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BACTERIAL ENZYME BIOSYNTHESES INHIBITION: A TOOL FOR ECOTOXICITY ASSAY

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ABSTRACT

The inhibition of biosyntheses of four bacterial enzyme systems by 39 chemicals (hydrocarbon fuels, oil spill dispersants, household detergents, drilling chemicals and crude oils) were examined as a tool for ecotoxicity assay. The enzyme systems were two extracellular inducible enzymes tryptophanase and □ - glucosidase produced by *Escherichia coli* and *Bacillus licheniformis* respectively, one intracellular inducible enzyme □ - galactosidase in *E. coli* and the constitutive enzymes in Nitrobacter mediating the oxidation of nitrite to nitrate "nitritase". Ecotoxicity assay results were obtained within 5 hours after commencement of tests. Beta –galactosidase biosynthesis was the most insensitive to the 39 chemicals. Tryptophanase and □-glucosidase biosyntheses displayed similar but higher levels of sensitivities to the chemicals. "Nitritase" enzymes biosynthesis was the most sensitive to all the toxicants. The inhibition of constitutive enzyme systems such as the "nitritase" enzyme system may thus be considered as a tool for rapid ecotoxicity assays.

Key words: Constitutive, Inducible, Ecotoxicity, Extracellular Intracellular.

INTRODUCTION

Many toxicity tests are accomplished by using microorganisms as test organisms (Williamson and Johnson, 1981; Wang, 1984; Giesy *et al.*, 1988; Jardim *et al.*, 1990; Xu and Schurri, 1990). A good bioassay-testing organism must be sensitive and convenient to use (Wiliamson and Johnson 1981). Other preferred characteristics of such as organism are as follows;

- The organism is a representative of an ecological important group (in terms of taxonomy, trophic level or realized niche) to man or other important species.
- The organism is widely available, is amenable, genetically stable, so uniform population can be tested
- There is adequate background data on the organism (that is its physiology genetics, taxonomy, role in natural environment are well understood. Other factors include economic importance, type of test, sensitivity to toxicant, consistency in response to the toxicant (Buikema *et al*, 1982).

Fish have been the most popular test organism because they are presumed to be the best understood organism in the aquatic environment. However the standard test species have been expanded in recent times. Macroorganism like rats, snails, crabs, crayfish e.t.c. have been used for toxicity bioassays (Buikema *et al.*, 1982). With the high demand of rapid inexpensive and relatively simple screening test for evaluating the acute toxicity of chemicals in the environment the use of microorganism has recently gained wide attention: These microorganisms include bacteria, fungi, protozoa and algae. Baeteria are easy to handle and respond quickly and reproducibly to changes in their environment. They are inexpensive and need only small amount of test substance.

Bacterial are particularly important because of their unique role of trophic dynamics in aquatic and terrestrial ecosystems (Jonas, 1989). They provide a source of enriched particulate organic carbon by utilization of both dissolved and particulate organic carbon (de la Cruz, 1973). Bacteria are easy to standardize for toxicity in comparison to many eukaryotic organism (Bauda and Block 1985).

Bioassay tests employing bacteria are based on the inhibition of some microbial vital function. Examples include inhibition of nitrite utilization by *Nitrobacter* (Williamson and Johnson 1981; Wang 1984; Okpokwasili and Odokuma 1996), Microtox tests (Gusy *et al.*, 1988), Ames Salmonella assay (Vandermuelen, 1986), inhibition of inducible extracellular and intracellular enzymes –tryptophanase, □ - glucosidase and □ -galactosidase (Dutton *et al.*, 1990).

Beta –galactosidase is an intracellular enzyme produced by *Escherichia coli*. It confers on this organism the ablity to break down the disaccharide lactose into glucose and galactose. Tryptophanase, an inducible enzyme produced by *E. coli* extracellularly in the presence of the aminoacid L. tryptophan. Both □-galactosidase and tryptophanase production are controlled by

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different operons in *E. coli* (Magasnik 1988). Alpha glucosidase is an inducible extracellular enzyme produced by *Bacillus lincheniformis*. It confers on this organism the ability to breakdown maltose into glucose. The "nitritase" enzyme system (Wang 1984) is constitutive and is present in *Nitrobacter*. It confers on this organism the ability to oxidize nitrites into nitrates. Studies have been carried out on these enzyme systems (Williamson and Johnson 1981; Wang 1984; Magasanik, 1988, Dutton *et al*., 1990).

The objective of this study was to compare the toxicity of a wide range of chemicals, liquid hydrocarbon fuels, oil spill dispersants, domestic detergents, lubricating oils, drilling chemicals and crude oils to the biosyntheses of tryptophanase, □ - galactosidase, □ - glucosidase and "nitritase." The aim was to identify the most sensitive enzyme system to these toxicants. This enzyme system could serve as a rapid, inexpensive and relatively simple screening tool for evaluating the toxicity of chemicals in the environment.

MATERIALS AND METHODS

Toxicants.

Hydrocarbon fuels:

Liquid hydrocarbon fuels employed, as toxicants in this study were jet fuel, multipurpose Kerosene, gasoline (petrol) and diesel.

Oil Spill Dispersants:

Samples of dispersants employed and their sources were Corexit 9527 (Shell Petroleum Development Company Ltd Port Harcourt), Surflo OW-1, Prodesolv and Dispolene 36.5 (Nigerian Agip Oil Company Ltd Port Harcourt). Included as reference detergents were; Sodium Dodecyl Sulphate (SDS] Tween 80 (Sigma Chemical Company, St Louis MO) and Triton X -100 (Vickers Laboratories, Burley England)

Organic Solvents:

Organic solvents employed were n – hexane, n –heptane benzene, toluene and xylene. They were all obtained from Sigma Chemical Company St, Louis MO).

Drilling Chemicals:

Drilling Chemicals employed were Huile-Clean, Chaux (lime), Carbotrol A 9, Carbotex HW, Carbotec Sea , Carbovis and Carbomul Sea. They were all obtained from the Nigerian Agip Oil Company Ltd Port Harcourt.

Crude Oils:

Crude oil assessed for toxicity were Bonny medium, Bonny light and Brass Rivers. Others were Ughelli Quality Control Crude (UQCC), Qua Iboe and Trans Niger Pipeline (TNP). All Crude oils were provided by the Nigerian National Petroleum Corporation (NNPC) Port Harcourt, Nigeria.

Household Detergents:

Household detergents assessed for toxicity were Flex, Spencer, Teepol Rainbow and Apollo. They were purchased locally.

Test Bacteria:

Toxicity assays were conducted with *Escherichia coli*, *Bacillus licheniformis* and *Nitrobacter sp*. *E. coli* and *Nitrobacter sp.* were both isolated from the New Calabar River water in Rivers State Nigeria while *B. licheniformis* was isolated from the soil near the Microbiology laboratory in the University of Port Harcourt. To ensure adaptation to laboratory conditions all strains were maintained by continuous subculture every 48h into fresh media.

Characterization and identification of Test Bacteria.

Isolates were examined for colony,

TABLE 1: Effect Of Fresh (Parent) Crude Oils On The Inhibition of biosynthesis of four enzymes

	IC_{50}(mg/L)			
Crude oil	β-galactosidase	Tryptophanase	α-glucosidase	Nitritase
Qua Iboe	NT	150	85,000	15.0
Brass Rivers	NT	140	NT	40.0
Bonny light	NT	400	NT	10.0
Bonny medium	80,000	8.0	NT	60.0
TNP	NT	2.0	NT	100
UQCC	NT	100	NT	60
Antan Heavy	1.0	0.8	8,000	3.5
Antan Medium	1.0	0.8	8,000	70

NT (Not toxic)= above 1,000000 mg/L

morphology and biochemical characteristics. For *E. coli* and *B. linchenifornis*, the tests included Gram reaction, spore staining, oxidase test, oxidative/fermentative. (O/F) utilization of glucose, motility test, indole test Voges – Proskuaer, catalase test, urea utilization, aerobic and anaerobic growth, growth in 7% NaCl and at 55^0 to 60^0C, which were carried out according to the methods described by Cruickshank *et al.* 1975). For *Nitrobacter sp.*, Gram reaction, colony morphology and sole utilization of nitrite as energy source, were used for its identification according to the method of Colwell and Zambruski (1972). Identification of bacteria to species level followed the scheme in Laskins and Lechevalier (1977).

Preparation of Toxicant Concentrations:

Toxicant concentrations of 1000, 100, 10,1.0. and 0.1 mg/L were prepared. For liquid toxicants an amount equivalent to one thousand milligrams of liquid toxicant was weighed and transferred into distilled water (100ml of distilled water minus the equivalent volume of 1000mg of liquid toxicant). A similar procedure was repeated for other concentrations (100, 10,1.0, 0.1). For example an amount equivalent to 100mg of the liquid toxicant was weighed and transferred in to 100ml of distilled water minus the equivalent volume of 100mg of the liquid toxicant to produce 100mg/1 of the toxicant.

Preliminary range finding test were undertaken. Organisms were exposed to logarithmic concentrations of toxicants. The least toxicant concentration that produced total inhibition of the enzyme system (or death of organism) was taken as 100% inhibition (the highest toxicant concentration). Also the highest toxicant concentration that could produce no inhibition of enzyme synthesis was taken as the lowest toxicant concentration. Toxicant concentrations were the produced between these two limits.

Concentrations varied according to the toxicity of the toxicant to 50ml of distilled water, O.1g of solid toxicant (or its equivalent in volume for liquid toxicants) was added. The volume was made up to100 ml to produce a concentration of 1000 mg/L serial tenfold dilutions of this stock were produced till required minimum concentration (concentration that will have no effect on the enzyme biosynthesis) was achieved.

Toxicity Assay Protocol:

The method of Dutton *et. al.* (1990) was employed for assays for the biosyntheses of □ – galalctosdase, tryptophanase and □ -glucosidase. They had the following basic steps in common: (1) cell growth (2) cell washing (3) cell exposure to toxicant, (4) induction of enzyme biosynthesis and (5) measurement of enzyme activity. Nitritase biosynthesis assay also followed the same steps except that the measurement of enzymatic activity was done indirectly by measuring the loss of substrate (nitrite) as in APHA (1985).

Cell Growth and preparation

Escherichia coli was grown in nutrient broth overnight at 37^0C.

Cells were diluted with medium to A_{550} = 0.2. (Absorbance 0.2 at 550 nm). Medium without organisms was used as blank. Cells were allowed to grow to A_{5550} = 0.6 after which were washed thrice with 0.8% NaCl.

TABLE 2: Effect of weathering and biodegradation of eight crude oils on the biosynthesis of nitritase

	IC_{50} (mg/l)									
	Weathered crude oil (weeks)					Biodegraded crude oil (weeks)				
Crude oil	0	1	2	3	4	0	1	2	3	4
Bonny Medium	55	0.2	0.2	4.0	4.0	100.0	1.00	0.35	0.03	0.02
Bonny Light	40	50.0	6.0	0.075	0.06	40	0.30	0.09	0.07	0.10
Brass River	120	0.2	0.5	0.50	30.0	40	1.0	0.50	0.095	0.0075
Qua Iboe	105	0.9	0.11	0.15	0.08	118	9.0	0.70	0.11	0.01
UQCC	50	0.10	0.40	0.09	0.08	60	5.5	0.10	0.10	0.01
TNP	200	0.09	0.10	0.50	0.09	300	0.85	0.40	0.02	0.03
Antan Medium	70	0.4	5.0	0.20	10	70	2.0	4.0	0.01	0.09
Antan heavy	0.2	0.07	0.10	0.05	0.008	6.0	0.08	0.095	0.10	0.005

NT (Not toxic) = above 1,000000 mg/l

Exposure to Toxicant:

Cell (0.1 ml) of washed cell suspension at A_{550} = 0.6) were exposed to 0.9ml of toxicant and incubated for 30min.

Control:

Control contained cells plus medium with no toxicant.

Enzyme Induction:

To 1ml each of the reaction mixture, 0.1 m1 Isopropyl – □ –D thiogalactoside (IPTG) 0.1%) (w/v) (Sigma Chemical Company), 0.5 – buffer and 0.5ml growth medium were added. The mixture was incubated for 30 minus.

Beta –galactosidase Measurement

Since □ – galactosidase is intracellular, it was necessary to lyse the cells. This was performed by the addition of 0.1 ml 10% Sodium Dodedcyl Sulphate (SDS) 10% (w/v) to the reaction mixture. This was followed by the addition of 0.2 ml O- nitrophenyl – D-galactoside (ONPG) 0.4% (w/v) Sigma Chemical Company. The mixture was incubated until colour developed (15 min). The reaction was stopped with 1 ml or cold $N_{a2}CO_3$ (1M) and the absorbance at 542nm using a blank consisting of all reagents minus IPTG was determined (Dutton *.et al.*, 1990).

Typtophanase biosynthesis Assay

(i) L –tryptophan (250 mg/L) in KH_2PO_3 (13.6 g/L) adjusted to pH – 7.8.

(ii) Erhlich's reagent consisting of 6 parts of p – dimethylaminobenzaldehyde (5%, w/v/) Sigma Chemical Company, in 95% ethanol and 12 parts acid alcohol |(16 ml conc. H_2SO_4 in 200 ml 95% ethanol).

Cell growth and preparation

Escherichia coli was grown in case in hydrolysate without tryptophan (10g/1, yeast extract (5 g/L) and NaCl (19g/l) overnight at 37^{0}C.

Cell preparation was as for □ – galactosidase biosynthesis assay.

Exposure to Toxicant:

Cell (O.1 ml of washed cell suspension at A550 = 0.6) were exposed to 0.9ml toxicant and incubated for 30.min.

Enzyme induction

To the reaction mixture 0.4 ml buffer, 0.1 L –tryptophan and 0.5 ml nutrient both were added. The mixture was incubated for 120 min.

Tryptophanase Measurement

One milliliter of Erhlich's reagent was added to the reaction mixture and incubated for 15 min. Absorbance at 560 nm using a blank consisting of all reagents minus L –tryptophan, was determined.

Alpha –glucosidase Biosynthesis Assay

Maltose (4% w/v) and autoclaved p –nitrophenyl □ –D – glucoside (PNG) 0. 5% (w/v) were used. This solution was filter-sterilized (0.22um pore size) and stored at 4^{0}C in the dark.

Cell Growth, Preparation and Enzyme Induction:

Bacillus licheniformis – was grown in tryptone soya broth without dextrose overnight at 37^{0}C. Cell preparation and toxicant exposure were as in □ – galactosidase biosynthesis assay.

To the reaction mixture 0.4 ml Z – buffer, ($N_{a2}HPO_4$ $7H_2O$ 16.1g/L NaH_2 PO_4 H_2O, 5.5. g/L KCL, O.75 g/l: and $MgSO_4$. $7H_20$ 0.25 g/L) 0.1 ml maltose and 0.5 fresh mediums were added. The mixture was incubated for 60 min.

Alpha –glucosidase Measurement

Ortho -nitrophenyl-D- galactoside (ONPG) (0.2ml) was added and the mixture incubated for 60 min. Reaction was stopped by the addition of 1ml Na_2CO_3. The absorbance of the mixture at 420nm was measured. A solution consisting of all other reagents minus maltose was used as blank for the reading.

"Nitritase" Biosynthesis Assay

The reagents used were as in method for nitrite determination APHA (1985). The other steps growth, cell preparation, exposure to toxicant, enzyme induction and nitritase measurement was as reported earlier (Okpokwasili and Odokuma, 1994).

Toxicity and Analysis

The method employed was adopted from Dutton *et al.*, (1990). One hundred militers of serial dilutions of each fresh crude oil were placed in 250 ml

TABLE 3: Effect of weathering and biodegradation of eight crude oils on the biosynthesis of Tryptophanase

	IC_{50} (mg/l)									
	Weathered crude oil (weeks)					Biodegraded crude oil (weeks)				
Crude oil	0	1	2	3	4	0	1	2	3	4
Bonny Medium	10.0	6.0	4.5	7,500	NT	10.0	0.6	700.0	90.0	NT
Bonny Light	500	6.2	30.0	80.0	NT	400	1.100	NT	100	NT
Brass River	80	2.5	8.5	0.09	NT	70.0	14.0	NT	10.000	NT
Qua Iboe	130	80.0	1.0	400	NT	130	9.5	NT	5.0	NT
UQCC	100	4.0	600	250	NT	100	1.000	30.0	105	NT
TNP	2.0	6.5	20000	80.0	NT	2.0	7.000	200	9.0	NT
Antan Medium	3.0	5.0	NT	2.000	NT	0.5	70.0	3.0	7.0	1,000
Antan heavy	0.8	60.0	NT	60.0	10.000	1.1	10.0	5.0	10.0	5,000

NT (Not toxic)=above 1,000000 mg/l

cotton wool- plugged shake flasks. The flasks were incubated for 4 weeks. Enzyme biosynthesis assay for nitritase, trytophanase □ – galactosidase and □ - glucosidase were carried out on each of the crude oil concentrations. Periodicity of assays was immediately after preparation of toxicant concentration (day 0) and thereafter every hour for four hours. This served as based line control data.

Water samples containing a mixed population of hydrocarbonoclastic microorganisms was obtained from the New Calabar River at a site located about 1 km southwest of the University of Port Harcourt.

Seeding of toxicant

To 75 ml of each crude oil concentration, 25 ml of the New Calabar River water was added.

All concentrations for the enzyme assay were prepared in triplicate. Preliminary range finding test was carried out where possible (because some toxicants did not inhibit the enzyme systems). To determine toxicant concentration causing between 10 and 100% inhibition. The degree of enzyme inhibition was determined by measuring absorbance with respect to control (assigned 0% inhibition). Control contained no toxicant. For "nitritase" biosynthesis assay, the ratio of the absorbance in control to that of toxicant multiplied by 100 was taken as percent toxicant inhibition. For the three other enzyme systems, the ratio of the absorbance of the toxicant to that of the control multiplied by 100 was taken as the percent toxicant inhibition. The mean of triplicate samples was obtained and used to plot a graph of percent inhibition versus concentration of toxicant. The chemical concentration giving 50% inhibition (IC_{50}) for the toxicants was derived from the line of regressions of the plot.

Stastical Analysis:

Results were subjected to student t-test and analysis of variance (Finney 1978).

RESULTS

In Table 3 the IC_{50} of, four hydrocarbon fuels and 5 organic solvents to the biosyntheses of four bacterial enzymes are presented. The IC_{50} of nitritase was the least indicating that nitritase biosyntheses was the most sensitive of the four enzymes. Beta - galactosidase synthesis was the least inhibited by both fuels and organic solvents. The high IC_{50} values showed this. Tryptophanase and □ - glucosidase biosyntheses displayed similar levels of significance at 95% probablity level. The four hydrocarbon fuels diplayed similar levels of toxicity to "nitritase" biosynthesis. With respect to organic solvents hexane was more toxic than heptane (increase in the length of the carbon chain of aliphatic hydrocarbon decreased their toxicity), and increasing methyl substitution in the aromatic ring led to a decrease in toxicity of the aromatic ring. Thus toxicity decreased as follows benzene >toluene > xylene.

The median inhibition concentration of four oil spill dispersants, 3 reference detergents and five household detergents are presented in table 4. There was a significant difference at 95% prohability level between the IC_{50} of "nitritase" and the IC_{50} of □ – galactosidase and tryptophanase when exposed to oil spill dispersants and household detergents.

There was no significant difference at 95% probability level between □ - glucosidase

and "nitritase" enzyme biosyntheses on exposure to these toxicants. However at 99.5% probability there was a significant difference between the inhibitions of both enzyme biosyntheses by these toxicants. These results suggest "nitritase" biosyntheses was the most sensitive to oil spil dispersants, reference detergents and household detergents. This was followed by □ - glucosidase and □ – galactosidase and tryptophanase biosyntheses which displayed similar levels of inhibition.

There was a significant difference between the IC_{50} of "nitritase" enzyme biosynthesis and the IC_{50} of the other 3 enzyme biosyntheses when exposed to household detergents. These results suggest that nitritase enzyme biosynthesis was more sensitive to the household detergents than the other three enzymes. These three enzymes displayed similar levels of sensitivities at 95% probability levels. Household detergents displayed similar levels of toxicity at 95% probability level to nitritase enzyme biosynthesis.

Table 5 shows the median inhibition concentration of seven drilling chemicals and 8 crude oils to the biosyntheses of four bacterial enzymes. The IC_{50} of "nitritase" biosynthesis was significantly (95% probability level)

TABLE 4: Effect of weathering and biodegradation of eight crude oils on the biosynthesis of β galactosidase

	IC_{50} (mg/l)									
	Weathered crude oil (weeks)					Biodegraded crude oil (weeks)				
Crude oil	0	1	2	3	4	0	1	2	3	4
Bonny Medium	NT	NT	NT	NT	NT	NT	NT	NT	10000	NT
Bonny Light	NT	1,000000	NT	80.0	NT	NT	NT	NT	NT	NT
Brass River	NT	7000	NT	NT	NT	NT	NT	NT	NT	NT
Qua Iboe	NT	10000	NT	NT	NT	NT	NT	NT	NT	NT
UQCC	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
TNP	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Antan Medium	4.5	20.0	NT	NT	NT	4.5	NT	NT	NT	NT
Antan heavy	1.1	2000	NT	NT	1,000000	4.0	NT	NT	NT	NT

NT (Not toxic)–above 1,000000 mg/l

TABLE 5: Effect of weathering and biodegradation of eight crude Oils on the biosynthesis of α -glucosidase

	IC_{50} (mg/l)									
	Weathered crude oil (weeks)					Biodegraded crude oil (weeks)				
Crude oil	90.0	1	2	3	4	0	1	2	3	4
Bonny Medium	30000	5,000	NT	NT	900000	07.0	NT	700.0	90.0	NT
Bonny Light	30000	0.6	NT	0.09	1.000	NT	NT	NT	0.85	NT
Brass River	90000	0.6	NT	NT	NT	NT	NT	NT	16.000	NT
Qua Iboe	NT	0.8	700000	NT	600000	NT	NT	NT	50000	NT
UQCC	NT	5.000	70000	NT	30.0	NT	NT	NT	NT	1,000
TNP	20.0	80.0	NT	NT	NT	NT	NT	10,000	NT	NT
Antan Medium	6000	0.07	NT	NT	NT	8000	700	80000	NT	NT
Antan heavy	0.025	0.50	NT	NT	7.7.0	60.0	3,000	600000	100	60.0

NT (Not toxic)–above 1000000 mg/l

smaller than the IC_{50} of the other 3 enzyme biosyntheses when exposed to these toxicants. These results suggest that "nitritase" bosynthesis was the most sensitive to the drilling chemicals. However both "nitritase" and tryptophanase displayed similar levels of inhibition when exposed to crude oils. Beta galactosidase and □ -glucosidase biosyntheses were less sensitive to the crude oils.

DISCUSSION

Results indicated that the constitutive enzymes mediating oxidation of nitrite to nitrate in *Nitrobacter* ("nitritase") biosynthesis was they most sensitive to the toxicants. This is accordance with similar observations made by Okpokwasili and Odokuma 1994, 1996a, 1996b. Alpha –glucosidase and tryptophanase biosyntheses displayed similar levels of inhibition by the toxicants however the inhibition of their biosynthesis was less then that of nitritase. Beta –galactosidase was the least sensitive. Inhibition of "nitritase" biosyntheses by the toxicants may be due to the high permeability of the outermembrane of *Nitrobacter* to these toxicants. The membrane been the site of the "nitritase" enzyme complex. The "nitritase" enzyme complex mediated respiration (Stanier *et al.*, 1980) and thus their inhibition affected the respiration process. The general insensitivity of □ – galactosidase biosynthesis (except to organic solvents) may be due to the intracellular nature of the enzyme and the subsequent inability of the toxicants to penetrate the outer membrane of *Escherichia coli*. Similar results obtained by Dutton *et al.*,(1990) revealed the relative insensitivity of □ – galactosidase biosynthesis to hydrophobes such as phenol. The relatively higher sensitivity of tryptophanase biosynthesis in the same *E.coli* may be attributable to the extracellular nature of the enzyme. Thus it easily comes in contact with the toxicants (the barrier of the outer membrane been overcome]. Similar results obtained by Dutton *et al.*, (1990) revealed a higher sensitivity of tryptophanase biosynthesis to sodium dodecyl sulphate (SDS) and poly chlorinated biphenyls (PCBs) than □ – galactosidase biosynthesis.

The absence of the outer membrane in *Bacillus licheniformis* (Koch and Schaehter, 1985) coupled with the extracellular nature of □ -glucosidase (Magasanik, 1988) offered relatively easy contact with the toxicants than □ – galactosidase in *E. coli*. This may have been responsible for the sensitivity of □ glucosidase biosynthesis compared to □ – galactosidase biosynthesis.

Normal hexane was more toxic than normal heptane for all four-enzyme biosyntheses. The higher water solubility of n- hexane over n – heptane four all four enzyme biosyntheses may have been responsible (Teh, 1974). The more water soluble the toxicant the easier it is to react with the enzymes or even penetrate the outer membrane of the organisms. Water solubility may have also been responsible for the decreasing toxicity with increase in methyl substitution in the benzene molecule (i.e toxicity decrease in the following trend; benzene > toluene > xylene). Water solubility also decreased in the same pattern. This was exhibited in the biosyntheses inhibition pattern of □ – galactosidase, tryptophanase and □ glucosidase. However the sensitivity of "nitritase" biosynthesis to these methyl substituted benzene derivatives was in the reverse pattern (i.e toxicity decreased as follows; xylene > toluene > benzene). Similar results were obtained by Pettibone and Cooney (1988). They observed that organotins with larger organomoieties (ethyl through butyl), di and tri-subtituted compounds were more toxic to bacteria than mono substituted compounds. They suggested that their mode of action though not clearly understood may be the disruption of the cytoplasmic membrane function due to steric hindrance. This increased with increase in substitution. The same mechanism may be in operation in *Nitrobacter* in its response to benzene, toluence and xylene.

Most toxicants did not show toxicity to the all the four enzymes at once indicating that each toxicant had different enzyme – operon system it affected (Dutton *et al* .,1990).

CONCLUSION

The constitutive enzyme mediating oxidation of nitrite to nitrate in *Nitrobacter* "nitritase" was the most sensitive enzyme - operon system to all the 39 toxicants tested. The biosyntheses of the inducible extracellular enzymes tryptophanase in *E. coli* and □-glucosidase in *B. licheniformis* displayed lesser degrees of sensitivities to

the toxicant. The enzyme operon system mediating the biosynthesis of the inducible intracellular enzyine □ – galactosidase in *E. coli* was the most insensitive to all the toxicants. These results suggest that in considering tools for toxicity testing microbial enzyme –operon system mediating the biosynthesis of constitutive enzymes may offer an alternative for ecctoxicity testing.

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