Growth and extracellular enzyme activity responses of a multi-enzymic strain of *Serratia* sp. in a simulated diesel-contaminated system

ABSTRACT

Aim: The present study was aimed at elucidating the alternative metabolic preferences of *Serratia* sp. strain DW2 that permitted its survival in a diesel-contaminated environment. **Study design: We adopted a** A 4 x 4 x 3 completely randomized design was adopted for**of** a full-factorial experiment **for** in this**e** study. **Place and duration of study**: The study was conducted at the Department of Microbiology, University of Calabar, Nigeria, during the **months of** March and June year?. **Methodology**: In this study, *Serratia* sp. strain DW2 was isolated from Douglas Creek water of the Qua Iboe Estuary, along the Qua Iboe terminal at Ibeno, Nigeria, as a significant biological contributor to the decontamination process through inherent ability to utilize diesel oil hydrocarbons. This paper elucidated the growth and responses of the bacterial lipase, caseinase and gelatinase activities to diesel-oil hydrocarbon contamination. **Results**: Range finding test results showed that the bacterium could grow in the presence of water soluble fraction (wsf-D) concentration between 0.0042 and 0.0335 µg/mL, albeit with increasing lag time and decreasing specific growth rate when compared with growth in glucose-Bushnell-Haas broth. Lag time changes were not significantly influenced by exposure time but changes in specific growth rate were. Gelatinase activity was most susceptible to toxicant onslaught but was least affected by exposure time. Conversely, lipase activity was the most affected by exposure time. Toxicant concentration/exposure time interaction of a two-way analysis of variance model for caseinase activity was not significant ($P > .05$) but those for lipase and gelatinase activities were. **Conclusion**: The bacterium survived diesel toxicity by exploiting its lipase and gelatinase activities for provision of alternative sources of carbon, energy and nitrogen to drive ecosystem decontamination in the event of refined petroleum contamination.

Keywords: *Serratia* sp. strain DW2; ,Toxicity, Water soluble fraction of diesel; ,Exposure time; **Two-way ANOVA.**

1 INTRODUCTION

Microbial growth is a positive result of an assortment of multiple growth-sustaining biochemical and/or enzymatic reactions and occurs in a continuum, particularly, in natural environments; recycling through the various phases of growth such that the organisms self-perpetuate in their natural environments. In polluted systems, stressors like hydrocarbons and toxic metals contribute to hampering microbial growth and sundry metabolic and ecological activities, in addition to other naturally occurring factors like oxygen tension, temperature, buffering capacity and salt content [1, 2]. In the Qua Iboe estuary of Akwa Ibom State, Nigeria, where extensive oil production and transportation activities occur, the adjoining creeks are chronically contaminated or polluted with petroleum hydrocarbons owing to seepages, spills and sabotages [3]. The pollution worsens by reason of its location on the coast where recreational activities dominate thus accounting for the intense drive for bioremediation. Several microorganisms especially bacteria and fungi, comprising both yeasts and molds, have been reported to have inherent abilities to degrade hydrocarbon pollutants, thus facilitating the decontamination process at the coasts [4, 5]. These have been exploited in the controlled remediation of polluted sites through either biostimulation or bioaugmentation [6]. In the event that targeted bioremediation is not implemented at the polluted site, autochthonous organisms with inherent abilities to degrade petroleum hydrocarbons are reported to drive the self-decontamination or attenuation process in the polluted system. A major method of survival for successful remediation of the site by bacteria is the use of alternative source of carbon and energy at the site to enable the remediating microorganisms build up sufficient number of cells for the remediation process.

In this study, **we found** a preponderant species of *Serratia* in a diesel contaminated water system was found in **of** Douglas Creek, Qua Iboe estuary, Nigeria. Its significantly higher percent abundance in the creek prompted the investigation into the nature of metabolic activities that caused its selective preponderance in the site. Initial site characterization put the concentration of water soluble fractions of petroleum at .03 µg/mL. The investigation sought to know the extent of inhibition of the contaminant on

Comment [u1]: Preliminary study? If this include in the study please do not include in introduction. But you can describe any microbe that has same function with serratia

the dominant metabolic activities of the bacterium as an indirect way to understand the basis for its superior abundance in the polluted ecosystem.

2 MATERIALS AND METHODS

2.1 Isolation and identification of bacterium

The bacterium was isolated from Douglas Creek water sample along the Qua Iboe terminal (QIT) of Mobil Producing Nigeria Unlimited, on Bushnell-Haas broth (HiMedia M350, Mumbai-India) supplemented with 0.1% diesel oil as sole source of carbon and energy and solidified by 1.5% agar-agar (Oxoid), after pH had been adjusted to 7.0 ± 0.2. The bacterium was identified by morphological and biochemical characterizations using the MICROGEN ID Kit (Microgen Bioproducts Limited, UK) along with their identification system software. Molecular characterization by 16S rRNA sequencing followed the protocol described in Ekpenyong *et al*. [7].

2.2 Growth in glucose minimal medium

Control growth of the bacterium was evaluated in Bushnell-Haas broth medium supplemented with 1% D-glucose (Merck) after pH adjustment to 7.0 ± 0.2. Medium was dispensed in 9.8 mL volumes into 50 mL Erlenmeyer flasks and sterilized by autoclaving at 121ºC for 15 min. D-glucose was selected to inhibit, partially, prodigiosin biosynthesis which would interfere with optical density measurement for biomass determination at 600 nm [8, 9]. Cooled sterilized media were inoculated with 2% (v/v) 18 h-old tryptic soy broth culture and incubated at room temperature (28 ± 2ºC) on a shaker agitating at 150 rpm for 24 h. Cells were harvested by centrifugation at 8,000 rpm for 15 min and cells washed with phosphate buffer (0.05M, pH 7.0) and concentrated bacterial suspension prepared in the same buffer. Lower concentrations of cells were prepared through a series of 8-10-fold dilutions from the concentrated suspension. Optical densities of different concentrations were measured at 600 nm wavelength and corresponding dry weight and viable cell counts determined as per Asitok and Ekpenyong [10]. Calibration curve was plotted by regressing $\log_{10}$ transformed number of viable cells against dry weight and absorbance.

2.3 Extracellular enzyme production, quantification and activity assay

Lipolytic potential of the bacterium was conducted on Tween 80-minimal medium with composition as detailed in Iboyo *et al.* [11]. Total protein was quantified by the Bradford method of protein detection and quantification using bovine serum albumin as standard, after centrifugation, membrane filtration (0.22 µM Millipore) and dialysis of fermentation broth. Lipase activity assay was conducted as per the protocol by Iboyo *et al*. [11]. One unit of lipase activity was defined as the amount of enzyme that released 1 mM of *p*-**nitrophenol** nitrophenol per min under the assay conditions.

Caseinolytic potential of the bacterium was evaluated on skimmed-milk minimal agar medium [12]. Enzyme activity assay was conducted on dialyzed fraction after total protein quantification, according to the protocol of Vasquez et al. [13] using azocasein as substrate. One unit of caseinolytic activity was defined as the amount of crude enzyme required to digest 1 mg of azocasein in one minute under the assay conditions [14].

Gelatinolytic potential of the bacterial strain was evaluated by using Todd-Hewitt agar plates (Difco) supplemented with 3% gelatin. Plates were incubated for 24 h at room temperature (28 ± 2ºC) and colonies with opaque zones were selected as gelatinase-positive isolates. Gelatinase-positive isolates were confirmed by gelatin liquefaction test [15]. Enzyme activity assay was conducted with the dialyzed fraction of culture supernatants obtained after centrifugation (8,000 rpm for 15 min) and sterilization by membrane filtration (0.22 µM) using the protocol described in Zeng *et al*. [16] with minor modifications. Briefly, an azo dye-impregnated collagen (0.25 g) was washed in 50 mL of 0.05 M Tris-HCl buffer (pH 7.8 ± 0.2) containing 0.001 M $CaCl_2$ to obtain a final concentration of 5 mg/mL. The protein solution was left to stand for 90 min at 37°C and then centrifuged at 1,500 x g for 10 min. The residue was re-suspended in 50 mL of the same buffer and 0.75 mL aliquots transferred into 1.5-mL Eppendorf tubes. Triplicate tubes were incubated at 37°C on a rotary shaker (100 rpm, 15 min) followed by addition of 0.25 mL of culture supernatant. The mixture was incubated for 4 h at 37°C on a shaker and reaction stopped by placing tubes on ice-water bath [17]. Cold sample was centrifuged at 1,500 x g for 5 min and absorbance of supernatant measured at 550 nm. Absorbance was fed into gelatinase calibration curve using glycine as primary amino acid standard. One unit of gelatinase activity was defined as the amount of enzyme that

liberated 1µmole of glycine from gelatin in one min under the assay conditions. Specific activities of all three enzymes were calculated by dividing volumetric enzyme activity by total protein and expressing as U/mg.

2.4 Range-finding test of water soluble fraction of diesel on *Serratia* sp. strain DW2

2.4.1 Preparation of water soluble fraction of diesel

Water soluble fraction of diesel (wsf-D) was prepared according to the method of Asitok *et al*. [12], except that diesel served as hydrocarbon source. The resulting wsf-D served as 100% toxicant solution. Total petroleum hydrocarbon (TPH) in the soluble fraction was determined by gas chromatography.

2.4.2 Range finding experiment

To establish which concentration(s) of the soluble fraction could support growth and which would inhibit it, a range finding test was conducted. The 100% stock solution of wsf-D, containing 8.58 µg/mL total petroleum hydrocarbon (TPH), was diluted to obtain lower concentrations using a series of 2-fold dilutions. Lowest concentration of 8.39×10^{-3} µg/mL was obtained at 1024-fold dilution. An aliquot of 4.5 mL of filter-sterilized (0.22 µM-Millipore) toxicant was aseptically mixed with 4.3 mL of sterilized (121ºC for 15 min) Bushnell-Haas broth in 50 mL Erlenmeyer flasks which were subsequently inoculated with 0.2 mL (10^5 cfu/mL) of twice-washed (phosphate buffer 0.05 M, pH 8.0) 18-h old tryptic soy broth bacterial culture. Final toxicant concentration in each flask was half the concentration mixed with broth. Flasks were incubated on orbital shaker operating at 150 rpm for 120 h. Cells were harvested, at 24 h interval, by centrifugation at 8,000 rpm for 15 min and cells washed with phosphate buffer (pH 7.0). Optical densities of growth at different wsf-D concentrations were measured at 600 nm wavelength and corresponding viable cell counts determined after adequate dilutions of cell suspension.

2.5 Toxicity studies

2.5.1 Growth toxicity studies

Each growth-enhancing concentration obtained from the range-finding test was tested against exponentially growing cells of the bacterium and activities of lipase, caseinase and gelatinase were determined. To evaluate the toxicity of wsf-D on lag time and specific growth rate of the bacterium, 0.5 mL of washed bacterial suspension (10^8 cfu/mL) was mixed with 0.5 mL of each of 4 toxicant concentrations (A = 0.0042 µg/mL, B = 0.0084 µg/mL, C = 0.0168 µg/mL, D = 0.0335 µg/mL) and incubated at room temperature for 15, 30, 45 and 60 min. A positive control (100% growth or activity) was set up with 0.5 mL of washed cell suspension + 0.5 mL of phosphate buffer. The negative control (0% growth or activity) contained 0.5 mL washed bacterial suspension + 0.5 mL 100% (8.58µg/mL) wsf-D concentration. The arrangement was prepared in triplicates resulting in a 4 concentration x 4 exposure time x 3 replicates = 48 experimental runs. Second, all the mixture preparations were aseptically transferred to 9 mL sterilized glucose-Bushnell-Haas broth in 50 mL eErlenmeyer flask in an inoculation hood and incubated at room temperature for 48 h. Lag time and specific growth rates were monitored and compared with those of control.

2.5.2 Enzyme toxicity studies

For enzyme toxicity studies, the method described by Usharani and Muthuraj [18] was employed. The effect of the 4 concentrations of wsf-D on caseinase activity was determined by adding 1 mL of each concentration to 1 mL of the cell-free supplements in the activity assay mixture as outlined and incubating at 30°C in a water bath for 15 to 60 min. Caseinolytic activity toxicity assay was conducted according to Asitok *et al*. [12] while lipase toxicity study was conducted by adding 1 mL of each toxicant concentration of the wsf-D to the activity assay mixture as described in Iboyo *et al*. [11]. Gelatinase toxicity assay was performed by adding 1 mL of each toxicant concentration to the assay mixture in Zeng et al. [16]. Incubations for all enzyme activity studies were at the assay conditions for the respective enzymes.

Residual activities of all enzymes were calculated as ratios of enzyme activities in the presence of different concentrations of the toxicant (inhibited activity) at the various exposure periods to the uninhibited enzyme activity of the control.

2.6 Statistical analysis

Correlation and two-factor analysis of variance was conducted on data using SPSS version 20 for Windows (IBM, USA). Significant means were separated by Bonferroni post hoc multiple comparison test at a significance level of 1%.

3 RESULTS AND DISCUSSION

The bacterium was identified as a red-pigmenting Gram-negative **facultatively**facultative anaerobic rod-shaped bacterium that utilized glucose fermentatively. The red pigment was cell-associated but soluble in aqueous solutions. Molecular characterization by 16S rRNA sequencing identified the bacterium as a strain of *Serratia* with 100% sequence homology with *Serratia sp.* ZJ-I (GenBank Accession No. JQ954966A). The bacterium demonstrated tremendous abilities to elaborate extracellular enzymes like caseinase, lipase (Plate 1) and gelatinase (Plate 2). Plate 1 reveals the large precipitation zone around the red-pigmenting bacterium on Tween 80-minimal medium. Red pigmentation (prodigiosin production) is a common identifying phenotype of the genus *Serratia* [19]. Plate 2, on the other hand, shows the hydrolysis of solidified gelatin in the presence of *Serratia* sp. strain DW2. Gelatin liquefaction is a confirmatory test for gelatinase production among bacteria [15].

The composition of the water soluble fraction of the diesel (WSF-D) used in the study is shown as Figure 1. The chromatogram shows that diesel hydrocarbon fraction was dominated by nC_{11} to nC_{29} fractions. Total petroleum hydrocarbon (TPH) was determined as 8.58 µg/mL.

The bacterium, *Serratia* sp. strain DW2, demonstrated significant growth with very minimal red-pigmentation in Bushnell-Haas minimal broth medium supplemented with D-glucose. Growth rate of the bacterium in this medium was 2.145 h^{-1}, with a lag time (time to first doubling of bacterial population) of 4.5 h. Baseline specific caseinase activity of the bacterium was 4.06 U/mg and those of lipase and gelatinase were 3.73 U/mg and 4.85 U/mg respectively. Figure 2 shows the relationship that existed

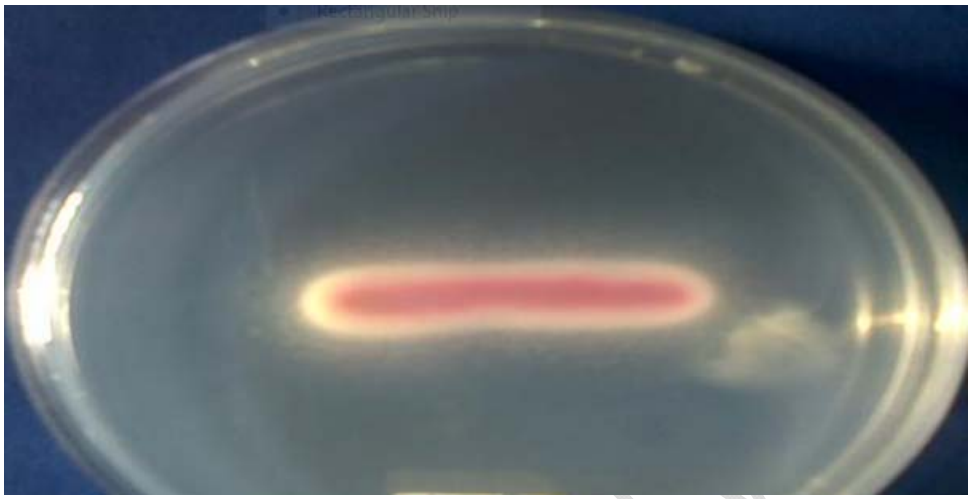

Plate 1 Lipolytic potential of *Serratia* sp. strain DW2 on Tween 80-minimal agar medium

Plate 2 Gelatin liquefaction test of *Serratia* sp. strain DW2. **A** – Positive liquefaction in test; **B** – No liquefaction in control tube

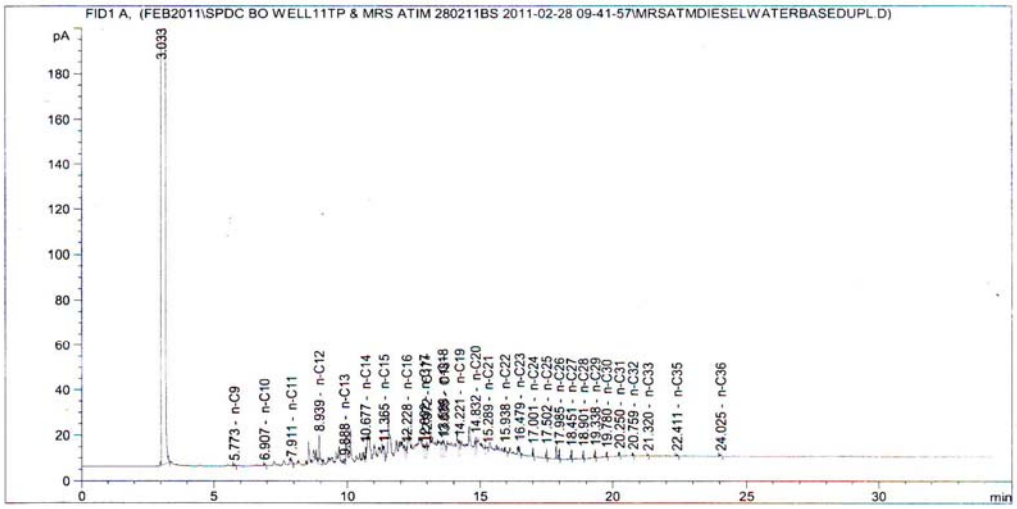

Figure 1 Gas chromatogram of total petroleum hydrocarbons (TPH) in water soluble fraction (WSF) of diesel

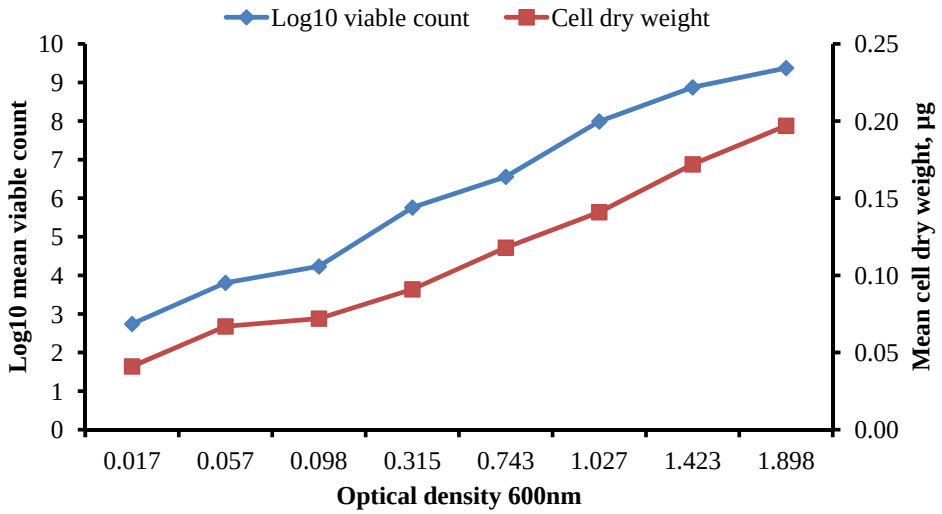

Figure 2 Relationship among optical density, viable count and cell dry weight of *Serratia* sp. strain DW2 grown in glucose-Bushnell-Haas broth

among cell dry weight, optical density and viable cell count. Linear regression analyses of data showed that optical density had a better relationship with cell dry weight than with viable cell count with respective linear model goodness-of-fit, R^2, values of 0.9729 and 0.9187 (P = .001). This discrepancy derives from the fact that cell dry weight, unlike viable cell count, measures both life and dead cells. This is similar to optical density measure which does not discriminate between live and dead cells in its ability to obstruct transmission and/or absorption of light through a defined path length. The ideal thing to have done would be to simply measure bacterial growth with optical density [20], however, in toxicity studies such as this, viable counts data are more expressive of bacterial responses than cell dry weight [21] because only live cells respond to toxicant onslaught.

Growth of the bacterium in a medium contaminated by diesel was observed to be grossly inhibited. A range finding test result presented as Figure 3 revealed that the bacterium could grow in a medium supplemented with a range of TPH concentration from 0.0042 to 0.0335 µg/mL above which cells started to die as indicated by the two linear positions on the plot.

Utilization of petroleum hydrocarbons as sources of carbon and energy by *Serratia marcesens* and other species had earlier been reported by Rajasekar [22] and Xia *et al*. [23]. The first linear portion correlated significantly, strongly and positively (P = .031 < .05; R = .962, R^2 = .939, adjusted R^2 = .909; MSE = .409) with WSF-D-TPH concentrations and suggested growth of the bacterium. The second linear portion correlated significantly, strongly but negatively (P = 1.63E-07 < .0001; R = -.998, R^2 = .997, adjusted R^2 = .996; MSE = .123) with WSF-D-TPH concentrations suggesting bacterial death. The difference in significance levels, adjusted R^2 and mean square error (MSE) values indicate that WSF-D-TPH is more of a growth-inhibiting substrate that a growth-promoting one, suggesting that respective variations in growth and death was substrate concentration-dependent. Since survivability of bacteria was critical to successful natural attenuation of the ecosystem, we measured diesel toxicity to the bacterium using the concentrations that supported growth since this concentration of TPH was observed in the ecosystem where the bacterium was isolated (data not shown).

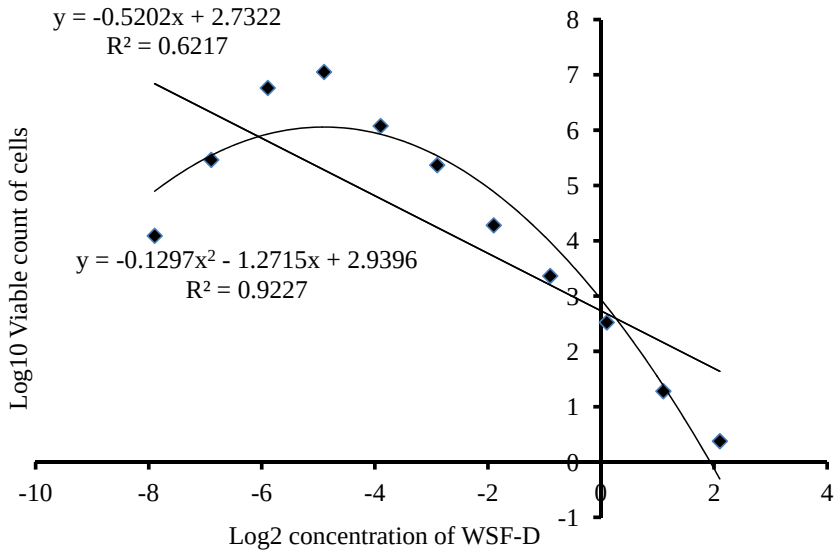

Figure 3 Range finding test of Serratia sp. strain DW2 to varying concentrations of water soluble fraction of diesel. An overall linear model R^2 = .622 while an overall second order polynomial model R^2 = .923.

Toxicity was conducted bearing in mind the obvious influence of time on toxicity mediation, therefore, toxicant concentration and exposure time served as independent factors. These factors were earlier reported as influencing factors in diesel toxicity studies involving *Citrobacter amalonaticus* strain Y1FSW [24]. The dual effects of wsf-D-TPH and exposure time on lag time, specific growth rate and residual activities of lipase, caseinase and gelatinase were evaluated. Figure 4 shows the influence of wsf-D and exposure time on growth lag time and specific rate of growth. The figure reveals that change in lag time increased as concentration of wsf-D-TPH increased but barely changed with exposure time. Higher concentrations with longer exposure time resulted in larger changes in specific growth rate. A very significant and interesting revelation in the figure is the influence of exposure time on the relationship between lag time and specific growth rate. The results revealed that the longer the exposure time, the worse the relationship as expressed by the wide separation between the data points. Figure 4 shows the influence of toxicant concentration and exposure time on residual activities of lipase, caseinase and gelatinase. These results showed that residual activities of lipase and gelatinase decreased with increasing toxicant concentration moving further and further away from 1. On the contrary, the toxicity pattern of residual caseinase activity barely changed with increases in exposure time. The figure revealed that gelatinase was the most susceptible of the three enzymes to toxicant onslaught but that lipase activity was the most susceptible to changes in exposure time.

Attempt made to analyze these effects using multivariate analysis of variance model failed because results of the bivariate correlation of the five dependent variables (DVs) revealed multicolinearity where all correlate pairs were strongly correlated above r = 0.80 (Asitok and Ekpenyong, 2019) and so two-way analysis of variance (ANOVA) was separately conducted for each DV. Table 1 presents the results of Pearson's bivariate correlations and shows that all the correlations were significant at P = 0.01. Correlation between the two growth variables was positive and those between any two enzyme activity variables were also positive. However, all correlations between either of the two growth variables and any enzyme activity were negative. The least correlated pair (r = 0.819) was that between change in lag time and change in specific growth rate. Change in lag time was calculated by subtracting the lag time of the bacterium after exposure to WSF-D-TPH from that in the control. A similar calculation was made for change in specific growth rate.

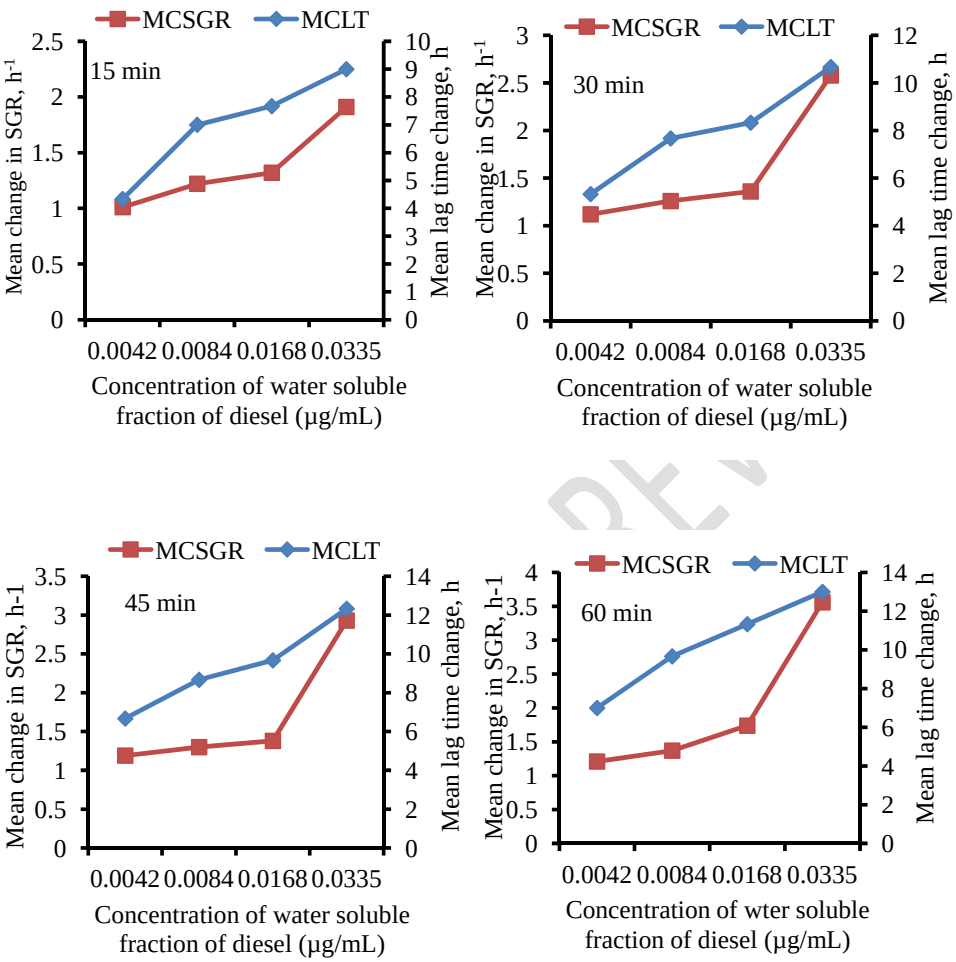

Figure 3 Effect of water soluble fraction of diesel on growth of *Serratia* sp. DW2 as a function of exposure time. **MCLT**-Mean change in lag time; **MCSGR**-Mean change in specific growth rate; **SGR**-Specific growth rate

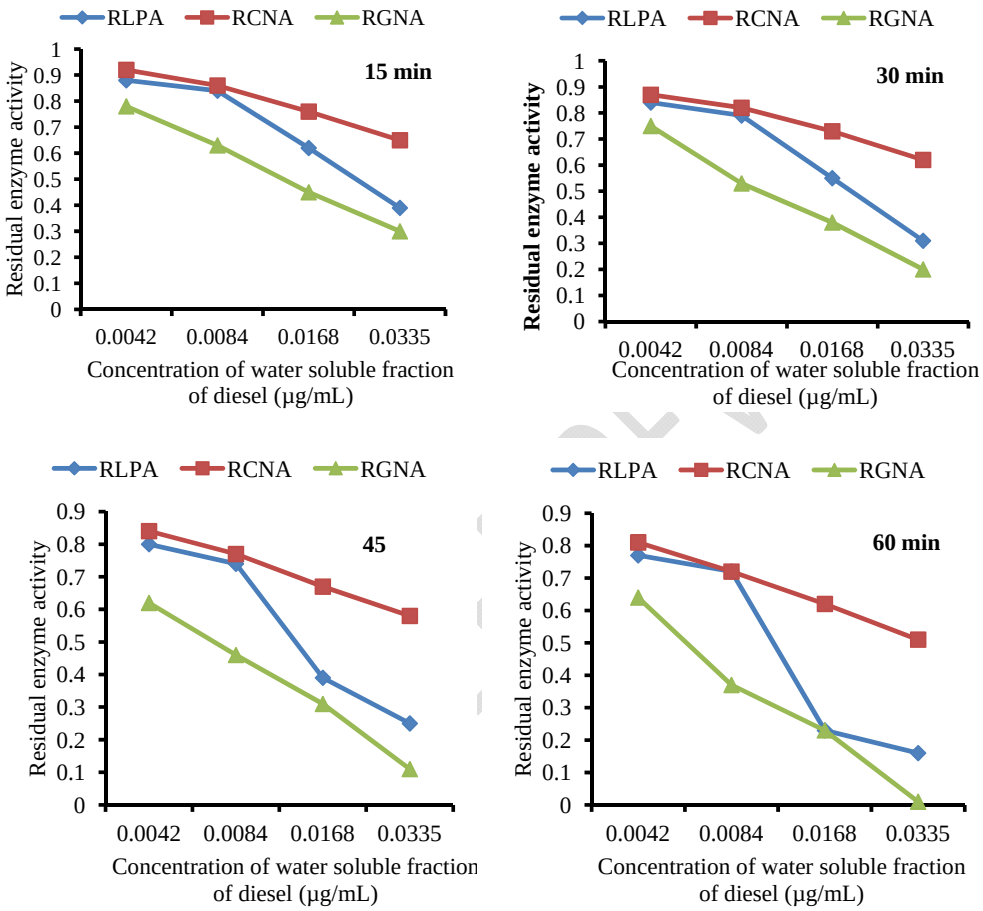

Figure 4 Effect of water soluble fraction of diesel on *Serratia* sp. strain DW2 enzyme activities as a function of exposure time. **RLPA**-Residual lipase activity; **RCNA**-Residual caseinase activity; **RGNA**-Residual gelatinase activity.

Table 1 Pearson's bivariate correlation of bacterial responses to the toxic effects of water soluble fraction of diesel and exposure time

	Change in specific growth rate	Residual lipase activity	Residual caseinase activity	Residual gelatinase activity	Change in lag time
Change in specific growth rate	1				
Residual lipase activity	-0.830**	1			
Residual caseinase activity	-0.863**	0.963**	1		
Residual gelatinase activity	-0.852**	0.939**	0.977**	1	
Change in lag time	0.819**	-0.882**	-0.933**	-0.951**	1

** Correlation is significant at the *P* = 0.01 level

Highest correlation (r = .977) was observed between residual gelatinase activity and residual caseinase activity suggesting that both enzymes were affected by the toxicant to roughly the same degree. Residual enzyme activity was calculated as a ratio of the specific activity of the enzyme after toxicant exposure to that in the control without prior toxicant exposure.

The Levene's test of equality of variance was not significant: F (15, 32) = 1.810, P = .078, for change in lag time suggesting that the homogeneity assumption was met. Two-factor ANOVA conducted on the data revealed that the interaction between WSF-D-TPH concentration and exposure time did not make significant contribution to changes in the lag time of the bacterium, F (9, 32) = 1.106, P = .387, η^2 = .237. However, the main effect of WSF-D-TPH concentration was significant, F (3, 32) = 139.095, P < .0001, η^2 = .929. From an overall model goodness-of-fit R^2 of .949, toxicant concentration contributed 92.9% of the fit. The main effect of exposure time was also significant, F (3, 32) = 56.429, P < .0001, η^2 = .841 suggesting that exposure time made 8.8% less contribution to the adequacy of the model than WSF-D-TPH concentration.

The assumption of homogeneity for change in specific growth rate (P = .273), residual lipase activity (P = .243), residual caseinase (P = .132) and residual gelatinase activities (P = .120) were not also violated. Two-way analysis of variance for residual caseinase activity revealed that the interaction between concentration of WSF-D-TPH and exposure time was not significant, F (9, 32) = 1.983, P = .075, η^2 = .358. However, the main effects of toxicant concentration and exposure time made significant (P < .0001) contributions of 99% and 95.7% respectively to the changes observed in residual caseinase activity of *Serratia* sp. strain DW2. The interaction effects between WSF-D-TPH concentration and exposure time were significant for change in specific growth rate (R^2 = 99.7%, P < .0001), residual lipase activity (R^2 = 92.2%, P < .0001) and residual gelatinase activity (R^2 = 76.7%, P < .0001). The Bonferroni multiple comparison post hoc tests for specific growth rate changes, residual lipase activity and residual gelatinase activity revealed that mean differences of all pairwise comparisons were significant (P < .05) .

4 CONCLUSION

Serratia sp. strain DW2 **was** has been isolated from the hydrocarbon-impacted Douglas creek waters of Qua Iboe Estuary, Nigeria and demonstrated significant ability to utilize petroleum hydrocarbons as sole source of carbon and energy thus contributing to the natural attenuation of the ecosystem. The bacterium also demonstrated significant abilities to elaborate caseinolytic protease and gelatinase for nitrogen cycling as well as lipase for carbon cycling and ultimate ecosystem health. However, growth of the bacterium was reduced in the presence of tolerable concentrations of soluble fractions of diesel through inhibition of enzyme activity. Interactions between toxicant concentration and exposure time did not significantly influence changes in lag time and residual caseinase activity; however, changes in the specific growth rate of the bacterium were significantly dependent on such interaction effect. Exposure time was found to interfere with the relationship between changes in lag time and specific growth rate. The bacterium is recommended as a model **bacterium** for natural attenuation studies in the ecosystem.

REFERENCES

1. Imron MF, Titah HS. Optimization of diesel biodegradation by *Vibrio alginolyticus* using Box-Behnken design. Environ Eng Res 2018; 23: 374-382. https://doi.org/10.4491/eer.2018.015

2. Dixit H, Lowry M, Mohsin U, Moond M, Kumar S, Chokriwal A, Gupta S. Growth optimization and comparative analysis of diesel oil degradation potential of *Bacillus* sp. isolated from petroleum contaminated soil of Barmer, Rajasthan. Int J Recent Sci Res 2018; 9: 24730-24737. https://doi.org/10.9790/2402-1009010108

3. Prasad RG, Anuprakash MVVS. Pollution due to oil spills in marine and control measures. IOSR J Environ Sci Toxicol Food Technol 2016; 10: 1-8 https://doi.org/10.9790/2402-1009010108

4. Itah AY, Essien JP. Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny, Nigeria. World J Microbiol Biotechnol 2005; 21: 1317-1322. https://doi.org/10.1007/s11274-004-6694-z

5. Ekpenyong MG, Antai SP, Essien JP. Quantitative and qualitative assessment of hydrocarbon degrading bacteria and fungi in Qua Iboe Estuary, Nigeria. Res J Microbiol 2007; 2: 415-425 https://doi.org/10.3923/jm.2007.415.425

6. Das N, Chandran P. Microbial degradation of petroleum hydrocarbon pollutants: an overview. Biotechnol Res Int 2011; Article ID 941810. https://doi.org/10.4061/2011/941810

7. Ekpenyong MG, Antai SP, Asitok AD. A *Pseudomonas aeruginosa* strain IKW1 produces an unusual polymeric surface-active compound in waste frying oil-minimal medium. Int J Sci 2016; 5: 108-123. https://doi.org/10.18483/ijSci.1064

8. Fender JE, Bender CM, Stella NA, Lahr RM, Kalivoda EJ, Shanks RMQ. Serratia marcescens quinoprotein glucose dehydrogenase activity mediates medium acidification and prodigiosin inhibition by glucose. Appl Environ Microbiol 2012; 78: 6225-6235. https://doi.org/10.1128/AEM.01778-12

9. Haddix PL, Jones S, Patel P, Burnham S, Knights K, Powell JN, LaForm A. Kinetic analysis of growth rate, ATP and pigmentation suggests and energy-spilling function for the pigment prodigiosin of *Serratia marcescens*. J Bacteriol 2008; 190: 7453-7463 https://doi.org/10.1128/JB.00909-08

10. Asitok, AD, Ekpenyong, MG. Comparative analysis of determination methods of glyphosate degradation by *Trichoderma asperellum* strain JK-28: a multivariate statistical approach. J Agric Ecol Res Int 2019; 19: 1-14. https://doi.org/10.9734/jaeri/2019/v19i130070

11. Iboyo, AE, Asitok, AD, Ekpenyong, MG, Antai, SP. Selection of *Enterobacter cloacae* strain POPE6 for fermentative production of extracellular lipase on palm kernel oil processing effluent. Int J Sci 2017; 6: 1-22. https://doi.org/10.18483/ijSci.1482

12. Asitok, AD, Antai, SP, Ekpenyong, MG. Water soluble fraction of crude oil uncouples protease biosynthesis and activity in hydrocarbonoclastic bacteria; implications for natural attenuation. Int J Sci 2017; 6: 5-21. https://doi.org/10.18483/ijSci.1344

13. Vazquez SC, Hernandez E, MacCormack MP. Extracellular proteases from the Antartic marine *Pseudoalteromonas* sp. P96-47 strain. Rev Argent Microbiol 2008; 40: 63-71.

14. Coelho DF, Saturnino TP, Fernandes FF, Mazzola PG, Silveira E, Tambourgi EB. Azocasein substrate for determination of proteolytic activity: re-examining a traditional method using bromelain samples. BioMed Res Int 2016; Article ID 8409183. https://doi.org/10.1155/2016/8409183

15. Ekpenyong, M, Asitok, A, Odey, A, Antai, S. Production and activity kinetics of gelatinase by *Serratia* sp.SLO3. Nig J Biopest 2016; 1: 70-82.

16. Zeng J, Teng F, Murray BE. Gelatinase is important for translocation of *Enterococcus faecalis* across polarized human erythrocyte-like T84 cells. Infect Immun 2005; 73: 1606-1612. https://doi.org/10.1128/IAI.73.3.1606-1612.2005

17. Papa MFD, Hancock LE, Thomas VC, Perego M. Full activation of *Enterococcus faecalis* gelatinase by a C-terminal proteolytic cleavage. J Bacteriol 2007; 189: 8835-8843 https://doi.org/10.1128/JB.01311-07

18. Usharani, B, Muthuraj, M. Production and characterization of protease enzyme from *Bacillus laterosporus*. Afr J Microbiol Res 2010; 4: 1057-1063.

19. Elkenawy, NM, Yassin, AS, Elhifnawy, HN, Amin, MA. Optimization of prodigiosin production by *Serratia marcescens* using crude glycerol and enhancing production using gamma radiation. Biotechnol Rep 2017; 14: 47-53. https://doi.org/10.1016/j.btre.2017.04.001

20. Karamba KI, Ahmad SA, Zulkharnain A, Yasid NA, Ibrahim S, Shukor MY. Batch growth kinetic studies of locally-isolated cyanide-degrading *Serratia marcescens* strain AQ07. 3 Biotech, 2017. https://doi.org/10.1007/s13205-017-1025-x

21. Ekpenyong, MG. Antai, SP. Cadmium toxicity on species of *Bacillus* and *Pseudomonas* during growth on crude oil. Trends Appl Sci Res 2007; 2: 115-123 https://doi.org/10.3923/tasr.2007.115.123

22. Rajasekar, A. Biodegradation of petroleum hydrocarbon and its influence on corrosion with special reference to petroleum industry. In: Rajasekar, A, Maruthamuthu, S, Ting, Y-P, Balasubramanian, R, Rahman, PKSM, editors. Bacterial degradation of petroleum hydrocarbons. Switzerland: Springer Nature; 2012. https://doi.org/10.1007/978-3-642-23789-8_13

23. Xia, M, Liu, Y, Taylor, AA, Fu, D, Khan, AR. Crude oil depletion by bacterial strains isolated from a petroleum hydrocarbon impacted solid waste management site in California. Int Biodet Biodegrad 2017; 123: 70-77. https://doi.org/10.1016/j.ibiod.2017.06.003

24. Nkanang, AJ, Antai, SP, Asitok, AD, Ekpenyong, MG. Assessment of diesel oil toxicity on some hydrocarbonoclastic bacteria isolated from Iko River estuary in the Niger Delta. World J Pharma Med Res 2018; 4: 48-55.