International Journal of

Biological Chemistry

ISSN 1819-155X

Academic
Journals Inc.

www.academicjournals.com

International Journal of Biological Chemistry 1 (1): 54-61, 2007
ISSN 1819-155X

pH-dependent Zinc Toxicity Differentials in Species of *Penicillium* and *Rhodotorula* During Oil Biodegradation

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Abstract: The influence of pH (5.5, 7.0 and 8.5) on the response patterns of species of *Penicillium* and *Rhodotorula* to increasing concentrations of zinc during biodegradation of Nigerian light crude oil was assessed by the carbon dioxide (CO_2) evolution technique, over a 16-day period. Mean rates of CO_2 evolution decreased with increasing pH in control cultures of *Penicillium* sp., but increased with increasing pH in cultures containing 1000 mg L^{-1} of zinc. However, analysis of variance of data revealed that there was no statistically significant (p>0.05) difference among the metal concentrations, suggesting tolerance of this species of *Penicillium* to high levels of zinc. In contrast, mean rates of CO_2 evolved in cultures containing *Rhodotorula* sp. decreased with increasing pH in both control and test cultures containing zinc. Efficiency of inhibition was minimal at pH 8.5 in cultures containing *Penicillium* sp., but maximal at the same pH in those containing *Rhodotorula* sp. Interestingly, low concentrations of zinc ($\leq$10 mg L^{-1}) stimulated CO_2 evolution in *Rhodotorula* sp. at pH 5.5 and 7.0, but not 8.5, suggesting pH-dependent low level zinc requirement of the oil biodegradative enzymes of the organism.

Key words: pH, zinc, CO_2 evolution, stimulation, *Rhodotorula* sp.

INTRODUCTION

One third of organically polluted sites are also contaminated with metals (Roane and Pepper, 1997). Studies on the effects of metals on organic pollutant biodegradation are not extensive, especially in aquatic environments, but the available few demonstrate that metals have the potential to inhibit pollutant biodegradation.

The impacts of metals (Cadmium, Nickel, Zinc, Mercury and Chromium) on litter decomposition, methanogenesis, acidogenesis and biomass generation have all been studied (Lin, 1993; Bardgett and Saggar, 1994; Knight *et al*., 1997). Benka-Coker and Ekundayo (1998) reported on the impact of zinc, lead, copper and manganese on crude oil biodegradation by a *Micrococcus* sp. and a *Pseudomonas* sp. Biodegradation, measured by microbial growth, was reduced most by zinc and least by manganese.

Biodegradation of petroleum hydrocarbons involves the participation of diverse taxonomic groups of microorganisms particularly bacteria and fungi (Atlas, 1981; Atlas and Cerniglia, 1995), but the relative importance of bacteria versus fungi in aquatic hydrocarbon degradation is a matter of controversy (Bartha and Atlas, 1977). *Penicillium*, *Aspergillus*, *Rhodotorula*, *Candida*, *Saccharomyces*, *Sporobolomyces* and *Geotrichum* are fungal genera frequently encountered in oil-impacted aquatic systems (Atlas, 1981; 1991; Ijah and Antai, 2003).

There have been reports on metal stimulation of bacterial biodegradation processes under favourable environmental conditions of pH, temperature and aerobiosis (Lin, 1993), but Sandrin and Maier (2003), observed that such stimulatory effects of metals on biodegradation occurred only when consortia, but not single microbial cultures were used for degradation processes. They argued that stimulation was a result of differential toxicity effects of the tested metals.

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This research reports on the influence of pH on the response patterns of species of *Penicillium* and *Rhodotorula* to increasing concentrations of zinc during biodegradation of Nigerian light crude oil.

MATERIALS AND METHODS

The Nigerian light crude oil used in this study was obtained from Mobil Producing Nigeria Unlimited, Ibeno, Akwa Ibom State, Nigeria. Filter-sterilized oil was left at room temperature (28±2°C) until required.

Preparation of Metal Stock Solution

Concentrated stock solution of zinc was prepared by dissolving 1 g of zinc chloride ($ZnCl_2$) in 5 mL aliquot of acid mixture (H_2SO_4: HNO_3) at a ratio of 3:1 V/V, both acids having a pH of less than 2. The preparation was then diluted to 1 L with sterile deionised distilled water (ddH_2O). The stock solution was then analyzed with UNICAM 939/959 flame Atomic Absorption Spectrophotometer (Asuquo *et al*., 2004). Working concentrations of 1, 10, 100 and 1000 mg L^{-1} of the heavy metal were prepared by serial dilutions of the stock solution (Zhang and Crow, 2001) in sterile ddH_2O.

Isolation and Screening of Fungal Cultures with Crude Oil Utilizing Abilities

The mold and yeast cultures used for the study were isolated from intertidal (epipellic) sediment samples obtained from Qua Iboe River estuary, Nigeria, on Mineral Salts agar Medium (MSM) of Zajic and Supplisson (1972) with composition as listed in Ekpenyong and Antai (2006). Fifty micrograms per milliliter (50 µg mL^{-1}) of each of penicillin G and streptomycin was incorporated into the medium to inhibit interfering bacteria. The medium pH was adjusted to 5.8. The vapour phase transfer method of Thijsee and van der Linden (1961) was adopted for the isolation. Discrete colonies were purified on yeast extract-malt extract agar. Fungal pure cultures were identified according to the schemes of Barnett and Hunter (1987) and Barnett and Pankhurst (1974).

The isolates were screened for utilization of Nigerian light crude oil using both the agar (for mold) and broth (for yeast) screen tests of Okpokwasili and Okorie (1988). Turbidity in tubes and early development of colonies on plates were indicative of oil utilization.

Influence of pH on Zinc Toxicity to Species of Penicillium and Rhodotorula During Biodegradation of Crude Oil

The influences of varying pH levels namely 5.5, 7.0 and 8.5, on the toxicity of varying concentrations of zinc to a species each of *Penicillium* and *Rhodotorula* during oil biodegradation were assessed by the carbon dioxide evolution technique of Cornfield (1961). The preparations and incubation were as described by Ekpenyong and Antai (2006). Amounts of CO_2 evolved during biodegradation were calculated using the formula of Stotzky (1965). The residual hydrocarbon in the flasks after 4, 8, 12 and 16 days was determined by the gravimetric method of Odu (1972). Mean rates of CO_2 evolution as well as inhibition efficiencies of the various concentrations of zinc at the different pH levels tested, were determined from the amounts of CO_2 evolved during the study.

Statistical Analysis

Data obtained from the study were subjected to Analysis of Variance (ANOVA) to ascertain the influences of the variables on CO_2 evolution at 95% confidence limit.

RESULTS

The yeast and mold cultures were identified as species of *Rhodotorula* and *Penicillium*, respectively. The yeast grew well in the broth medium and the mold also performed well in the agar screen test.

Cumulative amounts of CO_2 evolved at all the pH levels tested increased gradually, albeit not proportionately, throughout the sampling period in both the controls and metal test cultures. In control cultures of *Penicillium* sp., 68.4 mg of CO_2 was evolved at pH 5.5, 65.4 mg at pH 7.0 and 63.4 mg at pH 8.5, whereas in cultures of *Rhodoturula* sp., 61.3 mg of CO_2 was evolved at pH 5.5, 57.7 mg at pH 7.0 and 47.6 mg at pH 8.5 (Fig. 1-3).

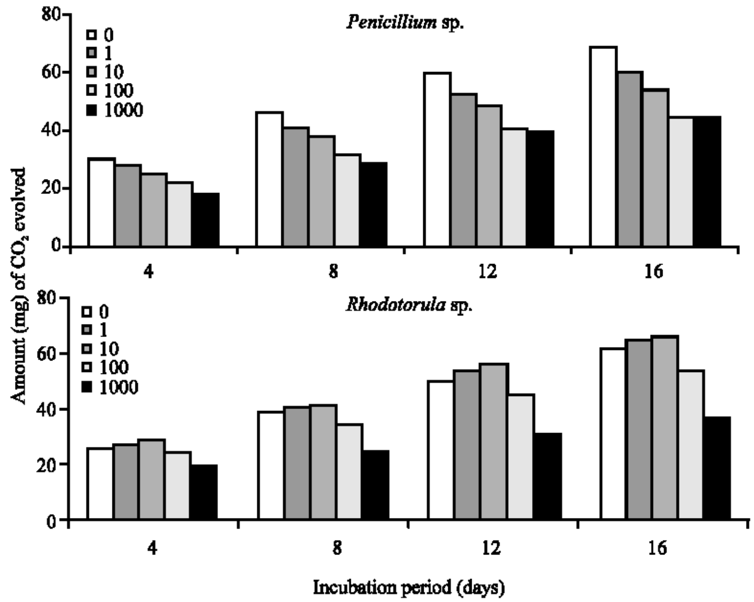

Fig. 1: Influence of zinc on the amount of CO_2 evolved during biodegradation of crude oil at pH 5.5

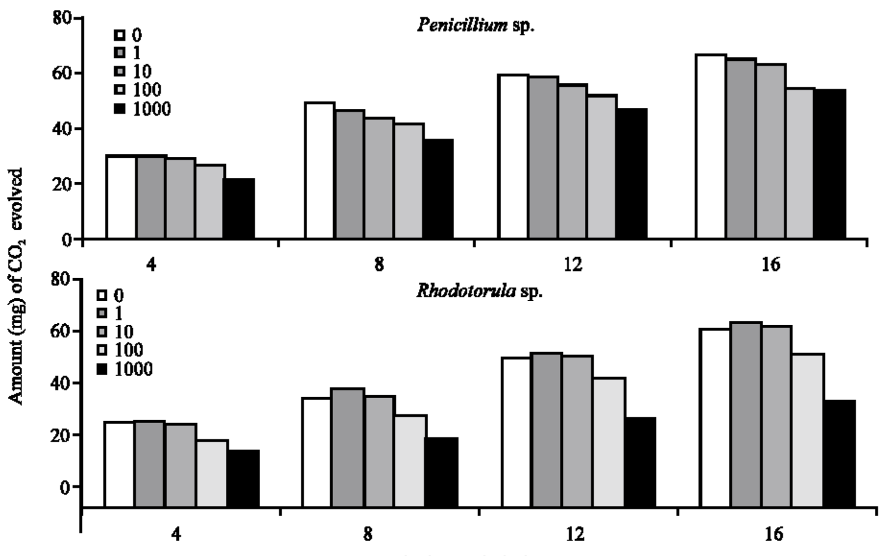

Fig. 2: Influence of zinc on the amount of CO_2 evolved during biodegradation of crude oil at pH 7.0

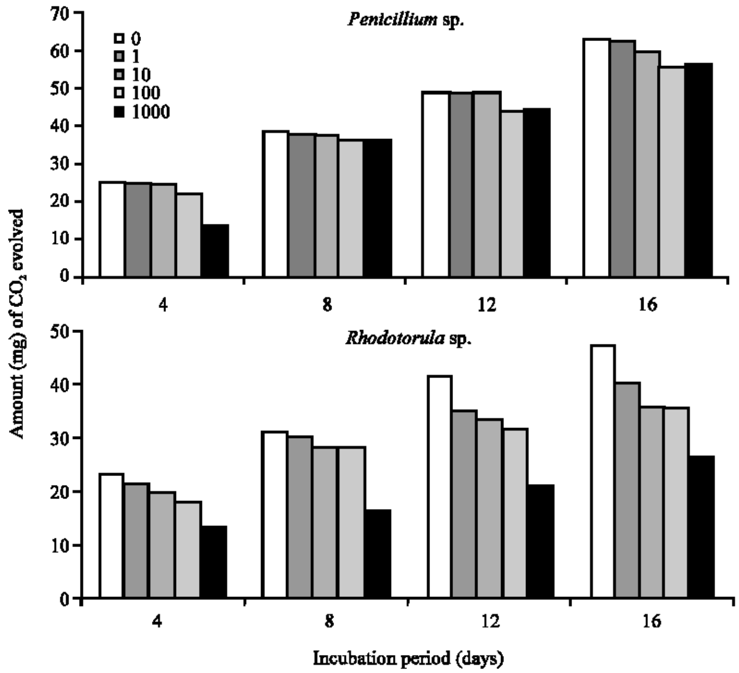

Fig. 3: Influence of zinc on the amount of CO_2 evolved during biodegradation of crude oil at pH 8.5

Table 1: Influence of zinc on mean rate of CO_2 evolution during biodegradation of crude oil at varying pH levels

	Zinc concentrations (mg total zinc L^{-1})				
pH	0.0	1.0	10.0	100.0	1000.0
A					
5.5	5.58±1.2	5.01±1.2	4.53±1.0	3.86±1.0	3.49±0.6
7.0	5.46±1.1	5.34±1.1	5.11±1.2	4.67±1.1	4.03±0.7
8.5	4.82±1.0	4.77±0.9	4.69±0.9	4.33±0.8	4.30±0.7
B					
5.5	4.91±0.9	5.12±1.1	5.28±1.1	4.35±1.0	3.19±1.0
7.0	4.59±1.0	4.82±1.1	4.67±1.1	3.87±0.8	2.85±0.8
8.5	4.05±1.0	3.67±1.1	3.40±1.0	3.20±0.8	2.19±0.6

A: *Penicillium* sp. B: *Rhodotorula* sp.

In Table 1, the mean rate of CO_2 evolution in cultures containing *Penicillium* sp. was observed to be highest in the control at pH 5.5, nevertheless mean rates at pH 7.0 under the influence of 1 mg L^{-1} and 10 mg L^{-1} total zinc were higher than those in the control at pH 8.5. In cultures of *Rhodotorula* sp., mean rates of CO_2 evolution in test cultures containing 1 and 10 mg L^{-1} total zinc were higher than their corresponding control cultures at pH 5.5 and 7.0. While the mean rate of evolution of carbon dioxide in the control culture of *Rhodotorula* at pH 8.5 was 4.05 mg day^{-1}, that at pH 5.5 in cultures containing 100 mg L^{-1} of zinc was 4.35 mg day^{-1}. The results of the analysis of variance (ANOVA) of data, assessing the influence of pH on the mean rate of CO_2 evolution and the toxicity of zinc to the organisms are presented in Table 2. Inhibition Efficiency (IE) of zinc concentrations on CO_2 evolution process in *Penicillium* was most profound at pH 5.5 with as much as 35.1% inhibition in the presence of 1000 mg L^{-1} zinc. The highest inhibition was observed at pH 8.5 to be 11.4% in the presence of 100 mg L^{-1} total zinc (Fig. 4). Maximal inhibition of CO_2 evolution in *Rhodotorula* sp. was observed at pH 5.5 in the presence of 1000 mg L^{-1} zinc, with an IE of 40.5%.

Table 2: Two-way analysis of variance (ANOVA) showing effect of pH and zinc concentrations on mean rate of CO_2 evolution process in (A) *Penicillium* sp. and (B) *Rhodotorula* sp.

Source of variation	SS	df	MS	F	p-value	F crit
A						
Rows	0.51088	2	0.25544	2.440548	0.148781	4.45897
Columns	3.62496	4	0.90624	8.658482	0.005268	3.837853
Error	0.83732	8	0.104665			
Total	4.97316	14				
B						
Rows	4.186813	2	2.093407	43.29096	5.12E-05	4.45897
Columns	7.120427	4	1.780107	36.81202	3.38E-05	3.837853
Error	0.386853	8	0.048357			
Total	11.69409	14				

Table 3: Two-way analysis of variance (ANOVA) showing effect of pH on inhibition efficiency of zinc concentrations on CO_2 evolution process in (A) *Penicillium* sp. and (B) *Rhodotorula* sp.

Source of variation	SS	df	MS	F	p-value	F crit
A						
Rows	0709.1117	2	354.5558	08.724486	0.016753	5.143253
Columns	2914.170	3	971.39	23.9028	0.000977	4.757063
Error	0243.835	6	040.63917			
Total	3867.117	11				
B						
Rows	778.1267	2	389.0633	32.75557	0.000591	5.143253
Columns	684.3533	3	228.1178	19.20543	0.001769	4.757063
Error	071.26667	6	011.87778			
Total	1533.747	11				

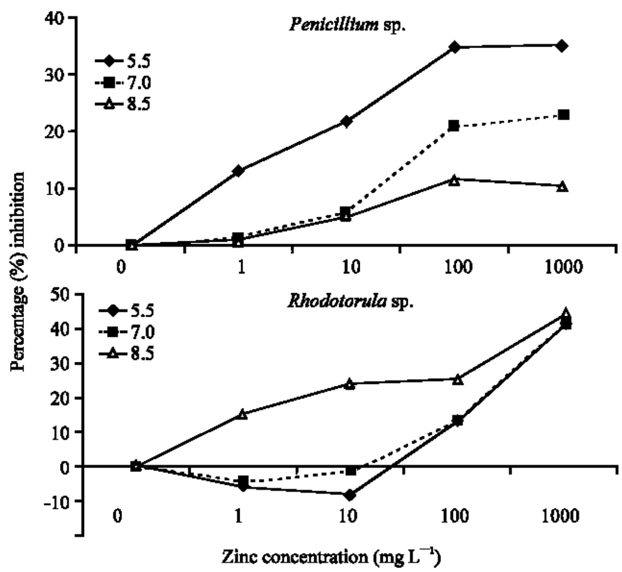

Fig. 4: Zinc inhibition efficiency of CO_2 evolution during biodegradation of crude oil at varying pH levels

Negative inhibition efficiency values were observed at pH 5.5 and 7.0 in the presence of zinc concentrations ≤ 10 mg L^{-1}. Least inhibition of -8.3% was observed at pH 5.5 in the presence of 10 mg L^{-1} total zinc and IE value of -1.0% at pH 7.0.

At zinc concentration of 1 mg L^{-1}, IE values of -5.7 and -4.0% were observed at pH 5.5 and 7.0, respectively. Generally, efficiency of inhibition increased with increasing pH (5.5 < 7.0 < 8.5) in *Rhodotorula* sp. (Fig. 4). Statistical analysis showing relationship between pH, zinc concentrations and inhibition efficiency of CO_2 evolution process of both organisms is presented in Table 3.

DISCUSSION

The mold, *Penicillium* sp., did not grow well in mineral salts broth medium, especially when the broth screen tubes were agitated, but both the broth culture and the condition of agitation favoured the yeast, *Rhodotorula* sp. Minimal shredding (breakdown) of the overlaid oil was observed in tubes containing *Penicillium* sp. The mold grew as fuzzballs in the broth cultures producing little or no visible turbidity along the column of the medium. Smucker and Cooney (1981) reported that filamentous fungi grow as heavy mycelial mats or fuzzballs in liquid culture and so turbidimetric methods are not accurate measures of growth. The selection of the mold for toxicity studies was therefore based primarily on the number of colonies developed on Crude oil/Mineral Salts agar Medium. *Rhodotorula* sp. produced good visual turbidity and impressive optical density reading at 540 nm wavelength and displayed extensive shredding (breakdown) of overlaid oil resulting in loss of oil viscosity attendant upon oil utilization (Ekpenyong and Antai, 2006).

The predominant heavy metal in Qua Iboe Estuary, Nigeria, where the test organisms were isolated is zinc (data not shown). In the control cultures (without zinc) of both organisms, it was observed that *Penicillium* sp. was more tolerant of alkaline environments than *Rhodotorula* sp. This was evidenced by the fact that cumulative amounts of CO_2 evolved decreased from 61.3 (pH 5.5) to 47.6 mg (pH 8.5) in *Rhodotorula* sp., but only dropped from 68.4 (pH 5.5) to 63.4 mg (pH 8.5) in *Penicillium* sp. In test cultures containing zinc, it was observed that *Rhodotorula* sp. produced higher cumulative amounts of CO_2 than *Penicillium* sp. at pH 5.5, at least up to 100 mg L^{-1} total zinc. The higher cumulative amounts of CO_2 evolved in cultures of *Penicillium* sp. in the presence of 1000 mgL^{-1} of zinc would be attributable to the mycelial growth of the organism in culture media (Baldrian *et al*., 2000). *Rhodotorula* sp. produced higher cumulative amounts of CO_2 in test cultures containing 1 and 10 mg L^{-1} of zinc than in the controls (without zinc), at pH 5.5 and 7.0. At pH 5.5, zinc concentration of 10 mg L^{-1} favoured the production of higher amounts of CO_2 than 1 mg L^{-1}, while the reverse scenario was observed at pH 7.0.

The essence of a successful bioremediation exercise rests on the amount of time needed to restore normalcy at the pollutant-impacted area. Mean rates of CO_2 evolution were observed to increase with increasing alkalinity in the presence of 1000 mg L^{-1} of zinc in cultures containing *Penicillium* sp., but no generalized pattern of toxicity response was observed at lower concentrations of the metal. In contrast, increasing alkalinity only resulted in reductions in the mean rates of CO_2 evolution in *Rhodotorula* sp. at all the tested metal concentrations. This suggests either the acidophilic nature of this species of *Rhodotorula* or that zinc speciated to a form more toxic to this *Rhodotorula* sp. at the alkaline pH (Sandrin and Maier, 2003). Franklin *et al*. (2000) earlier showed that even relatively small changes in pH (e.g., from 6.5 to 5.7) can reduce metal toxicity.

The higher cumulative amounts of CO_2 evolved at pH 5.5 and 7.0, coupled with higher mean rates of CO_2 evolution in test cultures of *Rhodotorula* sp. containing zinc concentrations ≤ 10 mg L^{-1} resulted in negative inhibition efficiency values. This suggests stimulation rather than inhibition of the biodegradation process by small amounts of zinc at these pH values. The lack of similar stimulatory results at pH 8.5 indicates significant ($p<0.05$) influence of pH on such observation. Metal stimulation of organic biodegradation has been reported by Kuo and Genthner (1996), albeit, in studies involving microbial consortia. Sandrin and Maier (2003) argued that such stimulation was a result of differential toxicity effect wherein a second population more sensitive to the metal stress competes with the population expressing the activity of interest. The stimulation of crude oil biodegradation by low amounts of zinc at acidic and neutral pH levels in pure cultures of *Rhodotorula* sp. would probably be attributable to the trace requirement of this redox-inactive former of tight complexes (Nies, 1999) by the hydrocarbon decarboxylase enzyme systems of the yeast.

Apart from the progressive inhibition of biodegradation processes with increasing metal concentrations, low concentrations of certain metals, at appropriate pH levels, could stimulate organic pollutants biodegradation processes, even in pure cultures.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude the efforts of Mobil Producing Nigeria Unlimited, Ibeno, Nigeria for providing the crude oil used for the study. They also thank Mr. Emmanuel Bassey of the Environmental department of the Institute of Oceanography, University of Calabar, Nigeria, for performing the hydrocarbon analysis of the samples.

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