Gas Phase Degradation of VOCs Using Supported Bacteria Biofilms

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Abstract

Herein we report the use of *Pseudomonas putida* F1 biofilms grown on carbonized cellulosic fibers to achieve biodegradation of airborne VOCs in the absence of any bulk aqueous phase media. It is believed that direct exposure of gaseous VOC substrates to biomass may eliminate aqueous phase mass transfer resistance and facilitate VOC capture and degradation. When tested with toluene vapor as a model VOC, the supported biofilm could grow optimally at 300 ppm toluene and 80% relative humidity, with a specific growth rate of 0.425 day $^{-1}$. During long-term VOC biodegradation tests in a tubular packed bed reactor, biofilms achieved a toluene degradation rate of 2.5 mg g $_{DCW}$ $^{-1}$ h $^{-1}$ during the initial exponential growth phase. Interestingly, the *P. putida* F1 film kept biodegrading activity even at the subsequent stationary non-growth phase. The supported biofilms with a biomass loading of 20% (wt) could degrade toluene at a rate of 1.9 mg g $_{DCW}$ $^{-1}$ h $^{-1}$ during the stationary phase, releasing CO $_2$ at a rate of 6.4 mg g $_{DCW}$ $^{-1}$ h $^{-1}$ at the same time (indicating 100% conversion of substrate carbon to CO $_2$). All the specific degradation rates are much higher than what can be gleaned from previously reported work. It also demonstrates the feasibility of biofilm growth and direct gas phase degradation of VOCs without requiring any bulk aqueous phase.

1. Introduction

Volatile organic compounds (VOCs) constitute a group of organic chemicals having low T_b (generally less than 250) and have been concerned widely for adverse impacts on environment and human health (McDonald et al., 2018; Sheu et al., 2020). Due to rapid urbanization and industrialization, there has been a rapid increase of VOC emission within the last century (He et al., 2019). Globally, VOC emissions contribute significantly to environmental pollutions and climate changes, generating photochemical smog and destroying stratospheric ozone. Exposure to VOCs can also cause severe health consequences (Almomani et al., 2021; Bravo et al., 2017; Hein et al., 2018; Matějová et al., 2013). Short-term exposure to VOCs can cause headaches, dizziness, fatigue, nausea, and respiratory irritation, while long-term exposure can lead to damages to the kidney, liver, and central nervous system (Rumchev, 2004; Wu et al., 2007; Yang et al., 2020). VOCs, especially aromatic compounds, are often strongly recalcitrant to biodegradation in environment. A series of restrict legislations and standards have been formulated for VOC emission control worldwide; however, VOCs generated in manufacturing, agriculture and transportation industries still account for a large portion of gaseous pollutants (Schiavon et al., 2017). To date, a variety of technologies have been proposed for VOC treatment which can be categorized into two groups: VOC recovery (sorption, condensation, and membrane separation) and VOC degradation (incineration, catalytic oxidation, biodegradation, photooxidation, and non-thermal plasma oxidation) (He et al., 2019). Among these technologies, biodegradation is particularly appealing due to its environmentally-benign nature and high energy efficiency over other physical or chemical technologies (Estrada et al., 2015).

Previous research had demonstrated the feasibility of using conventional bioreactors that typically involved microorganisms hosted in liquid media for VOC degradation, in form of biofilters, biotrickling reactors, and

bioscrubbers (Mudliar et al., 2010; Delhoménie & Heitz, 2005; Detchanamurthy & Gostomski, 2012). However, most VOCs are hydrophobic with very limited solubility and suffer significant mass transfer resistance in aqueous media (Cheng et al., 2016; Khan et al., 2018). Accordingly, various bioreactor designs have been suggested and examined to intensify VOC biodegradation by promoting substrate and biomass interactions. That included the use of organic solvents to form two-phase partitioning bioreactors. To our opinion, these organic phases (including silicone oil, hexadecane, and polymeric compounds) can help with the removal of VOC from gas phase, but do not necessary improve VOC solubility in aqueous media hosting the biomass, as they usually offer only limited partitioning coefficients between the two phases (Hernández et al., 2012; Muñoz et al., 2012; Muñoz et al., 2007). Another common approach is the use of membrane bioreactors in which porous membranes serve as contacting surfaces interfacing gas, liquid media and biomass for improved VOC solvation and degradation kinetics. However, the clogging and the high cost of the membrane supports generally limited large-scale applications of membrane bioreactors (Lebrero et al., 2013; Reij et al., 1998). An alternative strategy is to pretreat the VOCs and break them down to more soluble chemicals that are more vulnerable to biodegradation. Pretreatment technologies such as UV photooxidation and non-thermal plasma treatment have successfully been coupled with conventional bioreactors (Almomani et al., 2021; Schiavon et al., 2015). Such pretreatment processes may suffer from formation of toxic byproducts, high cost and complications in scaleup operations.

As far as mass transfer resistance concerned, VOC biodegradation may implicate gas phase diffusion, bulk liquid phase transfer, and cell membrane adsorption and diffusion. The liquid phase mass transfer resistance is often regarded the primary limiting factor (Mudliar et al., 2010; Delhoménie & Heitz, 2005; Detchanamurthy & Gostomski, 2012; Cheng et al., 2016). Therefore, we assume here that elimination of the bulk liquid phase would substantially improve biodegradation efficiency. The aim of this work is therefore to examine the feasibility of growing microbial biofilms on solid supports without any bulk liquid phase for VOC biodegradation. *P. putida* F1 and toluene are selected as the model bacterium and VOC, respectively. Factors regulating the growth of biofilm on the solid support and VOC degradation efficiency are examined. The long-term operational stability of the supported biofilm is also demonstrated in a tubular packed bed reactor.

2. Materials and Methods

2.1 Media and Chemicals

Minimalist salt medium (MSM) used for producing *P. putida* F1 was prepared in our lab. MSM solution lacking carbon source substrate was made by dissolving chemicals below to a solution of final concentrations as: $NaNO_3$ (1 g L^{-1}), KH_2PO_4 (1 g L^{-1}), K_2HPO_4 (1 g L^{-1}), $MgSO_4$ (0.2 g L^{-1}), KCl (0.7 g L^{-1}), NaCl (0.3 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}), EDTA (2 mg L^{-1}), $FeCl_2{\cdot}4H_2O$ (1.5 mg L^{-1}), H_3BO_3 (0.06 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (0.1 mg L^{-1}), $CoCl_2{\cdot}2H_2O$ (0.12 mg L^{-1}), $ZnCl_2$ (0.07 mg L^{-1}), $NiCl_2{\cdot}6H_2O$ (0.025 mg L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.02 mg L^{-1}) and $NaMoO_4{\cdot}2H_2O$ (0.025 mg L^{-1}). For MSM solid plate, extra agar (15 g L^{-1}) was added along with the above chemicals. For concentrated MSM, the concentration of the chemicals dissolved was doubled as MSM solution. Luria-Bertani (LB) media for producing *P. putida* F1 culture to anchor biomass onto cellulosic carbon fibers consisted of NaCl (10 g L^{-1}), peptone (10 g L^{-1}), and yeast extract (5 g L^{-1}). Toluene was purchased from Fisher Scientific (Waltham, MA). All the other chemicals were purchased either from Fisher Scientific or Sigma-Aldrich (St. Louis, MO).

2.2 Strain and Cultivation

Pseudomonas putida (Trevisan) Migula (ATCC 700007TM) was selected as a model bacterium in this study due to its ability of consuming toluene (Zylstra et al., 1988). The strain was preserved in a -70 degC refrigerator. To reactivate *P. putida* F1, a 2 L glass bottle containing 200 mL of MSM was inoculated with 1 mL of preserved strain and capped with a Teflon-lined silicon septum having a glass bulb with a small hole penetrating through. Toluene vapor was supplied continuously through the small hole on the glass bulb as the sole energy and carbon source (Claus & Walker, 1964). The bottle was then incubated in a shaker having 150 rpm and 30 under aseptic conditions for 24 hours. For further use, the activated *P. putida* F1 was inoculated onto an MSM solid plate and stored in a 4 degC refrigerator.

2.3 Preparation of Carbonized Cellulosic Fiber (CCF)

Woven gauze sponges (10 x 10 cm, 100 % cotton) were selected as the cellulosic material for carbonization as it has uniform structures and high purity and requires no pretreatments. It was later carbonized in a high-temperature tubular furnace (Sentro Tech., Model STT-1200-3.5-12, Cleveland, OH). A typical cellulosic carbon fiber (CCF) production process was described in our previous work (Wang et al., 2021). In summary, gauze sponges were placed in the high-temperature tubular furnace and then heated up to 250 for 3 hours with argon fed to the furnace at a flow rate of 500 mL/min. The temperature of the furnace was then increased to 650 for 4 hours and the feed was changed to a hydrogen and argon gas mixture using flow rates of 150 mL/min and 450 mL/min, respectively. Subsequently, the furnace was further heated up to 850 and was held for 30 min under argon only. The chamber was finally cooled down to room temperature at the end of the process. The strips of CCF were then removed from the furnace and weighted. The weights were adjusted to 0.17 g by removing excess CCF if needed.

2.4 Analytical Methods

For GC analysis of toluene and CO_2, the concentrations of toluene and CO_2 were analyzed in an Agilent 6890N Gas Chromatograph equipped with FID and TCD detectors (Agilant.inc.; Santa Clara, CA). A capillary HP-PLOT/Q column (Agilent, Santa Clara, CA) with a length of 30 m and a diameter of 0.530 mm was used for toluene and CO_2 determination. For toluene analysis, the temperatures of the oven, injector, and FID detector were maintained at 60 degC, 220 degC, and 260 degC, respectively. For CO_2analysis, the temperatures of the oven, injector, and TCD detector were maintained at 60 degC, 220 degC, and 250 degC, respectively.

Scanning electron microscopy (SEM) images of the CCF-supported biofilms were taken with a HITACHI S3500N microscope (Hitachi High Technologies America, Inc.; Schaumberg, Illinois), using 5-10 kV accelerating voltage. Sub-milligram selections of CCF were taken from samples. The sub-samples were then fixed, dried, and dehydrated according to reference (Hazrin-Chong & Manefield, 2012), after which they were coated with 2.5 nm of gold-platinum by the mean of a Cressington 108 Auto sputter coater (Watford, England).

2.5 Preloading *P. putida* F1 on CCF

MSM media was inoculated with *P. putida* F1 and incubated overnight. The OD of the culture was then adjusted to 0.5 for later use. For preloading *P. putida* F1 onto the CCF, five 50 mL centrifuge tubes containing 0.17 g of the CCF were autoclaved and then inoculated with 40 mL of the culture. Then four of the tubes were supplemented with either 2 mM sucrose, 2 mM cystine, 0.4 % (wt/v) citric acid, and 0.5 % (wt/v) NaCl (Chang et al., 2007; Dahlstrom et al., 2018). Finally, the tubes were statically grown (without shaking) for 48 hours at 30 for biofilm formation.

LB media was inoculated with *P. putida* F1 and incubated overnight. The OD of the culture was then adjusted to 0.5, 1.0, and 5.0, respectively, for later use. For anchoring *P. putida* F1 onto the CCF, four 50 mL centrifuge tubes containing 0.17 g of the CCF were autoclaved. Then three of the tubes were inoculated with 40 mL of one of the three LB based cultures. Finally, the tubes were statically grown (without shaking) for 48 hours at 30 for biofilm formation.

For *P. putida* F1 biomass density analysis, biomass concentrations in liquid phase were determined by measuring the turbidity of 1 ml of samples of media at 600 nm with a Varian Cary(r) 50 UV-Vis Spectrophotometer (Varian, Inc.; Lake Forest, CA). The two values were correlated using a regression line that was made with standards of*P. putida* F1 during log-phase growth which were diluted to specific turbidity readings and then dried at 90 in glass tubes overnight and weighed. A linear fit with an R^2 of 99.9 % was achieved with triple batches and found a 0.435 optical density correlated to 1 mg ml^{-1} of dry cell weight between an optical density of 0.2 to 1.

Biomass loading of supported *P. putida* F1 biofilms was obtained by monitoring dry weight of the samples. After being treated with either the MCM culture or the LB culture, the samples were removed from the

centrifuge tubes and washed twice using deionized water. The samples were then placed under 90 in an oven for overnight drying before being further conditioned at room temperature for 30 min. The dry weight of*P. putida* F1 on CCF was calculated based on mass balance using dry weight data of samples before and after preloading.

2.6 Gas-phase *P. putida* F1 growth in Stationary State

The testing chamber was built using an incubator (Barnstead Lab-Line, Inc.; Melrose Park, Illinois) which allows tubing connections. Toluene vapor was supplied into the testing chamber through the tubing and the waste gas was removed on another side. The testing chamber was also equipped with a humidifier (Great Innovations, LLC.; Miramar, Florida) that was used for adjusting the humidity. To verify the reliability of the testing chamber, two rounds of toluene concentration tests were conducted, and each round lasted for at least five consecutive days. For monitoring the toluene concentration, air samples were collected from the testing chamber and then analyzed via GC-FID/TCD.

For testing of effect of humidity, five different tests were conducted with relative humidifies (RH) of 40, 50, 60, 70, and 80 %. Each test lasted for five consecutive days, and the toluene concentration was set as 60 ppm. Before putting *P. putida* F1 loaded CCFs the into the testing chamber, each sample was washed with MSM media twice and then left for a short duration until no droplets came from the surface. For each test, six sets of *P. putida* F1 loaded CCFs were prepared, containing a set of control samples. Each set had three pieces of the well-prepared samples. A set of samples were removed from the testing chamber every day for monitoring the biomass growth.

For evaluation of toxicity of toluene substrate, two different tests were conducted with toluene concentrations of 300 and 1200 ppm. Each test lasted for five consecutive days, and the RH was set at 80 %. Before putting *P. putida* F1 loaded CCFs into the testing chamber, each sample was washed with MSM media twice and then drained briefly until no droplets came from the surface. For each test, six sets of*P. putida* F1 loaded CCFs were prepared, containing a set of control samples. And each set have three pieces of the well-prepared samples. A set of samples was removed from the testing chamber every day for monitoring the biomass growth.

For biofilm stability test, RH and toluene concentration were set as 80% and 60 ppm, respectively. Sample preparation and cultivation protocols were the same as described above unless specified otherwise. For toluene toxicity test, the toluene was only supplied for 6 hours every single day to avoid excess toluene accumulation. For nutrition limitation test, samples were washed by the concentrated MSM media solution for providing the biofilms with more nutrition.

2.7 Long-Term Biodegradation Using Tubular Packed Bed Reactor

For tests with tubular packed bed reactor (TPBR), three pieces *P. putida* F1 loaded CCFs were produced and then placed into a tubular packed bed reactor. The CCF-biofilm was washed with MSM media twice before being loaded. The TPBR was constructed using 100 ml glass column connected with air flow of 45 +- 5 ml/minute containing toluene contaminated air of 80 +- 5 ppm and RH of 80 % at 30 . Before putting in samples, the TPBR was set for pre-running for 72 hours until the toluene concentration and RH became stable. During 240-hour operation of TPBR, influent and effluent toluene and CO_2concentrations were monitored with GC-FID/TCD as described above.

3. Results and Discussion

3.1 Factors Affecting *P. putida* F1 Biomass Preloading on CCF

Carbonized carbon fiber was chosen in this study as biofilm support considering it has large specific surface area and hydrophobic surface for hydrophobic VOC adsorption (Wang et al., 2021). In addition, hydrophobic surfaces are also considered favorable for biofilm formation as well (Farber et al., 2019). However, preloading *P. putida* F1 onto CCF have been proven challenging. It has been reported that different carbon sources might affect the initial biofilm formation on material surface (Dahlstrom et al., 2018). Accordingly, three

types of carbon sources were tested. As can be seen from Table 1 that the biomass of *P. putida* F1 preloaded on CCF showed large differences when treated in MCM-based culture containing different carbon sources. Without any carbon source (thus no cell growth), the preloaded biomass (via adsorption) is 18 +- 5 mg_{DCW} g^{-1}(Dry cell weight per gram of CCF). However, in the presence of three different carbon sources into the MSM-based culture, the preloading of*P. putida* F1 reached 35 +- 3 (Citric acid), 73 +- 6 (Sucrose), and 29 +- 4 mg_{DCW} g^{-1} (Cystine), respectively. Previous research has also shown that alginate produced by*P. putida* F1 could promote biofilm formation (Chang et al., 2007). NaCl was selected as alginate production stimulant. However, that affected adversely the preloading of biomass, indicating the alginate might not necessarily promote affinity interactions between the CCF and cells.

Overall, sucrose promoted the highest preloading of *P. putida* F1 on CCF. We also observed that *P. putida* F1 could grow faster with sucrose than other carbon sources. So the higher preloading with sucrose could be a result of partitioning effect between the liquid media and CCF support. Apparently, higher solution phase cellular concentration will drive more cell assembling on CCF. We further tested this consideration with LB culture. As a full nutrient medium, LB media can provide *P. putida* F1 with sufficient nutrition and energy sources. To test the effect of cell concentration, LB-based culturing was controlled with a broader cell concentration range (with OD varied from 0.5 to 5.0). As sown in Table 2, cell concentration is positively correlated with the preloading of biomass. With OD 5.0, the preload was as high as 121 +- 8 mg_{DCW} g^{-1}. Biomass preloading was then conducted with OD 5.0 culturing in subsequent sample preparations in this work.

3.2 Effect of humidity on the growth of CCF-supported biofilm

Water is essential to cell growth. In the absence of a bulk liquid phase media, cell growth has to rely on moisture absorption from the gas phase. Tests were conducted to examine the growth pattern of supported biofilm under different relative humidity (RH). Figure 1A illustrates the growth curve over a five-day period with 80 % RH and 60 ppm toluene. After a one-day lag phase, the sample entered an exponential phase with a specific growth rate of 0.149 day^{-1} and finally had a total biomass gain of 73 $mg_{DCW}g^{-1}$. Decreased RH resulted in reduced growth and less biomass accumulation. Figure 1B˜E illustrate the growth with 70, 60, 50 and 40% RH, respectively. At 70% RH, the samples experienced an extended lag period before the growth phase was reached. Under this condition, the biomass has a specific growth rate of 0.100 day^{-1} during the exponential phase and finally final biomass gain was 33 mg_{DCW} g^{-1}, which is substantially less compared to 80% RH. Decreasing the humidity further resulted in smaller specific growth rates during the exponential growth phase, which are 0.103 day^{-1} at 60% RH, 0.054 day^{-1} at 50% RH, and 0.062 day^{-1} at 40% RH. Besides, the corresponding total biomass gains were 33, 28, and 28 mg_{DCW} g^{-1}, respectively. These results indicate that water supply regulates the growth rate of *P. putida* F1. Similar RH dependency was also reported by Jin et al in a study of supported fungal film prepared for α-pinene treatment, which showed an optimal RH of 85 % (Jin et al., 2007).

SEM images also verified the growth of *P. putida* F1 on CCF. Figure 2 shows the SEM images taken for different biofilm preparations. As shown in Figure 2B, the sporadically anchored *P. putida* F1 cells can be observed on the surface of the CCF, while the surface of untreated samples remained smooth and clean (Fig. 2A). Biofilm growth under 80 % RH and 60 ppm toluene showed more mature biofilm development which formed a good coverage of the CCF support (Fig 2 C and D). Especially, Fig. 2D shows the texture of extracellular polymeric substances (EPS) (Nguyen et al., 2014) grown under the gaseous phase growth mode.

3.3 Effect of toluene concentration on the growth of CCF-supported biofilm

Theoretically, higher substrate concentration can promote faster biodegradation kinetics before substrate saturation is reached. However, biodegrading microbes were believed suitable for culturing at low VOC concentrations (< 1000 ppm) due to VOC toxicity (González-Martín et al., 2021). We examined biofilm growth with gaseous VOC concentrations ranged from 60 to 1200 ppm. Compared to growth with 60 ppm toluene (Fig. 1A), when the toluene concentration was increased to 300 and to 1200 ppm showed faster

growth rate (Fig. 3). As shown in Figure 3, no apparent lag phase could be observed, and the specific growth rate was calculated as 0.425 day^{-1} at 300 ppm toluene (Fig. 3A). The growth rate at 1200 ppm decreased from that to 0.348 day^{-1}. The final biomass gains for the two tests were 72 and 65 mg_{DCW} g^{-1} for 300 and 1200 ppm toluene concentration, respectively. These results showed that increasing toluene concentration above 1000 ppm might inhibited the biomass growth, agreeing with what reported previously with aqueous culturing studies (Alagappan & Cowan, 2003; Choi et al., 2008; Reardon et al., 2000).

A comparison of growth kinetics of were *P. putida* F1 observed for different cultivation modes is presented in Table 3. Compared to our optimal specific growth rate of 0.425 day^{-1}, the specific growth rate of *P. putida* F1 with same toluene concentration (300 ppm in liquid phase) were estimated as 1.52 and 2.12 day^{-1}, when analyzed using Monod model and Andrews model, respectively (Abuhamed et al., 2004; Reardon et al., 2000). It is noteworthy that it is difficult to compare directly between gaseous and liquid phase growth modes though, considering differences in substrate concentration (300ppm gas phase toluene corresponds to ˜80 ppm in water at sorption equilibrium). Nevertheless, we may conclude that the 0.425 day^{-1} specific growth rate is comparable to liquid phase growth kinetics (if estimated for 80 ppm using Monod model and data summarized in Table 3).

3.4 Long-term Toluene Biodegradation Potentials

To exam long-term biodegradation potentials, a tubular packed bed reactor (TPBR) with a continuous toluene feeding stream was built. Before packing supported biofilms in the TPBR, the reactor was tested for 24 hours to achieve a stable operation condition. The operation condition set for TPBR was 80 % RH, with a gas feeding rate fixed at 45 ml/min. Toluene biodegradation rate and CO_2 production rate were monitored during the tests (Figure 4). The biomass loadings were also measured before and after the long-term operation. Compared with the initial biomass loading of 163 $mg_{DCW}g^{-1}$, the final biomass was measured as 203 mg_{DCW} g^{-1} on CCF, and the final biomass gain was 40 mg (about 25% mass gain) over 10 days. A total toluene consumed of 47 mg. Therefore, the yield coefficient of biomass was calculated as 0.85 g/g, which agrees well with what have been previously reported (ranged 0.58 ˜ 1.28 g/g) (Abuhamed et al., 2004; Reardon et al., 2000).

At the beginning of the operation (0 ˜ 12 h), the biofilm experienced a lag phase. After that, apparent degradation of toluene took place (as reflected from the difference between the inlet and outlet toluene concentration, 12˜36 h, Fig. 4). At the same time, the outlet CO_2 increased from 202 ppm to 369 ppm. Degradation proceeded after 36 h but with a reduced reaction rate, as indicated by the narrowed gaps between feed and outlet concentrations (both toluene and CO_2). The first few hours after the lag phase should correspond the a exponential growth phase of the biofilm, for which toluene consumption and CO_2 generation rates were calculated as 2.5 mg g_{DCW}^{-1} h^{-1} and 5.8 mg g_{DCW}^{-1} h^{-1}, respectively (based on initial biomass loading of 16%-wt). Reactions exceeding 72 h appeared to be stable throughout the operation, and should echo a stationary degradation phase. The inlet and outlet toluene concentrations were around 90 ppm and 70 ppm, respectively. In the stationary phase, supported biofilms loading increased to 20%-wt, achieved a degradation rate of 1.9 mg g_{DCW}^{-1} h^{-1} and releasing 6.4 mg $g_{DCW}^{-1}h^{-1}$ CO_2 at the same time (indicating 100% conversion of toluene to CO_2).

Overall the direct gaseous phase biodegradation showed exciting enhancement in reaction efficiency if compared to degradation rates reported previously by others. The growth phase degradation rate, measured as 2.5 mg $g_{DCW}^{-1}h^{-1}$ in this work, is as high as 10-fold of what has been observed for liquid media growth-associated biodegradation rate of toluene (ranged from 0.17 to 0.46 mg g_{DCW}^{-1} h^{-1} (Díaz et al., 2008; Muñoz et al., 2008). The nongrowth-associated stationary degradation phase is a comparable reaction mode to the latex coating film of *P.putida* F1 as reported previously by Estrada et al. (Estrada et al., 2015), which enabled degradation rates (in the rage of 0.034 to 0.321 mg $g_{DCW}^{-1}h^{-1}$) that were also much lower than what is observed in this work (1.9 mg $g_{DCW}^{-1}h^{-1}$).

3.4 Biofilm Growth Limiting Factors

The above observation clearly indicated that *P. putida* F1 biofilm could enable both growth-associated and

non-growth associated degradation of toluene. The higher growth-associated degradation rate prompt us to investigate the factors that limit the duration of the exponential growth of CCF-supported *P. putida* F1 biofilm. As mentioned earlier, toluene toxicity may inhibit biomass growth, and there is a possibility for concentrated toluene accumulation under continuous feed operations considering that CCF could adsorb well toluene (Wang et al., 2021). In addition, insufficient nutrients (no nutrition amendment during the operation) could also be limiting.

To evaluate the effect of toluene accumulation, biofilm growth was tested in the chamber reactor under 80 % RH, but with limited toluene feed (supplied with 60 ppm toluene intermittently 6 h per day). In this case, excess toluene adsorbed onto the surface of CCFs would be released or degraded timely. As can be seen from Figure 5A, the *P. putida*F1 growth pattern did not change from growth with constant toluene concentration (Fig. 1A). There was a lag phase followed by an exponential phase, and finally entered a stationary phase. That process appeared to be slower, but only slightly extended. The biomass gains of*P. putida* F1 in this test was 45 $mg_{DCW}g^{-1}$, which was lower than 73 $mg_{DCW}g^{-1}$ obtained under optimal condition. This can be attributed to the less amount of toluene supply in the chamber during the growth of *P. putida* F1. This result indicates that toluene accumulation is not a factor that limits growth period of *P. putida* F1.

To test the nutrient limitation hypothesis, samples were pretreated with concentrated MSM media. Microbial growth (Fig. 5B) experienced a longer exponential phase and entered the stationary phase after 4 days. The biomass gain in this test was 136 $mg_{DCW}g^{-1}$, which was about two times greater than that we observed under standard conditions. The much increased biomass growth also become detectable with eyes (Fig. 6). As can be seen in Figure 6, there were more white accumulation appearing on doubled nutrient treated CCF than the samples normally treated. However, since the growth duration was not apparently extended, excess nutrition simply increased the overall growth rate.

In fact, previous research conducted by other researchers have also indicated biofilms did not keep growing after three or four days. One potential reason for the halt in growth could be metabolite accumulation in biofilms, as toluene degradation with the *P. putida* F1 could accumulate a mutagenic metabolite benzyl alcohol (Bordel et al., 2007). From our observations, this could be the primary regulating factor, but will require more systematic investigation to confirm and eventually extend the growth-associated biodegradation operations.

4. Conclusion

P. putida F1 was successfully preloaded onto CCFs and applied for airborne toluene degradation. Studies showed that the *P. putida*F1 was able to grow on the CCF support and eventually formed mature biofilms under feeding with gaseous toluene. The CCF-supported *P. putida* F1 biofilms can grow at the RH range from 40 to 80% and the biomass grow faster at higher humidity levels. The optimal specific growth rate of *P. putida* F1 in gas phase was actually comparable to that in liquid phase. The CCF-supported *P. putida* F1 enable both growth-associated and non-growth associated biodegradation activities. Operated without any bulk liquid medium phase, the biofilm achieved direct degradation of gas phase VOC at rates of about one-order of magnitude higher than what has been previously reported for liquid culturing or immobilized cells. These results suggest that liquid phase mass transfer is a significant limiting factor for traditional biodegradation reactions, and biofilms operated in gas phases can promote efficient substrate-cell interactions and thus intensifying biotransformation reactions.

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