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Biochemical Oxygen Demand Exertion and Glucose Uptake Kinetics of *Azotobacter* in Crude Oil Polluted Medium

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Nitrogen fixing bacteria are important because they contribute substantially to the N_2-supplying potential of the soil as well as other ecosystems. *Azotobacter* spp. are the most ubiquitous non-symbiotic, aerobic N_2-fixing bacteria in many ecosystems and their relative importance in N_2-fixation has been recognised (Dicker and Smith, 1980). Bacteria found in soils where there are persistent oil spills, petroleum products and other pollutants are exposed to many potentially damaging oxidative reactions which give rise to membrane lipid peroxidation (Dinis *et al.*, 1993).

Soil contamination arising from crude oil spills is one of the most limiting factors to soil fertility and hence crop productivity (Klokk, 1984). After an oil spill, many plants die along with the symbiotic N_2-fixers, thus leaving *Azotobacter* as the most viable aerobic N_2- fixers as source of nitrates through biological nitrogen fixation. This study was conducted to determine the short term effect of Bonny light crude oil on glucose uptake and biochemical oxygen demand (BOD) exertion of *Azotobacter* in a BOD bottle. BOD values are usually a measure of the O_2 required for the carbonaceous oxidation of a non specific mixture of organic compounds for example crude oil. Hence the reduction in BOD *exerted* by *Azotobacter vinelandii* cultured in oiled medium, relative to control (non-oiled) gives an insight of the toxic injury caused by the crude oil.

MATERIALS AND METHODS

The modified method of Dicker and Smith (1980) was used to isolate an *Azotobacter vinelandii* strain from the top 5cm soil samples collected within a site that was contaminated by crude oil, four years before the experiment. The samples (~ 1g) were placed into different test-tubes that were previously sterilized in sodium hypochlorite solution. They were each brought to a volume of 10 ml with autoclaved *Azotobacter* medium containing 0.1 g/l of glucose and 10g/l of mannitol. The capped tubes were inverted several times and then allowed to stand for 24 hours before decanting into other sterile tubes. Following a 48 hour incubation, 1.0 ml aliquots were withdrawn from each tube and transferred into fresh sterile medium (in different 10-ml test-tubes) containing only mannitol as the carbon source. The tubes were incubated for 48 hours at 28

± 2°C after which they were serially diluted 1:10. At the 4th transfer of 1.0 ml aliquots, 0.5 ml of amphotericin β (10 mg/ml) was added to each medium to forestall the growth of fungi.

Solid agar (1.5% w/v) was prepared with the *Azotobacter* medium and then autoclaved. The resulting solution was allowed to cool to between 50°C - 45°C before pouring into sterile plates. Then 0.01 ml aliquots of the 4th serial dilution tubes were plated on different plates and incubated for 96 hours at 28 ± 2°C when discrete colonies were identified. Each colony was aseptically transferred into fresh sterile *Azotobacter* medium and the cells multiplied to a density of about 10^8/ml by incubating for another 72 hours at 28 ± 2°C.

Some cells grown in each of the vessels were harvested by centrifuging 50 ml aliquots at 12,000 x g for 15 minutes and then washed by centrifugation in the salt portion of the medium (3 volumes). The isolates from each medium were subjected to physiological, morphological and biochemical tests (Bergey's Manual, 1957) whereby one of the isolates tentatively identified as *Azotobacter vinelandii* was used for the subsequent experiments.

Four sets (A,B,C,D) of *Azotobacter* medium containing glucose as carbon source were prepared in 48 BOD bottles (12 per set) and they contained 0, 1.0, 1.5 or 5.0% (v/v) concentrations of Bonny light crude oil and 100 ml each of A. *vinelandii* inocula from the primary culture at exponential growth phase. The final volume in each BOD bottle was 200 ml and incubation was carried out at 28 ± 2°C for five days. Dissolved oxygen (DO) levels in each set were determined by titrating directly 200 ml duplicate samples at intervals of 24 hours, using the Alsterbeg Azide procedure (APHA, 1981). The various BOD values were calculated from the DO concentrations using the formular (see Table 1).

In determining the glucose uptake in the various media, *A. vinelandii* cells at exponential phase were harvested from the primary culture by centrifugation at 12,000xg for 15 mins. The cells were washed and suspended in *Azotobacter* medium devoid of any carbon source. Incubation flasks containing 0, 1.0, 1.5 or 2.0% (v/v) concentrations of crude oil in carbon-free *Azotobacter* medium were each inoculated with 50 ml of washed cells (106 cells/ml) in a total volume of 100 ml. Glucose was then added to give various concentrations of 1.6, 2.7, 3.5, 4.2 or 5.4 mg/ml and then mixed thoroughly, Two replicates of each were set-up and incubation was carried out at 28 ± 2°C for 15 hours with gentle swirling in a gallenkamp orbital shaker. At intervals of three hours, 0.1 ml aliquots (triplicate) were withdrawn from each medium and the remaining glucose determined by o - toluidine method (Cooper and McDanile, 1970). Glucose uptake was calculated as the difference between the initial concentrations in each medium at different time intervals. The experiments were run in replicate and the results pooled to obtain the mean ± SD. A one-way ANOVA was used to determine the effect of the different concentrations of the crude oil on glucose uptake amongst the *Azotobacter* cells in oiled media on one hand, and between cells in control media and oiled media on the other hand. The *p* value less than 0.05 was regarded as not significant.

RESULTS AND DISCUSSION

Table 1 shows the concentrations of DO and the calculated BOD_5 in the various media. The values of DO determined five minutes after crude oil application (zero time), and 24 hours later, prior to *Azotobacter* inoculation showed that crude oil reduced the level of dissolved oxygen, relative to control. Also the values at zero time remained the same over the 24 hour period for the respective oiled media, thus showing that this reduction was not time dependent. However, the result revealed that reduction in DO was dependent on the concentration of the crude oil applied. Hence at 5% (v/v) concentration of the crude oil in one of the media, about 3 times less DO was obtained, relative to the control (Table 1). Some chemicals or compounds such as inorganic or organic acids, miscible organic solvents or soluble salts, when mixed with water liberate or absorb "heat of solution or dilution", thereby causing a reduction in DO . It was possible that some components of the crude oil oxidatively reacted with the "constituents" of the medium, thus reducing DO level. Wardley - Smith (1976) reported that high concentration of crude oil (vapour) can cause suffocation by effecting a reduction in oxygen level in a given sample (of air). However, *A. vinelandii* is an active aerobic bacterium capable of surviving at a very low oxygen tension ($< 0.12\%$) (Meyerhof and Burk, 1928), by applying an intramolecular respiratory mechanism (Fife, 1943).

In spite of this, the rate of 0_2 utilization by bacteria parallels their growth rate in an aqueous environment (Tchobanoglous and Schroeder, 1987). Therefore, the daily assessment of BOD exerted by *A. vinelandii* in the various aqueous media was used to monitor growth. The values of the ultimate BOD (BOD_u) determined for the various media are shown in Table 2. This BOD_u is the total biochemical oxygen demand exerted at the 5th day by *A. vinelandii* while metabolizing the glucose (and possibly co-metabolism of the biodegradable components of the crude oil) in the medium. The BOD_u values of the various media were statistically different ($p < 0.05$). Using BOD_u as the index of growth, it becomes apparent that the growth of *A. vinelandii* decreased as the crude oil concentration applied increased, relative to control. The decreasing BOD_u in the respective oiled media shows the increasing negative effect of the oil concentrations on the aerobic microorganism. This shows that the toxicity of the crude oil was concentration - dependent (that is, a dose-response relationship) probably within a certain concentration range. Kuwait crude oil at low concentration (0.001 - 0.1 %, v/v) has no effect on some marine bacteria (Alexander and Schwarz, 1980). The result in Table 2 also shows that the high values of the first order rate constants K in the oiled media, relative to the control, tend to suggest enhanced biochemical activities that were probably directed to cell repair rather than cell proliferation. According to Tchobanoglous and Schroeder (1987), the first order rate constant depends on the bacterial culture, temperature, and the nature of organic pollutant in the system. However in this work, all the culture media at the same temperature ($28 \pm 2°C$) were inoculated with the same number of *A. vinelandii* from the same pure culture at exponential phase. Therefore the crude oil was responsible for the change in the first order rate constants in the oiled media and control.

Table 1. Dissolved oxygen and biochemical oxygen demand exerted by *Azotobacter* incubated in crude oil polluted media.

	Dissolved Oxygen (DO) mg/l				Corresponding BOD exerted (mg/l)			
	Crude oil concentrations (%, v/v)				Crude oil concentrations (%, v/v)			
Day	(A) 0	(B) 1.0	(C) 1.5	(D) 5.0	(A) 0	(B) 1.0	(C) 1.5	(D) 5.0
0	37.97	26.21	23.44	13.10	-	-	-	-
1	26.96	23.96	21.42	11.26	220.2	47.0	40.4	36.8
2	22.60	21.42	19.82	10.10	307.4	95.8	70.4	60.1
3	20.24	20.98	19.17	8.99	354.6	104.6	85.5	83.2
4	18.98	19.12	16.35	8.18	379.8	141.9	128.3	98.3
5	17.81	18.59	17.14	7.06	403.2	152.4	136.0	120.8

Table 2. First order coefficients of biochemical oxygen demand exertion by *Azotobacter* in various incubation media

Crude oil concentrations (%,v/v) in each medium	Ultimate medium BOD_u (mg/l)	Rate Constant (K) d^{-1}
0	215.3	0.35
1.0	95.3	0.77*
1.5	81.0	0.82*
5.0	66.3	0.68*

*Not statistically different ($p < 0.05$)

The different rates of glucose uptake by the microorganism in the various media are shown in Table 3. The data revealed an apparent stimulated glucose uptake (0.018 ± 0.005 mg/ml; n = 15) in the oiled media. The uptake was determined as the difference between the initial glucose concentrations in the various media at time zero, and that remaining at the end of lag phase which lasted for about 7 hours in the control and 18 hours in the oiled media. The lag phase was monitored by taking the optical density measurements of the various culture media at 546 nm every one hour. Using the 5.4 mg/ml single glucose concentrations (n = 5) in the various media containing 0, 1.0, 1.5, or 2.0% (v/v) concentrations of crude oil, this stimulated uptake was statistically significant ($p >$ 0.05). However, among the oiled media alone, this stimulated uptake was not

$$DO = \frac{V_T \times 0.2}{200} \times \frac{C_r}{0.025} \times \frac{1000}{1}$$

where 0.025 is the normality of the recommended thiosulphate titrant (APHA, 1981), V_T are the titre values obtained with C_r (0.042N thiosulphate) used, and 200 is the volume (ml) of culture medium.

statistically different, thus showing that the crude oil only has an initial stimulating effect, but such effect did not increase with increasing crude oil concentrations in the media.

Table 4 shows the mean transport constants K_t and the mean maximum potential glucose uptake velocity V_{max} of the *Azotobacter* inoculum in the various media. These values were deduced from the kinetic treatment of the data in Table 3, using the classical Michaelis-Menten enzyme reaction equation. Many uptake kinetics used this equation for systems, such as *A. vinelandii,* in which their transport proteins are mediated by some lipid membrane components (Maloney *et al.,* 1975). The concentrations of transport proteins in the various media were assumed unchanged during the glucose uptake experiment as this was carried out during the "lag phase". In all the media, the K_t values were not statistically different, showing that the crude oil has no effect on the transport proteins, but on the membrane integrity and probably on other membrane bound enzymes like the nitrogenase complex required for nitrogen-fixation. In the oiled media alone, the maximum potential uptake rates were not statistically different from one another, thus suggesting again that further increases in oil concentrations in the various media had no additive effect on glucose uptake rate. The mean V_{max} in non- oiled media (control) was significantly lower than the values obtained in the oiled media, thus showing that only the initial application of the crude oil has a stimulatory effect on glucose uptake. Differences in V_{max} in the control and the oiled media, but the same K_t in all the media show that the crude oil was not a competitive inhibitor to glucose uptake (Griffiths *et al.,* 1981). The stimulated glucose uptake in *Azotobacter vinelandii* cells incubated in crude oil polluted media could be due to the "solubilization" of the cell membrane by the soluble components of the crude oil. The difference in V_{max} in oiled media and non-oiled media and the unchanged transport constant (K_t) in both media lay credence to this view. The relationship between membrane fluidity and biological membrane passive permeation and mediated transport of solutes in microorganisms have been reported (De Gier *et al.,* 1971). For example, the transport of lactose (sugar) in *E. coli* was not controlled by its metabolism (Kaback, 1976) which means that metabolism does not change the transport (uptake) kinetics appreciably in both intact cells and membrane vesicles. Bearing this in mind, it is possible at least, that the observed increase in uptake rate (V_{max}) for *Azotobacter vinelandii* cells incubated in oiled media could be due to toxic damage done to the cell membrane. The "facilitated" glucose influx (diffusion) into the cells in the oiled media involved no input of energy, hence the uptake process was only by translocation .

Different membrane bound enzymes, especially in bacteria are involved in energy - coupling reactions and these enzymes respond to the composition and physical state of the membrane lipids (Dinis *et al.,* 1993). Hence the damage done to the intracellular membrane - bound enzymes could lead into loss of some biochemical activities such as nitrogen fixation. The increased glucose influx with low BOD_u (relative to control) indicates low metabolism or cell proliferation. The implication of this is that *Azotobacter* in the oiled media was using the metabolized glucose only for repair of damaged organelles e.g. the membrane.

Table 3. Rate of glucose uptake by *Azotobacter* inoculum in various media containing varying concentrations of both crude oil and glucose

Initial glucose conc. (mg/ml)	Glucose uptake per hour (mg/ml)			
	Control medium (no oil)	1.0% (v/v) oiled medium	1.5% (v/v) oiled medium	2.0% (v/v) oiled medium
1.6	0.080	0.090	0.091	0.091
2.7	0.110	0.130	0.122	0.126
3.5	0.112	0.130	0.131	0.135
4.2	0.120	0.141	0.140	0.141
5.4	0.130*	0.150*	0.160*	0.150*

*Mean ± SD; n = 5; others n = 3

Table 4: Transport constants K_t and maximum potential glucose uptake velocity V_{max} of *Azotobacter* in various media (results are Mean ± SD; n = 3)

Incubation Conditions	V_{max}	K_t
Non oiled media (control)	0.178 ± 0.05	1.92 ± 0.02
Media ± 1.0% (v/v) crude oil	0.208 ± 0.06	1.92 ± 0.04
Media ± 1.5% (v/v) crude oil	0.217 ± 0.08	1.92 ± 0.06
Media ± 2.0% (v/v) crude oil	0.215 ± 0.03	1.92 ± 0.03

Alexander and Schwarz (1980) reported that high concentration of Kuwait crude oil (> 0.1%) affected glucose uptake and utilization in some marine microorganisms. Also Griffiths *et al.* (1981), showed that the proportion of the glucose taken into the cells of an aquatic marine microorganism following an oil spill and that respired as carbon dioxide indicated that crude oil affected a biosynthetic mechanism and hence cell growth and proliferation.

Both the results of BOD exertion and glucose uptake kinetics by the *Azotobacter* grown under various conditions show that during the initial "lag" phase the BOD exerted was primarily due to oxidation of absorbed glucose and possibly some components of the crude oil, though without significant cell proliferation. Certain microorganisms could oxidize some hydrocarbons without deriving their growth materials from such metabolic activity (Gunkel and Gassman, 1980). However, *Azotobacter vinelandii* has the potential of surviving in crude oil polluted environment and even better when in "mutualistic" association with crude oil degrading bacteria (Onwurah, 1996). Hence, apart from the inherent toxicity of the crude oil to the aerobic bacterium, there is the tendency that it also has the

property of reducing dissolved oxygen level in aqueous solutions, especially those containing some chemical compounds that could react oxidatively with the oil components. The latter aspect could be regarded as another mechanism of toxic injury imposed by crude oil since dissolved oxygen is of fundamental importance in maintaining aquatic life.

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