from the gas to the aqueous phase. It is clear from the results that the silicone oil was not toxic to the cells. The log P value for silicone oil used in these experiments with a molecular mass of 3000 g/mol was estimated to be 2.93 *(21)*.

The mass transfer coefficient ($K_L a$) was calculated (Eq. 1) from the measured consumption of toluene, the composition of the headspace in the reactors, and the assumption that mass transfer–limited conditions were present.

$$\text{rate of toluene conversion} = (K_L a / H)(p_{\text{toluene}})(V_{\text{liq}}) \quad (1)$$

in which p_{toluene} is the partial pressure of toluene in the headspace and V_{liq} is the total liquid volume in the reactor. The results showed that the mass transfer of toluene from the gas increased by a factor of 2 in the presence of silicone oil (Table 2). No trend was found between the mass transfer rate and amount of silicone oil added to the reactor.

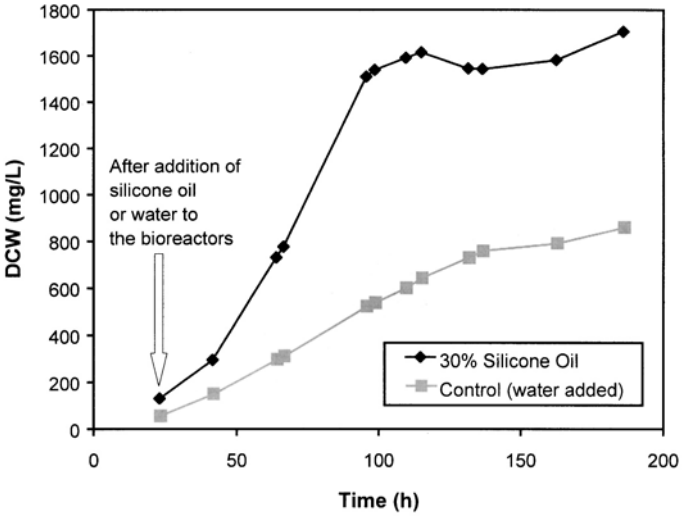

Fig. 6. Cell growth in SBRs with or without the addition of 30% (v/v) silicone oil.

Table 2
Average K_La' for Conversion of Toluene
by *P. putida* F-1 at Different Concentrations of Silicone Oil[a]

Silicone oil (%)	Experimental average K_La' ±SD	Control average K_La' ±SD
10	7.9 ± 1.6	3.7 ± 1.1
30	16.7 ± 6.6	5.3 ± 2.1
50	8.2 ± 3.0	3.1 ± 1.0

[a]Average K_La' was determined from samples taken in an interval of approx 40–140 h during the course of the experiment. The unit for the mass transfer coefficient ($K_La' = K_La/H$) is mol/(h·L·atm) and includes the Henry's law constant (H).

To confirm the enhancement of the mass transfer rate by the addition of silicone oil, the control and experimental reactors were switched so that the reactor normally used as control became the experimental reactor and vice versa. Within experimental error, the results were the same as before (data not shown).

Since this was a non-steady-state condition, it is important to estimate the amount of toluene that initially may accumulate in the silicone oil. An overestimated microbial uptake rate may be calculated if the accumulation is significant. Using a Henry's law constant of 0.071 atm/(L·mol) *(22)* for the toluene/silicone oil system, we can calculate that it would take 15–76 min to saturate the silicone oil if no microbial conversion existed. This time is considerably shorter than the fermentation time with silicone oil, which lasted approx 150 h.

The CSTR was operated for 35 d in a study conducted to investigate the effect of agitation rate. After an initial batch growth without silicone oil,

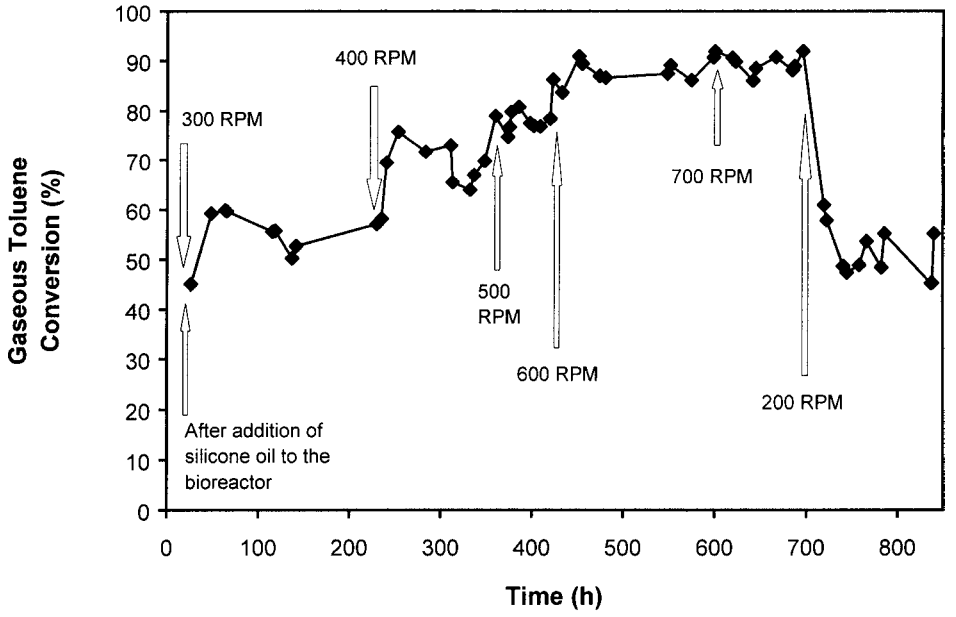

Fig. 7. Effect of agitation on toluene conversion in CSTR with 15% (v/v) silicone oil. Arrows indicate agitation changes (in revolutions per minute).

the liquid feeds were started and oil was added to the reactor. As expected, the conversion of toluene was higher at higher agitation rates, although the dependence was more apparent at a low agitation rate (Fig. 7). In these studies we also confirmed that it was possible to recycle the silicone oil after a gravity separation and filtration through a filter paper (Whatman, Clifton, NJ). Calculations using a simple mass balance over the CSTR showed that the maximum toluene loss in the silicone oil exiting the reactor was 3.5% of the gaseous toluene exiting the reactor.

Conclusion

Silicone oil is an organic cosolvent that efficiently enhances the conversion of toluene by *P. putida* F-1. We speculate that the increases seen may be attributed to an increase in effective transfer area between the toluene-rich and toluene-poor phases. This is more apparent at lower agitation rates where the gas holdup in the liquid is low. Toluene is absorbed into the silicone oil, and the silicone oil disperses the toluene into the aqueous medium. Subsequently, the availability of toluene increases, thus increasing the consumption of the gas by the microorganism. In our studies with low levels of toluene (average of 15 ppmv) in the headspace air, the calculated concentration of oxygen in the aqueous phase (2.5×10^{-4} mol of O_2/L) is 70 times greater than the calculated toluene concentration (3.5×10^{-6} mol of toluene/L) under equilibrium conditions *(23,24)*. Thus, the concentration of O_2 is eight times higher than is needed to completely oxidize the

toluene, assuming a theoretic oxygen-to-toluene molar ratio of 9. It is therefore safe to assume that the limiting reactant is toluene.

This process would be applicable for biologic conversion of other poorly water-soluble gases such as nitric oxide or synthesis gases. Since the silicone oil can be reused, it minimizes the generation of waste and the capital cost. Further research needs to be conducted to expand this process for industrial applications.

Acknowledgments

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