Comparative Adaptation of B. Subtilis and P. Aeruginosa in Diesel Supplemented Medium and Impact on Biodegradation Potential

Elvis Fosso-Kankeu, Sanette Marx, and Antonie Brink

Abstract—The degree of diesel blending is likely to influence its toxicity as well as the susceptibility and speed of biodegradation. It is therefore imperative to identify strains that can degrade diesel blend in record time, such as to minimize the impact on the environment. In this study the response of Bacillus subtilis and Pseudomonas aeruginosa to induced tolerance to diesel is comparatively analyzed and the implication on the biodegradation of blended diesel investigated using the gravimetric analysis method.

Results show that biodiesel promotes the degradation of diesel blend; however the induction of resistance affects differently the biodegradation potential of B. subtilis and P. aeruginosa. From two weeks to eight weeks induction time, there was increase of the biodegradation capabilities of B. subtilis, 213 % for B0, 24 % for B10 and 62 % for B50, while the biodegradation potential of P. aeruginosa decreased: 17% for B0, 60% for B10, 51% for B50.

The induction of tolerance to diesel could therefore be exploited to improve the biodegradation potential of B. subtilis, but further treatment will certainly be required for P. aeruginosa.

Keywords—Blended diesel, biodegradation, B. subtilis, P. aeruginosa, induced tolerance.

I. INTRODUCTION

PETROLEUM mainly consists out of saturated and unsaturated aliphatic and cyclic hydrocarbons. The ecotoxicological impact of fossil fuels on a marine environment is both biochemical and psychical. According to Baawain et al. [1] biochemically there is an accumulation of polyaromatic hydrocarbons (PAH’s) in marine organisms and can greatly affect the hematopoietic, immune, reproductive and neurological systems. Physically crude oil has a high viscosity and can cover the protective layers of microbial species [2].

Biodiesels would prove a better alternative to conventional diesels when considering aspects such as sustainability and ecological impact. From an ecological point of view the FAME and FAEE present in biodiesels tend to biodegrade a lot quicker than standard petroleum hydrocarbons.

In 2012 the South African Department of Energy announced the need to eventually consider the use or commercialization of blended diesel for economic and environmental reasons. The commercialization of blended diesel, entails the existence of possible environmental pollution risk related to the spill of blended diesel.

Microorganisms can be used to turn hazardous hydrocarbons into biomass and CO2 during a bioremediation process. Certain microorganisms in the field have the capability to build resistance against the inhibitory effects of petroleum based substances. Using Pseudomonas aeruginosa ZJU collected from Shengli oil field previous authors showed that Pseudomonas aeruginosa species not producing rhamnolipids could alter the gene producing the rhamnolipids when exposed to insoluble crude oil to produce more rhamnolipids [3]. Therefore Pseudomonas aeruginosa ZJU could build up resistance to the toxicity of crude oil and become more active in the degradation of diesel/biodiesel blends.

The main environmental concern considering conventional diesel is the low biodegradation rate in comparison with the alternative biodiesel source. Microbial species previously exposed to diesel can develop ability suitable for biodegradation of diesel and biodiesel. However, the development of such ability may vary among species. Hence the need to comparatively investigate such potential among microbial species.

II. METHODOLOGY

A. Diesel and biodiesel

The diesel used in this study was purchased from a local petrol pump station; the biodiesel was prepared using sun flower oil purchased from a local shop and an alkaline catalyst.

B. Induction of resistance

Stock cultures of Pseudomonas aeruginosa and Bacillus subtilis species from nutrient agar (composition in g.L-1; meat extract: 1.0; peptone: 5.0; yeast extract: 2.0; sodium chloride:8.0; Agar: 15.0) were inoculated in 50 ml nutrient broth (composition in g.L-1; meat extract : 1.0; peptone: 5.0;yeast extract: 2.0; sodium chloride:8.0), then incubated overnight at 30°C in a shaking incubator (160 rpm). The culture was transferred into 50 ml centrifuge tubes and
centrifuged at 4000 rpm for 5 min to harvest the cells from nutrient broth. The cells in the centrifuge tubes were washed several times with sterile distilled water to remove the residual broth, then the cells were suspended into 30 mL of Bushnell Haas (BH) medium. Equivalent volume of diesel was added to make up a final concentration of 70% and 1 % (v/v) diesel in the BH medium. The cells were exposed-1% (v/v) diesel for 2 and 8 weeks, while the exposure to 70 % (v/v) diesel lasted for 2 weeks. The mixtures were incubated at room temperature (25 °C) and gently mixed daily. The cells were transferred to fresh BH medium every 2 weeks.

C. Quantification of cells

The cells were quantified by determining the absorbance and by counting the colonies.

The absorbance was measured at 600 nm using the spectrophotometer. An aliquot of 1 ml of inoculum was serially diluted in sterile distilled water and 100 µl inoculated in freshly prepared agar plates, then incubated at 30°C for 24 h; the number of colonies was expressed as colony formed per unit (CFU).

D. Gravimetric analysis and extraction

To monitor the biodegradation rates of diesel blends by induced species; the species were added to 50 ml of Bushnell Haas (BH) medium containing 1 g of blended diesel (B0, B10, B50, and B100); the mixture contained in a 250 ml Erlenmeyer flask was incubated in an incubator with shaker (105 rpm) at 30 oC for 7 days.

The amount of biodegraded blended diesels was determined by adding 5 ml n-hexane to the remaining mixture in the 250 ml Erlenmeyer flasks which were transferred to a separation funnel. This process is carried out twice to ensure complete extraction. The separated extracts were treated with 0.4 g Na2SO4 which removes the excess water present in n-hexane. The treated extract was decanted, leaving the salt behind. The n-hexane was removed from extract in a rotary evaporator operating at 40 °C and under reduced pressure. The weight of the residual blended diesel was measured.

III. RESULTS AND DISCUSSION

A. Induction and adaptation

Both P. aeruginosa and B. subtilis were exposed to 1 % and 70% (v/v) of diesel mixture to induce metabolic reactions that will enhance their adaptation to the milieu. Cell growth was monitored during the 2 weeks and 2 months incubation period and expressed as CFU counts. A small decline of the growth of the two species was observed after two weeks, but the cells quickly recovered and reached the initial count or more after eight weeks for P. aeruginosa and B. subtilis respectively. Table 1 shows the CFU counts for both species.

<table>
<thead>
<tr>
<th>Exposure Time(Weeks)</th>
<th>P. aeruginosa</th>
<th>B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st set of experiment 1% diesel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>58 x 10^6</td>
<td>8 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td>16 x 10^7</td>
<td>14 x 10^7</td>
</tr>
<tr>
<td>8</td>
<td>2 x 10^7</td>
<td>6 x 10^7</td>
</tr>
<tr>
<td>2nd set of experiment 1% diesel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>43 x 10^6</td>
<td>1 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td>123 x 10^6</td>
<td>3 x 10^7</td>
</tr>
<tr>
<td>3rd set of experiment 70% diesel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2 x 10^7</td>
<td>22 x 10^6</td>
</tr>
<tr>
<td>2</td>
<td>130 x 10^6</td>
<td>4 x 10^6</td>
</tr>
</tbody>
</table>

The initial decline in viable cell growth in both species may be ascribed to the change of substrate and the possible inhibition of cells by the diesel which contains toxic trace elements such as heavy metals [4]. Hydrocarbons in fossil fuels are reported to alter microbial membrane structures by changing membrane fatty acids and protein composition [5]. It has been reported [4, 6] that contamination of diesel results in a less diverse microbial communities in the ecosystem due to the susceptibility of microorganisms to stressful conditions; but as observed in this study, microbial communities recover following adaptation [7].

There effect of diesel concentration was not conclusive as the inhibition of P. aeruginosa increased with the diesel concentration while the opposite trend was observed with B. subtilis. It was observed that P. aeruginosa was more susceptible to the presence of diesel than B. subtilis which is likely due to the fact that the latter are likely to form endospores when exposed to stressful conditions [8].

B. Effect of induction on the biodegradation potential

The % biodegradation were calculated as follow;

\[
\text{Biodegradation(\%)} = \frac{m_{\text{deg}}}{m_{\text{oil added}}} \times 100
\]

Where \( m_{\text{oil added}} \) is the mass (g) of the oil added to the Erlenmeyer flasks, \( m_{\text{deg}} \) is the mass (g) of the residual oil.

Biodegradation potential of induced B. subtilis and P. aeruginosa

The induced cells were used to degrade various grade of blended diesel; Figure 1 shows that the degradation rate increased with the concentration of biodiesel in the blend. Diesel contains complex mixture of normal, branched and cyclic alkanes, and aromatic compounds; while biodiesel is composed of methyl or ethyl esters of fatty acids with low structural complexity [9]. The rate and extent of degradation...
of diesel is often limited by the high level of carbon content and low level of nutrients essential for microbial growth; on the other site based on its composition, biodiesel is easily degradable [10]. This clearly explains why the addition of biodiesel to diesel resulted in the improvement of biodegradation in this study. However, it was observed that \textit{B. subtilis} perform better than \textit{P. aeruginosa}, which is probably due to the ability of the former to tolerate the toxicity of diesel.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Biodegradation of diesel blends by induced \textit{B. subtilis} & \textit{P. aeruginosa} (2 month induction, exposure to diesel at 1\% concentration)}
\end{figure}

\textit{Effect of diesel concentration on induction effectiveness}

To determine the influence of the concentration of diesel during induction, the cells induced in 1\% and 70\% diesel mixture were used for biodegradation of blended diesel. Figure 2a shows that the cells of \textit{B. subtilis} exposed to 70\% of diesel mixture achieved the highest biodegradation rate. This could be interpreted by the fact that higher concentration of diesel may comparatively stimulate more metabolic expression enabling better tolerance and degradation of diesel.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2a.png}
\caption{Biodegradation of diesel blends by \textit{B. subtilis} induced in 1\% and 70\% diesel mixture (2 week induction, exposure to diesel at 1\%, 70\% concentrations)}
\end{figure}

A different trend was observed during the use of \textit{P. aeruginosa} cells from similar induction conditions (1\% and 70\%). Figure 2b shows that except for B0, cells induced in 1\% diesel mixture performed better than the cells exposed to 70\% mixture. Tang et al. [3] also observed that \textit{P. aeruginosa} exposed to crude oil were not able to emulsify or biodegrade crude oil, there was a need in that case to expose the cells to glycerol for stimulation of the production of the surfactant rhamnolipid useful for biodegradation. However the cells induced in 70\% diesel mixture exhibited better degradation of B0; this clearly shows that the induction has improved the

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2b.png}
\caption{Biodegradation of diesel blends by \textit{P. aeruginosa} induced in 1\% and 70\% diesel mixture (2 week induction, exposure to diesel at 1\%, 70\% concentrations)}
\end{figure}

The comparison of Figure 2a and Figure 1, could elucidate the effect on induction time on the biodegradation potential. It can therefore be seen that, from two weeks to eight weeks induction time, there was increase of the biodegradation capabilities of \textit{B. subtilis}, 213\% for B0, 24\% for B10 and 62\% for B50. It is plausible that with time \textit{B. subtilis} species improve the mechanism to tolerate diesel oil, and metabolise proteins require for the breakdown of complex molecules of the diesel. The adaptation capability of \textit{B. subtilis} is clearly illustrated in table 1 where the decrease of cell growth after two weeks is followed by a recovery after eight weeks of exposure to diesel.

This trend was however not observed with \textit{P. aeruginosa}, Figure 2b and Figure 1 indicate that the longer induction time resulted in the decrease of the biodegradation capability of \textit{P. aeruginosa}. The biodegradation rate decreased as follow; 17\% for B0, 60\% for B10, 51\% for B50. According to Tang et al. (2007) stressful conditions in the crude oil may reduce the ability of \textit{P. aeruginosa} ZJU to produce surfactants required for crude oil degradation.
IV. CONCLUSION

Addition of biodiesel to diesel has promoted the degradation of diesel blend through a plausible co-metabolism. Induced species of B. subtilis and P. aeruginosa have exhibited different behaviour with regard to the adaptation and tolerance of diesel as well as the degradation of diesel blends. Compared to P. aeruginosa, B. subtilis was found to better adapt to diesel at relatively higher concentration and prolonged time; this has resulted to improved degradation potential.

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REFERENCES


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