Effect of Cyanobacterial Exudates on Aerobic Heterotrophic Bacteria

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Abstract: The effect of cyanobacteria exudates on aerobic heterotrophic bacteria was examined using cyanobacterial and bacterial consortia. Water and sediment samples were collected from Bodo creek for the isolation of aerobic heterotrophic bacteria. Physicochemical parameters of the ssamples were determined. The different species of bacteria isolated and identified using Analytical Profile Index (API) include; *Bacillus licheniformis*, *Bacillus megaterium* 2, *Bacillus subtilis*, *Enterobacter asburiae*, *Staphylococcus aureus*, *Corynebacterium kutscheri*, *Corynebacterium ulcerans*, *Staphylococcus saprophyticus*, *Aeromonas hydrophila* group 2, *Acinetobacter baumannii*, *Serratia ficaria*, and *Kocuria varians*. The cyanobacteria consortium obtained from Environmental Microbiology Laboratory, University of Port Harcourt consists of *Anabaena cicadae*, *Pseudonabaena minima*, *Laptolyngbya* sp., *Oscillatoriales cyanobacterium*, *Microcoleus* sp., *Mycrocystis holsatica*, *Mycrocystis elabens*, *Phormidium faveolaurum*, *Phormidium* sp., and *Synechococcus* sp. The total culturable bacteria counts (TCBC) increased progressively in the two treatment flasks troughout the experimental period. In flask A the TCBC increased from 3.0×10^3 cfu/ml to 10.3×10^8 cfu/ml, while in flask C it increased from 4.1×10^3 cfu/ml to 14.6×10^8 cfu/ml respectively. The difference in the bacterial counts in the two flasks was not statistically significant ($p>0.05$). The analysis of the cyanobacterial extract gave leucine, arginine, gluose, oxalic acid, malonic acid, propanoic acid, acetic acid and sucrose as exudates (metabolites). The cyanobacterial exudates obtained in this study can be utilized by bacteria which prove that cyanobacterial exudates can affect the growth rate of bacteria positively.
[Okerentugba Phillip Oritsegbubemi, Ejileugha Chisom, Okonko Iheanyi Omezuruike. **Effect of Cyanobacterial Exudates on Aerobic Heterotrophic Bacteria.** *Nat Sci* 2016;14(4):21-26]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 3. doi:10.7537/marsnsj14041603.

Keywords: Aerobic Heterotrophic Bacteria, Cyanobacteria, Effect, Exudates,

1. Introduction

Products released during metabolic processes of organisms are regarded as exudates and exudates released by one organism can be utilized by another organism along the food chain. Cyanobacteria, a group of photosynthetic prokaryotes secrete a variety of metabolites which affect other associated microorganisms including aerobic heterotrophic bacteria. Cyanobacteria in microbial mats are associated with aerobic heterotrophic bacteria and such association is sustained by the interaction among the individual species or community (microbial communities). The metabolites released by the higher microbial community are utilized by the lower microbial community in a process of syntrophy and through this interaction, they are able to attack a variety of compounds including complex compounds and they are resistant to harsh environmental conditions.

The exudates of cyanobacteria have been hypothesized to make up a higher percentage of bacterial carbon supply. Cyanobacteria discharge a diversity of organic particles and these cyanobacteria exudates can arouse or deter other members of the microbial community, heterotrophic bacteria inclusive (Kirkwood *et al.*, 2006).

The interaction of the cyanobacteria with the bacterial community through its photosynthetic products like oxygen assists the proliferation of the bacterial community while the carbon dioxide released by the bacteria through their metabolic activities is also utilized by the cyanobacteria. This may be the secret behind the strong association between these two groups of organisms.

Exudates from cyanobacteria may be amino acids, vitamins, sugars, sugar alcohols, alcohols, fatty acids, organic acids, and most of these products have been shown to be utilized by bacteria. Sugars especially, are known to be utilized by several bacterial species to derive nourishment for proliferation. Organic acids, fatty acids, sugar alcohols, including amino acids have also been shown to be utilized by bacteria. Some bacteria have been shown to grow faster on compounds known to be cyanobacteria exudates (Abed, 2010).

The use of cyanobacteria in cleanup processes to stimulate bacterial degraders is considered a cost effective means of bioremediation compared to the use of artificial fertilizers. Cyanobacteria have been used in waste water treatment processes and in bioremediation using microbial mats. Although cyanobacteria are known to be photosynthetic, the heterotrophic nature reported among some species

puts them in the list of potential hydrocarbon degraders. They are known to be attached with aerobic heterotrophic bacteria making them potential immobilizing agents on which bacteria can attach and carry out their degradation processes. The use of cyanobacteria as immobilizing agents is considered to be cost effective compared to the use of other artificial immobilizers.

The use of cyanobacteria in the cleanup of petroleum hydrocarbon polluted sites is regarded as a stimulation and augmentation process through their reported ability to release organic exudates coupled with their reported biodegradation potential. Thus, the effect of cyanobacteria exudates on aerobic heterotrophic bacteria was examined using cyanobacterial and bacterial consortia.

2. Materials And Methods

2.1. Study area: Samples were collected from Bodo creek in Bodo community, Gokana Local Government Area, Rivers State Nigeria. Ogoni land covers over 1000km^2 area of land and has been involved in oil exploration for several years. Ogoni land has also been a victim of several oil spills with or without proper remediation which makes it one of the environmentally devastated areas in Nigeria.

2.2. Sample collection: Ten samples each of water and sediment were collected. Water and sediment samples were aseptically collected from the brackish water of Bodo creek for the isolation of aerobic heterotrophic bacteria. Water samples were collected using sterile bottles while sediment samples were collected with Eckman grab sampler (Gaur et al., 2005). Samples were homogenized and transported to the laboratory for analysis.

2.3. Physicochemical analysis of samples: The following physicochemical parameters; electrical conductivity, pH, total dissolved solids, total suspended solids, total organic carbon, moisture content, phosphate, sulphate, nitrate, total nitrogen, and total petroleum hydrocarbon were determined using standard methods according to APHA, 2008.

2.4. Consortium of Cyanobacteria: The consortium of cyanobacteria used in this study was obtained from the Environmental Microbiology Laboratory, University of Port Harcourt, Port Harcourt, Nigeria. The cyanobacterial consortium consists of *Microcoleus* sp., *Anabaena cicadae*, *Pseudonabaena minima*, *Oscillatoriales cyanobacterium*, *Mycrocystis holsatica*, *Mycrocystis elabens*, *Phormidium faveolaurum*, *Phormidium* sp., *Synechococcus* sp., *Laptolyngbya* sp.

2.5. Culture medium: The consortium was cultured in BG-11 medium. The culture was incubated at room temperature near closed transparent glass window to allow rays of sunlight to reach culture. BG-11 medium used in this study is made up of the following composition: $NaNO_3$ (1.5 g), Na_2CO_3 (0.02 g), $MgSO_4.7H_2O$ (0.075 g). K_2HPO_4 (0.04 g), $CaCl_2.2H_2O$ (0,036 g), Citric acid (0.06 g), Ferric ammonium citrate (0.06 g), EDTA (0.01 g), Agar (for solid medium), trace metal mix 1.0 ml (H_3BO_3: 2.86 g, $MnCl_2.4H_2O$; 1.81 g, $ZnSO_4.7H_2O$; 0.222 g, $Na_2MoO_4.2H_2O$; 0.39 g, $CuSO_4.5H_2O$; 0.079 g, $Co(NO_3)_2.6H_2O$; 0.494 g, Distilled Water; 1000 ml), distilled water (1.0 L), pH 7.1.

2.6. Enumeration of total culturable aerobic heterotrophic bacteria (AHB): One gram of sediment and 1.0 ml of water were transferred into 9.0 ml of sterile distilled water respectively. 1.0 ml aliquot amount was aseptically transferred into 9.0 ml amount of sterile dilution blank (10^{-1} dilution). The samples were diluted in tenfold serial dilution up to 10^{-6} dilutions. Inoculation was carried out using spread plate technique with sterile adjustable pipette tips, 100 µl aliquot (0.1 ml) of inoculums from 10^{-5} dilution was inoculated onto the sterile disposable Petri dish which had been poured with nutrient agar respectively in triplicates. The plates were allowed to set and subsequently incubated in an incubator at 35 ± 2^{o}C for 24 hours. The plates were observed for growth after incubation period, colonies observed were counted using coulter colony counter. The colonies were randomly picked and their colonial morphology was recorded and was subsequently purified by sub culturing onto new agar plates. The isolates were characterized based on their cultural characteristics, cell morphology, and few biochemical tes ts. The organisms were further identified using the API Kits (Analytical Profile Index).

2.7. Enumeration of total culturable hydrocarbon utilizing bacteria (HUB): The composition of basal medium used for enumeration of HUB is as follows: NaCl, 10.0 g; $MgSO_4.7H_2O$, 0.29 g; KCl, 0.29 g; K_2HPO_4, 1.25 g; KH_2PO_4, 0.83 g; $NaNO_3$, 0.42 g, Agar, 15 g. The minimal salt medium (basal medium) components were dissolved in 1000 ml of distilled water, homogenized to form a uniform solution. Cooled to room temperature and the pH adjusted to 7.0 with 1N NaOH solution. The medium was autoclaved at 121^{o}C for 15 minutes. Cooled to 45^{o}C in a pre-set water bath. The medium was poured into several disposable Petri dishes and the surface was subsequently surface dried in an Oven at 45^{o}C. 100 µl aliquot of inoculums from 10^{-5} dilution was inoculated onto the plates in triplicates, sterile hockey stick (spreader) was used to spread the inoculums evenly on the plates. The plates were allowed to set at room temperature. Sterile filter paper soaked with crude oil (sterile) was placed on the Petri dish lid and the crude oil was supplied by vapour phase transfer. The plates were incubated at 30^{o}C for 5-7 days. After incubation

period, colonies observed were counted and recorded. The total cultural hydrocarbon utilizing bacteria counted were multiplied by reciprocal and reported as colony forming units per ml for water and colony forming units per g for sediment (cfu/ml or g).

2.8. Identification of microorganisms: The colonies were randomly picked and their colonial morphology was recorded and was subsequently purified by subculturing onto nutrient agar plates. The bacterial strains were gram stained. Different standard morphological, physiological and biochemical tests were performed using API 20 kits.

2.9. Examining the effect of cyanobacteria on bacterial growth: Two 250 ml Erlenmeyer flasks labeled flask A and C were used. 100 ml of brackish water was added in the flasks. To the first flask was inoculated with aerobic heterotrophic bacteria while the second flask was inoculated with both aerobic heterotrophic bacteria and cyanobacteria. Samples were taken at 5 days intervals for an experimental period of 30 days to check for possible difference in bacterial count.

2.10. Exudate analysis: Sample was taken from the cyanobacterial broth culture of high optical density for filtration using Whatman No. 1 filter paper. The filtrate and residual cell mass (biomass) were used for metabolite analysis using Gas Chromatography. The filtrate was analyzed using ethanol as the injection solvent. To the biomass and a culture of cyanobacteria on a solid medium, the extracellular matter was separated and extracted using modified Folch method. The cells were first washed with 2 ml methanol into a 50 ml Falcon tube to quench cellular metabolism. The total volume was adjusted to 5 ml with methanol and 10 ml chloroform was added and vortexed to break the cell wall and denature the enzymes. Additionally 3 ml water was added and vortexed making a methanol chloroform and water (MCW) ratio of 5:10:3. The mixture was filtered using a 0.22 µm filter into another 50 ml Falcon tube. The mixture was centrifuged at 1000 g for 2 minutes. The upper phase, inter phase and lower phase were collected and subsequently analyzed using GC-MS.

2.11. Data Analysis: The data generated were subjected to analysis of variance (ANOVA) at $p \leq 0.05$ (Steel and Torrie, 1980) using SPSS software (version 20.0, Chicago, USA). A correlation analysis was used to compare values of the experimental treatment and control group. A comparison was also considered statistically significant if the p value was ≤ 0.05.

3. Results And Discussion

The physicochemical properties of the water and sediment samples are presented in **Table 1**. The pH value of water and sediment were 7.39 and 6.93 respectively. In earlier studies, the optimal pH for hydrocarbons degradation ranged from 6 to 7 (Chikere and Ekwuabu, 2014). Other important parameters needed for biodegradation like phosphorus and nitrogen were also measured. Nutrients, particularly phosphorus and nitrogen, are very vital requirements for efficacious biodegradation of hydrocarbon contaminants. Nutrients supplementation is essential to improve biodegradation of crude oil pollutants (Chikere and Ekwuabu, 2014).

Table 1: Physicochemical analysis of water and sediment samples from Bodo creek

Parameters	Water	Sediment
pH	7.39	6.93
Sulphate	45.79 mg/L	14.41 mg/kg
Phosphate	2.19 mg/L	14.31 mg/kg
Total organic carbon	3.22 %	2.11 %
Nitrate	10.97 ppm	23.47 ppm
Total nitrogen	0.0021 %	0.006 %
Electrical conductivity	8.71 µS/cm	17.72 µS/cm
Total hydrocarbon content	878 mg/L	18343 mg/kg
Moisture content	NA	68.05 %
Total dissolved solids	650 mg/L	NA
Total suspended solids	1.030 mg/L	NA

Key: NA= Not Applicable

Table 2 shows the total hydrocarbon utilizing bacteria and Total heterotrophic bacteria counts. Highest TCHB counts was recorded for water having 12.1×10^7 CFU/ml while sediments had the highest TCHUB counts (5.9×10^5CFU/g). This is comparable to what was reported by Ibiene *et al.* (2011) and Chikere and Ekwuabu (2014). The sediment sample has a higher TCHUB count which relates to the higher THC observed in the sediment sample showing that the sediment have higher hydrocarbon contamination than the water which implies that the contamination is not recent most of the hydrocarbon have settled to the bottom of the water body.

Table 2: Total hydrocarbon utilizing bacteria and Total heterotrophic bacteria counts

Type of sample	TCHBC	TCHUBC
Water (CFU/ml)	12.1×10^7	4.6×10^5
Sediment (CFU/g)	9.7×10^7	5.9×10^5

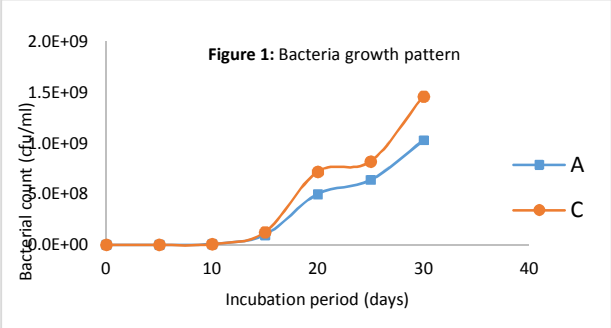
The bacterial counts obtained from the two setups are shown in **TABLE 3.** The bacterial count in flask C was observed to be higher than that in flask A.

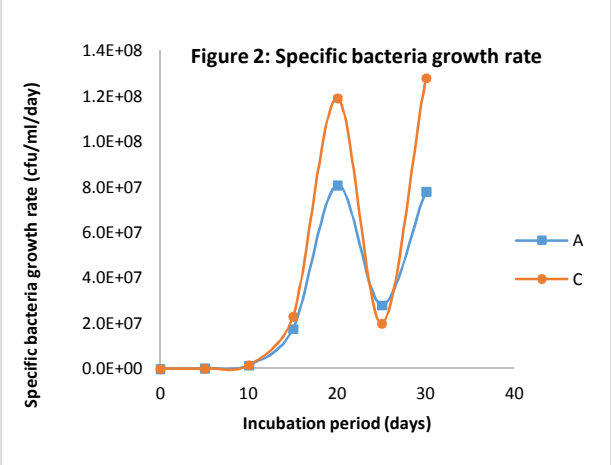
Table 3: Total Culturable Heterotrophic Bacteria Counts (CFU/ml)

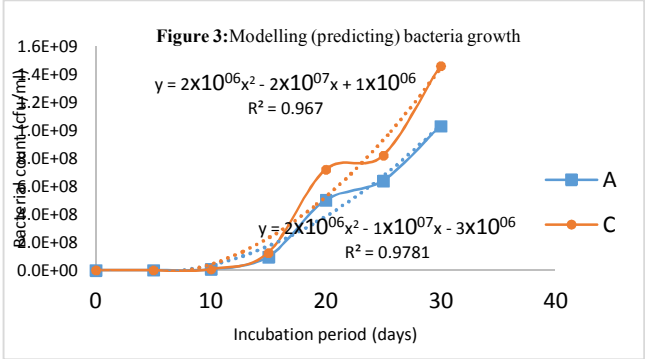
Time (days)	A	$Log_{10}A$	C	$Log_{10}C$
0	3.0×10^3	3.48	4.1×10^3	3.61
5	6.3×10^5	5.80	7.1×10^5	5.85
10	8.1×10^6	6.91	8.9×10^6	6.95
15	9.6×10^7	7.98	12.4×10^7	8.09
20	5.0×10^8	8.70	7.2×10^8	8.86
25	6.4×10^8	8.81	8.2×10^8	8.91
30	10.3×10^8	9.01	14.6×10^8	9.16

Key: Flask A= only bacteria, Flask C= bacteria and cyanobacteria

From the modeling in **Figure 1-4** the bacterial counts were observed to have a high percentage increase from days 10 to 15 and days 25 to 30. Although there are differences in the bacterial counts in flasks A and C, there was no significant difference in the bacterial counts when subjected to Student T-test. Nevertheless, there was difference in bacterial counts but this difference was not significant when analyzed statistically.







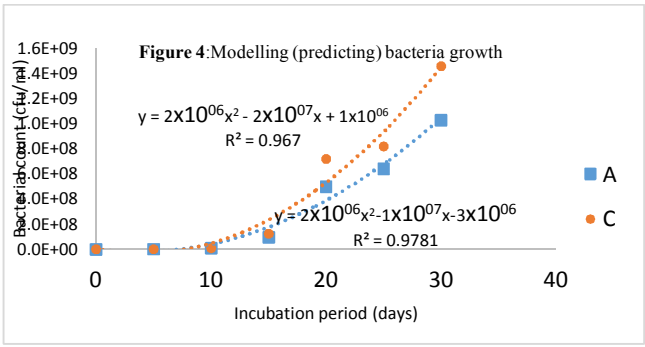


Table 3 shows the diversity of bacterial isolates. The different species of bacteria isolated and identified using Analytical Profile Index (API) include; *Bacillus licheniformis*, *Bacillus megaterium* 2, *Bacillus subtilis*, *Enterobacter asburiae*, *Staphylococcus aureus*, *Corynebacterium kutscheri*, *Corynebacterium ulcerans*, *Staphylococcus saprophyticus*, *Aeromonas hydrophila* group 2, *Acinetobacter baumannii*, *Serratia ficaria*, and *Kocuria varians* (Table 3).

Table 3: Bacterial diversity

Code	Identified organism
Sed1	*Bacillus megaterium* 2
Sed2	*Enterobacter asburiae*
Sed3	*Staphylococcus aureus*
Sed4	*Bacillus subtilis*
Sed5	*Corynebacterium kutscheri*
Sed6	*Corynebacterium ulcerans*
Sed7	*Staphylococcus saprophyticus*
W1	*Aeromonas hydrophila* group 2
W2	*Acinetobacter baumannii*
W3	*Serratia ficaria*
W4	*Kocuria varians*
W5	*Bacillus licheniformis*

The cyanobacteria consortium obtained from Environmental Microbiology Laboratory, University of Port Harcourt consists of *Anabaena cicadae*, *Pseudonabaena minima*, *Laptolyngbya* sp.,

Oscillatoriales cyanobacterium, *Microcoleus* sp., *Mycrocystis holsatica*, *Mycrocystis elabens*, *Phormidium faveolaurum*, *Phormidium* sp., and *Synechococcus* sp. The result of the cyanobacterial exudates analyses is given in **Table 5**. The exudates comprise of amino acids, organic acids and sugars. This is similar to previous finding by Daniel *et al.*, 2014. The sugars might have contributed to the increase in TCHB counts observed in setup C in accordance with the release of oxygen by cyanobacteria during photosynthesis which help the AHB in the setup C to proliferate and multiply faster than those in setup A. Oxygen level is very important for successful biodegradation of hydrocarbon by aerobic heterotrophic bacteria. The intake of oxygen from cyanobacteria by the AHB to release carbon dioxide during biodegradation which is utilized by cyanobacteria may be the reason behind the great interaction and mutualistic relationship between cyanobacteria and aerobic heterotrophic bacteria. Similar findings have been reported by Kirkwood *et al.*, 2006 and Abed and Koster, 2005.

Table 5: Cyanobacterial exudates **Organic Acids**

Exudates	**Types**
Amino acids	Leucine, Arginine
Organic acids	Oxalic acid, Malonic acid, Propanoic acid, acetic acid
Sugars	Glucose, Sucrose

4. Conclusion

The result of the cyanobacterial exudates (metabolite) analysis (characterization) had shown that cyanobacteria secrete exudates which can be utilized by aerobic heterotrophic bacteria. The findings of this present study lend credence to the fact that associated aerobic heterotrophs and cyanobacteria establish a well-organized and an effective consortium for the biodegradation of hydrocarbons as earlier reported (Radwan *et al.*, 2002; Abed and Keoster, 2005; Sanchez *et al.*, 2006; Abed, 2010).

From the bacterial count result, we conclude that the cyanobacterial exudates have effect on the bacterial growth rate but this effect is not statistically significant but it can be observed by the high bacteria counts obtained in the results. However, in line with Abed and Keoster (2005) this result demonstrates that "aerobic heterotrophic bacteria associated with cyanobacteria are most accountable for the biodegradation of petroleum composites rather than the cyanobacteria themselves". The associated cyanobacteria only play a supportive role through their metabolic activities and exudates secretion by providing organics that supports the major bacterial degraders.

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3/4/2016