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≪ **Research Paper** ≫

Biodegradation Kinetics of Diesel in a Wind-driven Bioventing System

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ABSTRACT

Bioremediation, which uses microbes to degrade most organic pollutants in soil and groundwater, can be used in solving environmental issues in various polluted sites. In this research, a wind-driven bioventing system is built to degrade about 20,000 mg/kg of high concentration diesel pollutants in soil-pollution mode. The wind-driven bioventing test was proceeded by the bioaugmentation method, and the indigenous microbes used were *Bacillus cereus, Achromobacter xylosoxidans,* and *Pseudomonas putida.* The phenomenon of two-stage diesel degradation of different rates was noted in the test. In order to interpret the results of the mode test, three microbes were used to degrade diesel pollutants of same high concentration in separated aerated batch-mixing vessels. The data derived thereof was input into the Haldane equation and calculated by non-linear regression analysis and trial-and-error methods to establish the kinetic parameters of these three microbes in bioventing diesel degradation. The results show that in the derivation of μ_m (maximum specific growth rate) in biodegradation kinetics parameters, K_s (half-saturation constant) for diesel substance affinity, and K_i (inhibition coefficient) for the adaptability of high concentration diesel degradation. The K_s is the lowest in the trend of the first stage degradation of *Bacillus cereus* in a high diesel concentration, whereas K_i is the highest, denoting that *Bacillus cereus* has the best adaptability in a high diesel concentration and is the most efficient in diesel substance affinity. All three microbes have a degradation rate of over 50% with regards to Pristane and Phytane, which are branched alkanes and the most important biological markers.

Key words : Passive bioventing, *Bacillus cereus*, *Achromobacter xylosoxidans*, *Pseudomonas putida*, Haldane equation

1. Introduction

The causes of oil pollutions include oil leakage from corroded underground oil tanks, oil pipe breakages, accidental oil leakage above ground and illegal discharge or buried waste oil, which result in severe environmental pollution such as oil leaks into the soil or groundwater. Take diesel pollution as an example: diesel primarily consists of alkanes and aromatic hydrophilic organic compounds, the carbon content of which is around C10-C40 and is non-polar in its physico-chemical properties. Therefore, oil products can be treated as hydrophobic non-ionic organic compounds. Use of the methods of physico-chemical treatment for the handling of oil-polluted soil is universal, but often results in secondary pollution, not to mention the added disadvantage of high energy costs and expenses. On the other hand, bioremediation is a low energy consumption remedial technology (Brown et al., 1996), which is often used as a remedy for pollution from gas stations and oil refineries (Kumar et al., 2011).

Bioventing converts the polluted soil into a ventilated environment by supplying oxygen and nutrients, which promotes the growth of aerobic microbes that primarily degrade oil pollutants (Leeson and Hinchee, 1997). In general, high concentrations of petroleum hydrocarbons in excess of 25,000 mg/kg, or heavy metals in excess of 2,500 mg/kg, in soils are considered inhibitory and/or toxic to aerobic microbes (USEPA, 2004). While under the green remediation concept, passive bioventing utilizes the wind-driven bioventing system to degrade diesel pollutants in cases of soil pollution. Therefore, a wind-driven bioventing system is established in this research to explore the efficiency of degrading high-concentration diesel by specific microorganisms in a bioventing system. The wind-driven bioventing

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test was proceeded by bioaugmentation, using indigenous microbes of *Bacillus cereus*, *Achromobacter xylosoxidan* and *Pseudomonas putida*, all of which were verified as having a good diesel degradation capability (Colla et al., 2014; Mariano et al., 2008; Moon et al., 1997; Nielsen et al., 2006; Szulc et al., 2014). Nine bacterial strains including *Bacillus subtilis* were isolated from the polluted stream which could degrade crude oil (Adebusoye et al., 2007). Szulc et al. (2014) demonstrated that bioaugmentation of *Pseudomonas putida* contributed to the highest diesel oil biodegradation efficiency, even the benefits of bioaugmentation was found in a diesel-polluted clay-rich soil (Masy et al., 2014). *Pseudomonas putida* was selected as a coculture to degrade benzene, toluene, ethylbenzene, and xylenes under various growth conditions (Shim et. al., 2005). However, it is often seen that pollutants in high concentration diesel or particular components are difficult to biodegrade and tend to suppress the activity of microbes leading to a slow degradation condition as well as multi-stage diesel degradation of a different rate.

2. Materials and Methods

2.1. Wind-driven bioventing pilot

In this study, a 106 Liters (53 cm in diameter and 63 cm in height) plastic barrel was used as the bioventing pilot for soil pollution. The soil used in the experiment was obtained from an unpolluted area in Taichung, Taiwan. The soil collected in this study contains sand 83%, silt 7% and clay 10%, which is categorized as sandy loam type, and the pH of soil is 7.95. The soil was then surrogated to a condition of about 20,000 mg/kg concentration of diesel pollution, and the contaminated soil in each bioventing pilot was surrogated with individual microbe, one blank set without surrogating microbe was used to compare the degradation efficiency. Each bioventing pilot consists of a 50 cm thick polluted soil layer and a 10 cm thick surface soil layer, the sources of both polluted soil and surface soil are identical. The bulk density of the soil is 1.4-1.5 g/cm^3, and total 120 kg soil was packed in the plastic barrel. In the center of the bioventing pilot, there was a PE casing with ventilation screen of 2 inches in diameter, which was surrounded by quartz sand in order to induce the air with oxygen into the

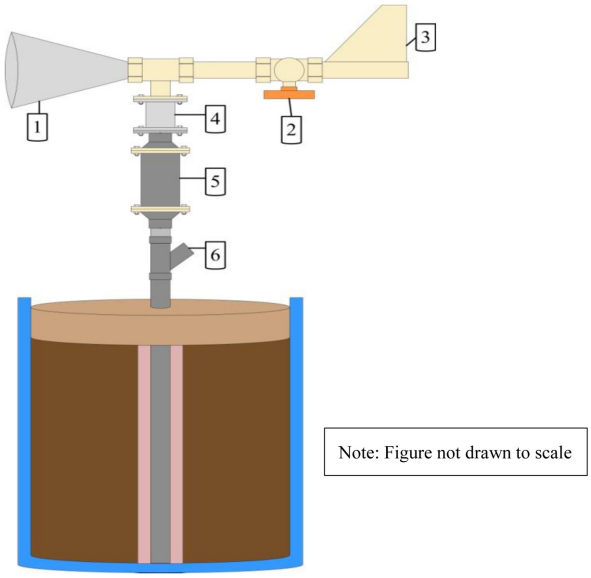

Fig. 1. Sketch diagram of wind-driven bioventing system.
1: Air inlet, 2: Flow-regulating valve, 3: Fin, 4: Rotary shaft, 5: Check valve, 6: Y-type air monitoring pipe.

polluted soil layer. Once the soil conditioning was completed, the voids around barrel were sealed to prevent air from entering, so as to simulate the underground anaerobic environment. The ventilation test was not initiated until the oxygen concentration was confirmed as being less than 15% by instrumental measurement. The self-developed wind-driven bioventing system used in the soil was connected to the duct to aerate the soil layer at a wind rate of 0.6 m/s. The bioventing system includes an air inlet, air flow-regulation valve, a wind fin, rotary shaft as well as the check valve that prevent VOCs emission. A diffusion radius test indicated a drastic increase in oxygen concentration. The illustration of wind-driven bioventing system is shown in Fig. 1.

2.2. Selection of microbes

Based on the previous research, three species of indigenous microbes with diesel degradation ability were selected for the study; *Bacillus cereus*, *Achromobacter xylosoxidans* and *Pseudomonas putida*. After activation, microbes were cultured in a commercially available Luria-Bertani (LB) liquid medium, and the amount of growth was assessed by optical density. Once the optical density maintained at 0.761, 0.698 and 0.564 for *Bacillus cereus, Achromobacter xylosoxidans* and *Pseudomonas putida*, respectively, the

quantity of microbes became stable, the microbes were transferred into M9 medium before introduced into the wind-driven bioventing pilots and aerated batch-mixing vessels.

2.3. Microbial growth medium

The microorganism culture medium used in this study include a LB medium, the formula includes Tryptone 10 g, yeast extract 5 g and NaCl 5 g, which were all mixed with 1 L water. The LB medium was then autoclaved to sterilize for 15 minutes at 121°C. Moreover, a M9 medium was prepared with 10.5 g broth powderin 1 L water and additional supplements including 1 M $MgSO_4$ 2.0 mL, 20% Glucose 10 mL, and 1 M $CaCl_2$ 0.1 mL; the above supplements were sterilized separately followed by filtration prior to being added to the medium.

2.4. Aerated batch-mixing vessels experiment

In order to understand the diesel degradation phenomenon in wind-driven bioventing pilots, each of the three indigenous microbes was also added to the soil containing high concentration diesel in an individual aerated batch-mixing vessel. The biodegradation experiments were conducted under controlled conditions. In the vessel test, there were no nutrients provided to avoid interference, but the air was supplied during the mixing. In this experiment, a 2 Liters PE barrel (15 cm in diameter and 15 cm in height) was used as the aerated batch-mixing vessel for treating 1 kg contaminated soil. The soil obtained was sterilized at 1.2 kg/cm^2 and 121°C for 15 minutes, and then diesel was surrogated and mixed to create a polluted soil at a target pollution concentration of about 20,000 mg/kg. Thereafter, the microbes (1% v/w soil) were added to the polluted soil for the aerated batch-mixing test in order to simulate the passive bioventing condition. The soil was mixed on a daily basis in order to maintain an oxygen concentration of 20% to provide sufficient oxygen for the microbes. During the mixing process, the water content was controlled at 8%-10% by spray to maintain the activity of microbes. At the beginning of the test, the soil samples were collected every three days until the degradation trend became slower, then the sampling schedule was changed to a weekly basis. The diesel of each soil sample was extracted by ultrasonic extraction and analyzed in duplicate by gas chromatograph and flame ionization detector (GC/FID), the concentration of diesel was determined based on the analytical results of C10-C25, Pristane (Pr) and Phytane (Ph) which are branched alkanes and the most important biological markers (Makeen et al., 2015).

2.5. Kinetic model and parameter calculation

As the growth of microbes is restrained during the process, if the petroleum hydrocarbons concentration is close to the bioventing limitation 25,000 mg/kg (USEPA, 2004) and thus causes the microbial growth rate equation, the Monod equation no longer applies (Han and Levenspiel, 1988), but only the modified Haldane equation can be used to determine the growth kinetics of microbes (Sponza and Isik, 2004; Yang and Humphrey, 1975). In this study, the Haldane kinetic model was used as the basis and was further integrated to obtain the matrix equation of concentration versus time using nonlinear regression analysis of experimental data sets adapted to the equation in order to obtain the relevant kinetic parameters. In the appropriate concentration range, the dynamic behavior of microbial growth can be presented by the Monod equation (Rittmann and McCarty, 2001) in the following equation (1):

$$\mu = \mu_m \frac{S}{K_s + S} \tag{1}$$

Where μ is specific growth rate (1/d); μ_m is maximum specific growth rate (1/d); S is timely substrate concentration (mg/kg); and K_s is half-saturation constant (mg/kg).

The specific growth rate represents the biomass produced per unit time per biomass. The half-saturation constant is the substrate concentration at half the maximum specific growth rate. The greater the half-saturation constant, the higher the substrate concentration required for the maximum specific growth rate, which means that the substrate affinity of this microorganism is low. On the contrary, the smaller the half-saturation constant, the higher the substrate affinity, which means that the microorganism is suitable for handling the specific matrix. The definition of specific growth rate (μ) and the specific substrate utilization rate are shown in equation (2). If equation (2) is combined with equation (1), then the differential equation of Monod is obtained as equation (3). Equation (3) is further

integrated with separate variables to obtain equation (4).

$$\mu = Y \cdot q = Y \cdot \frac{-dS}{X \cdot dt} \tag{2}$$

$$\frac{dS}{dt} = \frac{-\mu_m \cdot S \cdot X}{(K_s + S) \cdot Y} \tag{3}$$

$$K_s \cdot \ln\frac{S}{S_0} = \left(S_0 + \frac{X_0}{Y} + K_s\right) \cdot \ln\left(\frac{\frac{X_0}{Y} + S_0 - S}{\frac{X_0}{Y}}\right) - \left(S_0 + \frac{X_0}{Y}\right) \cdot \mu_m \cdot t \tag{4}$$

Where X is timely biomass concentration (mg/kg) and could be written as $X = Y(S_0 - S) + X_0$; S_0 is initial substrate concentration (mg/kg); X_0 is initial biomass concentration (mg/kg); Y is biomass-yield coefficient (mg-biomass/mg-substrate); and t is experimental time (d). Degradation rate and biomass productivity rate were used to give a normalized index for evaluating the degradation efficiency of each substrate and to determine the biomass, and degradation rate is calculated as $(S_0 - S)/t$ and biomass productivity rate is $(X - X_0)/t$.

Five substrate inhibition kinetic models were compared for experimental data by Edwards (1970), and the simplified Haldane kinetic model was also suggested to interpret biodegradation data with high concentration substrate. If the systemic substrate concentration is too high or there are multiple substrates, the microbes are susceptible to inhibition, therefore the definition of specific growth rate (μ) and the specific substrate utilization rate are then shown in equation (5). The microbial growth kinetics should be represented with the modified Haldane equation as shown in equation (6).

$$\mu = \frac{\mu_m \cdot S}{\left(K_s + S + \frac{S^2}{K_i}\right)} \tag{5}$$

$$\frac{dS}{dt} = \frac{-\mu_m \cdot S \cdot X}{\left(K_s + S + \frac{S^2}{K_i}\right) \cdot Y} \tag{6}$$

Where K_i is the inhibition coefficient (mg/kg). The higher value of K_i obtained in the Haldane equation, the higher the inhibition concentration, which means a smaller inhibition of the substrate. Equation (6) is integrated similarly to the above Monod equation. By separation of variable, the integrated form of equation (6) can be described as following:

$$\left(-\frac{K_S}{Y \cdot S_0 + X_0} - \frac{1}{Y} - \frac{Y \cdot S_0 + X_0}{K_i \cdot Y^2}\right) \cdot \ln\frac{Y \cdot S_0 + X_0 - Y \cdot S}{X_0} + \frac{K_S}{Y \cdot S_0 + X_0} \cdot \ln\frac{S}{S_0} + \frac{S_0 - S}{K_i \cdot Y} = -\frac{\mu_m}{Y} \cdot t \tag{7}$$

The above parameters S_0, S_t, X_0, X and t were obtained by direct measurements in aerated batch-mixing vessels experiments, and μ_m, K_s and K_i were obtained through non-linear regression analysis and trial-and-error methods. These parameters were calculated to evaluate the change of substrate concentration (S) versus time (Lin et al., 2007), the coefficient of determination, denoted R^2 was also determined.

Because eighteen components, including C10-C25, Pristane and Phytane, were used to calculate the total concentration of diesel, the parameters in Haldane equation for petroleum hydrocarbon compounds were calculated with the assumption of non-competitive type inhibition model in this study. Uncompetitive inhibition is the substrate inhibition where the inhibitor and the substrate are the same substance. In addition, the heterogeneity of diesel and microbes in soil were ignored for developing the kinetic model.

3. Results and Discussion

3.1. Wind-driven bioventing system

This research used a self-developed wind-driven bioventing pilot system to degrade the diesel pollution at a high concentration about 20,000 mg/kg, however the detection values of initial diesel concentration was 22,428 mg/kg. The microbe of *Achromobacter xylosoxidans* is proven to have a good diesel degradation capability, the results of this research are in conformity with the findings of Ho et al. (2012). In addition, *Bacillus cereus* and *Pseudomonas putida* demonstrate effective diesel degradation capability, the similar finding were reported by Colla et al. (2014) and Szulc et al. (2014), respectively. The results also showed a frequent microbial growth suppression in the process of degradation, as shown in Fig. 2. During the 35-day diesel degradation test, the blank set without growing microbes

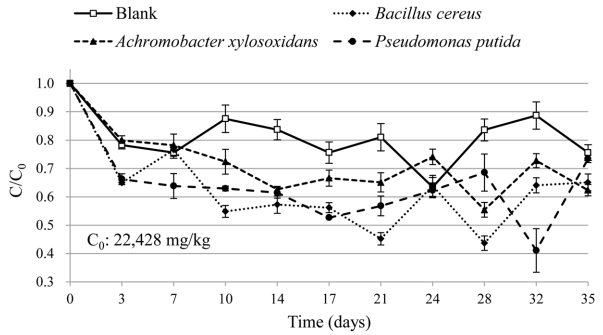

Fig. 2. Relative diesel concentration (C/C_0) vs. time with various microorganisms in the wind-driven bioventing system.

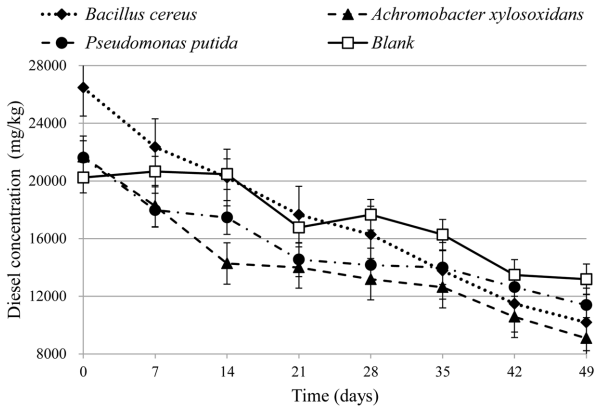

Fig. 3. Diesel concentration vs. time with various microorganisms in aerated batch-mixing vessels experiment.

showed a relative diesel concentration (C/C_0) of 0.78 on the third day, and no obvious degradation trend was observed ($C/C_0 = 0.75 – 0.90$). On the contrary, the *Bacillus cereus* set showed diesel degradation at the beginning and continued for 21 days then eventually slowed down. The lowest relative diesel concentration of 0.45 was observed in the degradation trend, while C/C_0 remained at around 0.44-0.65 after 21 days. The degradation of the *Achromobacter xylosoxidans* set is slower but more stable compared to others. The first degradation was observed after 14 days. In the degradation trend of the first 14 days, the C/C_0 decreased to 0.63. After that no further significant degradation was observed and the C/C_0 remained between 0.55-0.74. The *Pseudomonas putida* set was able to decompose the pollutants upon faster. The C/C_0 decreased to 0.66 on the third day of the test and continued for 17 days and then it slowed down. In the diesel degradation trend, the *Pseudomonas putida* microbe managed to reduce the relative diesel concentration to 0.53. All the data of the wind-driven bioventing pilot test were analyzed by using Mann Kendall test, during the bioventing process of diesel degradation, a two-stage degradation of different rate was observed.

3.2. Aerated batch-mixing vessels experiment

In the test period, control of water content ratio and the oxygen concentration detection in the sealed vessel was conducted daily and the soil samples collected regularly for a diesel concentration analysis. The aim was to observe the diesel degradation capability of the microbes. The data received from the test was input into the Haldane equation and calculated by non-linear regression analysis and trial-and-error method to establish the kinetics and parameters of these three microbes in the degradation of the diesel polluted soil. There was a total of 4 test sets supplied with oxygen through daily mixing. The test lasted 49 days, and the test results of the relative diesel concentration are shown in Fig. 3.

Although the diesel concentration 20,000 mg/kg was surrogated in soil, the detection values of diesel concentration in test set were above 20,000 mg/kg due to the heterogeneity of pollutants in soil. All the microbe test sets presented a two-stage degradation of different rates with the exception of the Blank set in the experiment. The first stage shows a result of degradation of compositions which are easy to be degraded by microbes, while other diesel compositions with a high molecular weight or branches were only degraded in the second stage. Although microbes were not added to the Blank set, there was an obvious trend of decreasing concentration levels due to the volatilization of low carbon quantity of compositions in the diesel. In contrast, the *Bacillus cereus* set presented the best diesel concentration degradation at 61.5% with a more stable degradation trend as well.

3.3. Degradation kinetics parameter deduction

The kinetic parameters μ_m, K_s and K_i of each microbe test set using the Haldane equation in equation (6) were derived and is listed in Table 1. Both the experimental data of diesel concentration (S) vs. time (t) of all microbe sets in the aerated batch-mixing vessels are given in Fig. 4, and the fitted value curve is created by using the Haldane kinetic model, which is shown in equation (7). A two-stage degradation trend of diesel concentration vs. time was observed.

Table 1. Kinetic parameters of diesel biodegradation by three indigenous microbes

Strains	Degradation stage period (days)	Degradation rate (mg/kg/day)	Maximum specific growth rate, μ_m (1/day)	Half-saturation constant, K_s (mg/kg)	Inhibition coefficient K_i (mg/kg)	R^2
Bacillus cereus	0-28	529.1	0.138	1,000	2,800	0.9891
Bacillus cereus	28-49	148.3	0.075	4,500	400	0.9942
Achromobacter xylosoxidans	0-14	363.9	0.10	1,000	1,900	0.9995
Achromobacter xylosoxidans	14-49	290.2	0.20	2,000	460	0.9900
Pseudomonas putida	0-28	265.8	0.08	4,000	2,300	0.9977
Pseudomonas putida	28-49	213.1	0.09	2,000	800	0.9818

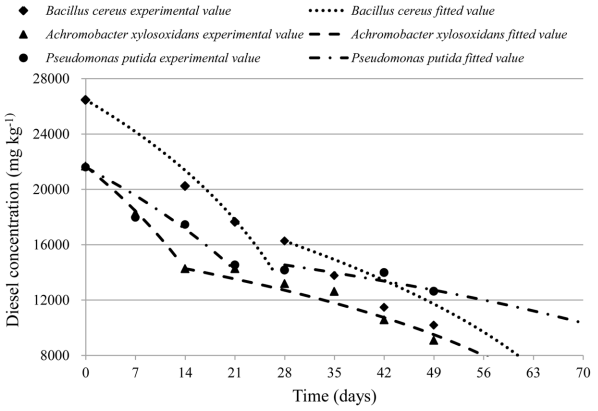

Fig. 4. Diesel concentration data and Haldane kinetic model fitted values of three strain sets in aerated batch-mixing vessels experiment.

The *Achromobacter xylosoxidans* set first presented second stage degradation on day 14, while the *Bacillus cereus* set and *Pseudomonas putida* set presented the same on day 28. The highest degradation rate in the first stage appeared in the *Achromobacter xylosoxidans* set at a level of 529 mg/kg/d, which represents a lower half-saturation constant K_s, in *Achromobacter xylosoxidans* set than the other sets and a higher substrate concentration that is required for the growth of microbes. Therefore, the second stage degradation of *Achromobacter xylosoxidans* set was observed earlier than in the *Bacillus cereus* and *Pseudomonas putida* sets. Furthermore, the maximum specific growth rate of the second stage is 0.20 and is relatively higher, representing that the *Achromobacter xylosoxidans* set can degrade the diesel concentration more rapidly if the diesel pollution concentration is less than 15,000 mg/kg in the second stage.

The *Bacillus cereus* set had the highest final diesel degradation rate at 61% on the 49th day, used less substrate than *Achromobacter xylosoxidans* and was also relatively less suppressed. However, after the first stage degradation, the maximum specific growth rate decreased, denoting that the *Bacillus cereus* set can consistently degrade its diesel concentration at a diesel pollution concentration above 20,000 mg/kg. The *Pseudomonas putida* set presented better kinetic parameters at the second stage than that of other sets but with the lowest final diesel degradation rate, possibly caused by the poor degradation of the high molecular weight diesel compositions in the *Pseudomonas putida* set.

Gas chromatographs of diesel components during biodegradation in different stages are shown in Fig. 5. Both the *Bacillus cereus* and *Pseudomonas putida* sets presented the second stage of degradation on test day 28 and shared similar degradation trends. All three test sets displayed a decreasing trend of low carbon number compositions (C10-C15) in the GC chromatographs during the first stage. It is noted that the organics of the low carbon number compositions had already decomposed prior to the beginning of second stage, and the major peaks drastically decreased. During the process of diesel biodegradation, the low carbon number and straight-chain hydrocarbon compositions evidently decreased after the first stage degradation. From the GC chromatographs of the day 49, all test sets presented over 50% degradation efficiencies for the recalcitrant Pristane (Pr) and Phytane (Ph).

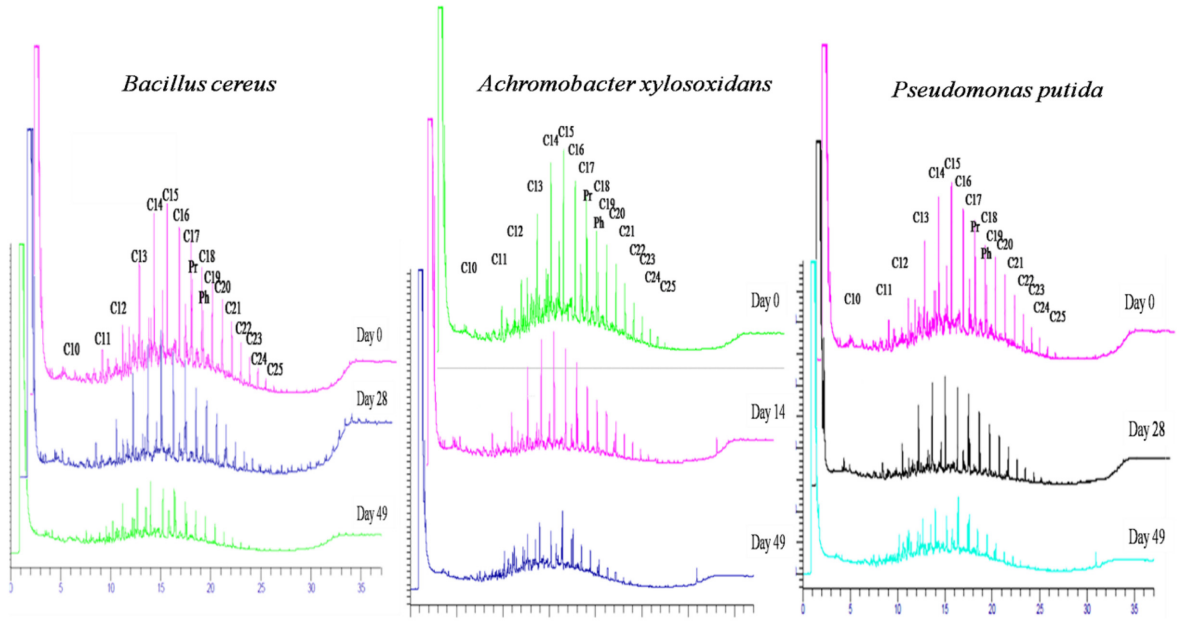

Fig. 5. Gas chromatographs of diesel components during biodegradation in different rate stages.

4. Conclusion

From the test results, it can be concluded that the activity of aerated microbes can be increased by providing sufficient oxygen in the wind-driven bioventing system to facilitate diesel degradation capabilities using specific microbes. However, after three weeks the diesel degradation trend slowed and developed a different degradation trend at different rates. The results of the aerated batch-mixing vessels test show that the diesel degradation rate of all microbe test sets was 13%-27% higher than that of the Blank set, denoting that adding proper diesel degradation microbes is relatively effective in remedying diesel-polluted soil. From the GC/FID chromatograph, it was discovered that all the three microbe sets were unlikely to degrade C14-C20 compositions, Pristane and Phytane until the second stage of the degradation process where the microbes start degradation process rapidly. The results of kinetics deduction show that the K_s in the trend of the first stage degradation of *Bacillus cereus* in a high diesel concentration is the lowest, while K_i is the highest, denoting that *Bacillus cereus* has the best adaptability in a high-diesel concentration and is the most efficient in a diesel degradation.

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