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Application Report

Report from

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TECHNICAL BIOCATALYSIS TUHH

Assessing biodegradation of oil with marine bacteria by monitoring O_2 and CO_2 concentrations online in a closed loop

Introduction

In April 2010, the Deep-Water-Horizon (DWH) well explosion at the Gulf of Mexico caused the worst oil spill in the deep sea to date. About 5 million oil barrels flowed during 86 d into the sea1. The effects on the marine habitat and the fate of the oil are not well understood. Bacteria play a major role in the degradation of petroleum in marine spills. The high pressure, high salinity and low temperatures in that environment make it difficult to study the oil-degradation mechanisms in situ. It is therefore important to study the mechanisms of the biodegradation of oil in the laboratory. For that purpose, we have built high-pressure reactors (400 bar) and low-pressure reactors (1 bar) where it is possible to compare the biodegradation rates of mineral oil at room condition (1 bar, 20°C) vs. those in the deep sea around the DWH well (150 bar, 4°C).

Because of the hydrophobic nature of the oil, at least two immiscible phases appear when mixed with seawater and bacteria, making it impossible to sample the system representatively. Moreover the low molecular weight components of the oil are volatized during sampling and that affects the experiment. Currently, we have been quantifying the degradation of oil indirectly by offline analysis of bacterial concentrations or by sacrificing one reactor for gas chromatography analysis per time point, which is labour intensive. In order to quantify the extent of oil biodegradation in real time, it is necessary to measure one or more of these parameters: bacterial, oxygen, carbon dioxide or oil concentration online. A continuous measurement of oxygen and carbon dioxide can be correlated to the disappearance of the oil and to the growth of the bacteria.

Research objective

The objective of this research was to monitor the rate of oil biodegradation by newly isolated marine bacteria using the oxygen and carbon dioxide BlueInOne analyzer (BlueSens gas sensor GmbH, Herten, Germany). Since the biodegradation is a rather

slow process a direct measurement of oxygen or carbon dioxide changes is not possible. Therefore, a closed loop reactor was designed in order to monitor cumulative oxygen consumption and carbon dioxide production.

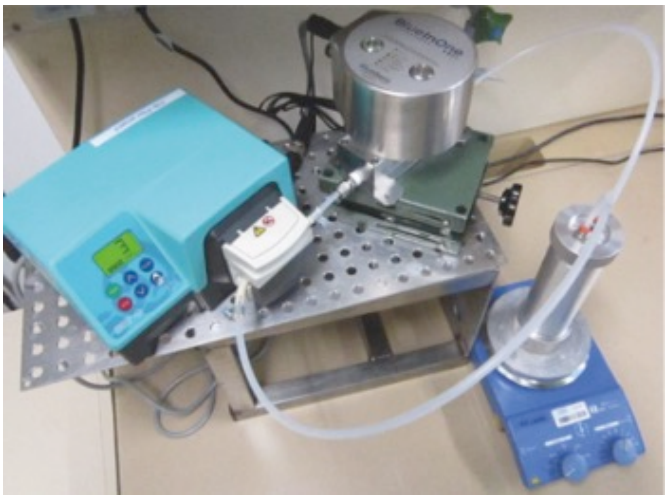

Figure 1: Closed-loop setup used for monitoring of the CO_2 and O_2 concentration during the incubation of marine bacteria with oil

Setup description

One of the low-pressure reactors was adapted with airtight connections and was connected to the BlueInOne Cell analyzer via flexible tubing (Figure 1). To transport the gas in closed-loop modus through the analyzer, a peristaltic pump recirculated the air at 3 rpm. The reactor was stirred with a magnetic stirrer. In the same way an airtight 3-port-Schott flask was connected to the analyser. Two ports were used for the gas recirculation; the third port was used for taking samples for determining the concentration of bacteria (Figure 2). A general scheme of the setup is shown in Figure 3.

Materials and methods

The oil-degrading bacterial strains used in this study were kindly provided by Prof. Joe Lepo and Prof. Wade Jeffrey (University of West Florida). They were isolated from sea samples taken in expeditions to the Gulf of Mexico before (reference strains) and after (test strains) the DWH explosion. The strains were used as inocula for the experiments. The fermentations were carried out in batch mode.

Figure 2: Closed-loop setup used for monitoring of the CO_2 and O_2 and the bacterial concentration during the incubation of marine bacteria with oil

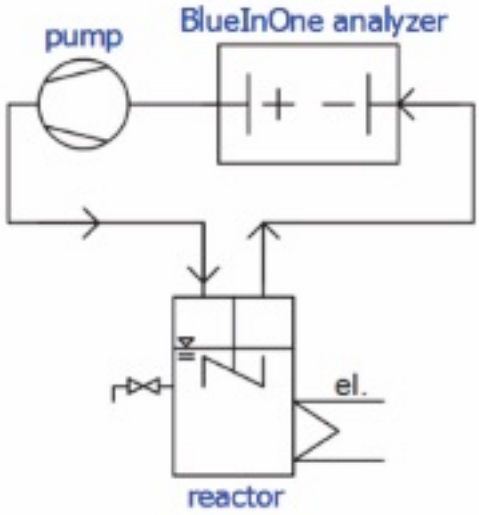

Figure 3: Diagram of the reactor setup with the BlueInOne analyzer and pump connected in a closed recirculation loop in the gas phase. A liquid sampling port as used in the setup of Figure 2 is depicted

In order to overcome oxygen limitation, the reactor was partially filled up to 1/4th of its volume with minimal medium and the rest with air. Sweet Louisiana crude oil or some of its main compounds: naphthalene, xylene, toluene and hexadecane were used as the only carbon source in 0.03% to 0.1% (v/v) concentrations. Finally 10% of bacterial inoculum was added. The O_2 and CO_2 values were

recorded with the BlueVis software. Samples were collected at the start and end points or in case of the setup with the sampling port, throughout the experiment in order to determine the number of bacteria. The BlueInOne analyzer was calibrated with air, when the analyzer requested it and the calibration values were automatically saved. Data storage was reliable and user-friendly.

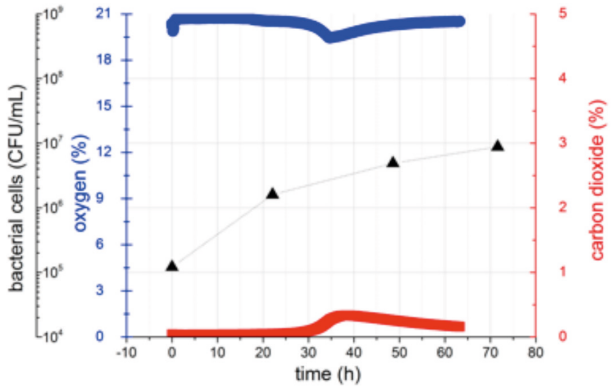

Figure 4: CO_2 (■), O_2 (●) and bacterial (▲) concentration of the -incubation of strain DWHO6A in minimal medium with 0.1% (v/v) oil

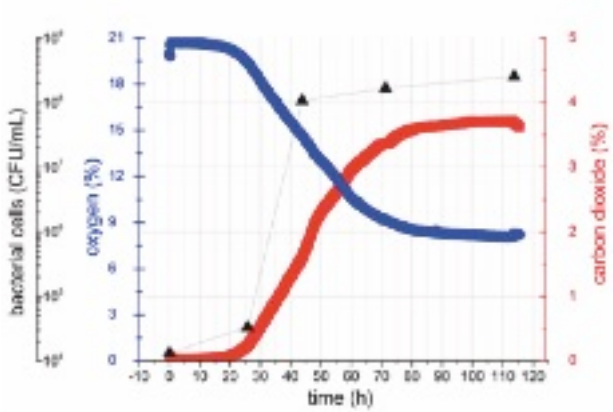

Figure 5: CO_2 (■), O_2 (●) and bacterial (▲) concentration of the -incubation of strain GM2 in minimal medium with 1 mM hexadecane

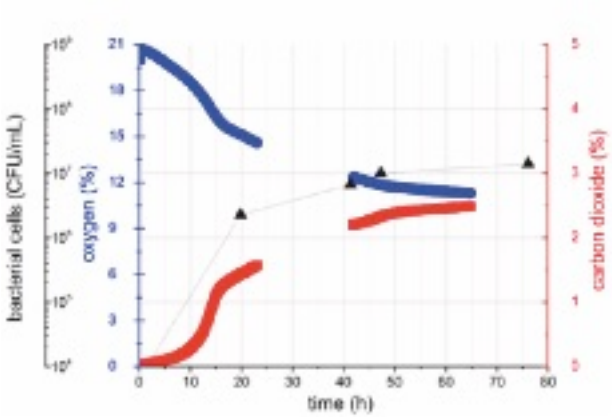

Figure 6: CO_2 (■), O_2 (●) and bacterial (▲) concentration of the -incubation of strain DF8 in minimal medium with 1 mM hexadecane

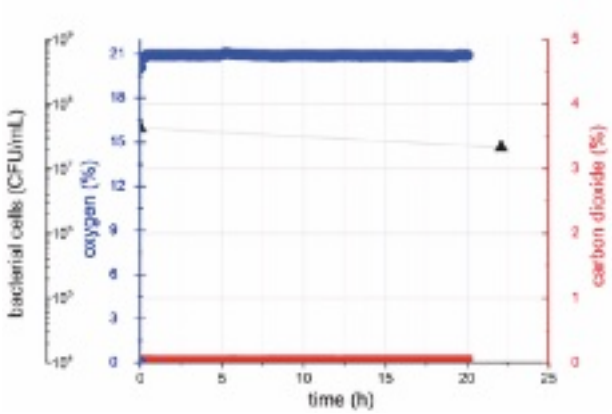

Figure 7: CO_2 (■), O_2 (●) and bacterial (▲) concentration of the -incubation of strain DS4P5 in minimal medium with 0.1% (v/v) Louisiana oil

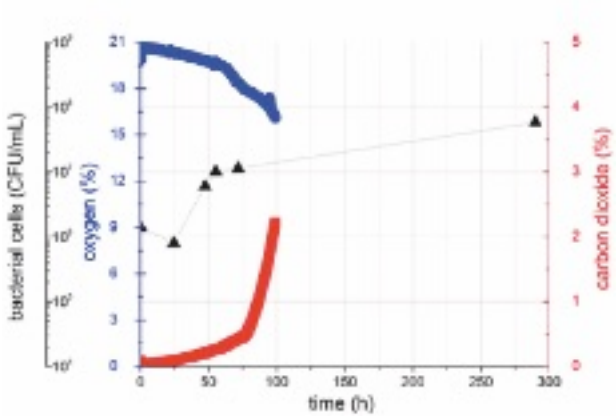

Figure 8: CO_2 (■), O_2 (●) and bacterial (▲) concentration of the -incubation of strain DWHO6B in minimal medium with 1 mM toluene

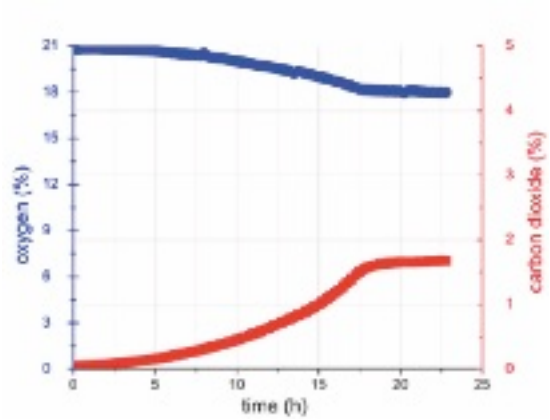

Figure 9: CO_2 (■), O_2 (●) concentration of the incubation of strain SY1
in minimal medium with 1.8 mM naphthalene

Results

Growth of oil-degrading bacteria
in oil and oil-compounds

The strain DWHO6A could degrade Louisiana oil, hexadecane, xylene and toluene on agar. In liquid medium the strain DWHO6A was able to biodegrade Louisiana oil in mineral medium as seen from the bacterial cell number of Figure 4. The system was leaking through the silicon hoses, leading to diffusing of air into the reactor. This can be observed by the increase in the oxygen concentration to its initial value of 21% after 35 h. For the next experiments the setup was air-tight, the hoses were changed to PVC material and with this, the leak was sealed.

The strain GM2 is milky coloured in appearance when grown on agar plates. GM2 showed good growth in minimal medium with hexadecane (Figure 5), oil and moderate growth with naphthalene. GM2 reached the highest bacterial cell numbers during this investigation.

The strain DF8 grew well on selective mineral medium agar supplemented with Louisiana oil and hexadecane. The results of the incubation of strain DF8 in liquid medium containing 1 mM hexadecane are shown in Figure 6. The strain DS4P5 could not degrade any of the oil components and showed residual growth on agar plates. Using Louisiana oil as carbon source this strain did not grow and the oxygen and carbon dioxide values remained constant (Figure 7). The BlueInOne analyzer helped us to corroborate the information from the bacterial density.

The strain DWHO6B is orange when grown on naphthalene agar plates and can degrade Louisiana oil, naphthalene, toluene and xylene. This strain was the only one that could degrade naphthalene in a medium supplemented with 3% NaCl, which is the concentration of salt in the open ocean. This strain can degrade toluene in liquid mineral medium as shown in Figure 8 and it can also grow well at 145 bar. Both pressure regimes gave similar bacterial cell values, but the O_2 and CO_2 cannot be analysed at the high pressure. It would be interesting to know if the rates of degradation of toluene are similar. The fermentation shown in Figure 8 was not recorded completely due to a communication error between the analyzer and the computer, but this strain could grow to $1x10^8$ with toluene as only carbon source. The degradation capabilities of this interesting strain should be further studied.

The strain SY1 (Figure 9) was able to degrade naphthalene without any lag phase, but consumes less oxygen than strains GM2, DF8 and DWHO6b do with hexadecane and toluene.

Conclusion

Spilled oil can be degraded by marine bacteria. Oxygen is a key substrate for aerobic microbial growth and biodegradation. Carbon dioxide and biomass are the major products of the biodegradation and can be used to estimate the amount of oil that has been degraded. The degradation capabilities of some deep-sea and surface-isolated bacteria could be tested and some promising strains were found. Sampling in this multiphase system was impossible and this was circumvented by the measurements with the BlueInOne analyzer. This accelerated the screening of the new strains. The use of the BlueInOne analyzer provided valuable, online and real-time information of the biodegradation of oil.

Moreover we proved that the BlueInOne analyzer can be used to measure batch fermentations with rather low metabolic rates by recirculation of the exhaust flow into the fermenter. This new application is attractive for laboratory research because the analyzer can be connected to any type of reactor or fermenter via a couple of hoses. Its application is high versatile, exact and a cost effective solution for monitoring oxygen and carbon dioxide.

Outlook

Further experiments, changing various parameters like temperature and type of strains can be conducted using the BlueSens system efficiently. This BlueInOne analyzer would also be highly useful for conducting long-term biodegradation studies.

A further useful application of the BlueInOne analyzer would be to take samples from the gas in the pressurized reactors, decompress it, and supply it to the analyzer. These data would be extremely useful for comparing the metabolism of the bacteria between atmospheric and deep-sea pressures.

References

1.
Marcia K. McNutt, Rich Camilli, Timothy J. Crone, George D. Guthrie,
Paul A. Hsieh, B. Ryerson, Omer Savas and Frank Shaffer.
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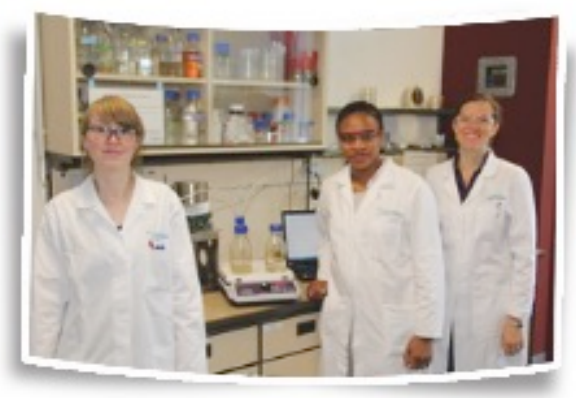

The main focus of the group environmental biotechnology led by Prof. Müller of the Institute of Technical biocatalysis lies in the elucidation of novel pathways in the biological degradation of environmental pollutants. For this purpose new bacteria with the ability to degrade problem-causing substances are isolated from environmental samples and the intermediates in the degradation pathways are determined. For interesting new reaction steps the corresponding enzymes are purified, cloned, sequenced and characterized.

Biodegradation is the key to understand the environmental fate of chemicals released into the environment either intentionally (e.g. pesticides) or by accidents (e.g. oil spills). For many chemicals the biological degradation pathways are not known yet. For others the degradation is only known under certain conditions. Therefore, research is conducted to close these gaps in our knowledge, and to find safe solutions for environmental problems.