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Enzymological Responses in Channa Punctatus Exposed to Sub-Lethal Concentration of Hexaconazole (Contaf) In Paddy-Cum-Fish Ecosystem

KEYWORDS: Hexaconazole, Channa puntatus, malate dehydrogenase (MDH), lactate dehydrogenase (LDH), peroxidase (Pox).

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ABSTRACT *Pesticides usage in agricultural fields to control pests is extremely toxic to non target organisms like fish and affect fish health through impairment of metabolism, sometimes leading to mortality. Present study is an attempt of investigating enzymological responses in Channa puntatus exposed to fungicide Hexaconazole used against Rhizoctonia solani in rice plants in paddy-cum-fish ecosystem. Native polyacrylamide gel electrophoresis have been used to analyze malate dehydrogenase (MDH), lactate dehydrogenase (LDH) and peroxidase (Pox) isozymes in different tissues like gill, heart, liver, kidney, muscle and spleen of the Channa punctatus in order to study the tissue specificity of these isozymes. LDH and MDH isozymes are major system found in liver and kidney and associated with environmental stressors gets elevated. Peroxidase, is related to antioxidant activity against free radical toxicity induced by fungicides, was found highly expressed in gill and heart. Lactate and malate dehydrogenase showed increased activity in spleen and kidney indicating detoxification and tissue damage induced by pesticide.*

Introduction

Rice and fish are one of the main staple foods for most people of Asia, where food deficiency is one of the crucial issues. Rice-fish farming is one of the best options to increase the food production from limited land through ecological agriculture (Jintong, 1996; Sugen, Zhechun and Jicheng, 1996; De la Cruz, 1994). The Kharif crop is constantly confronted with a wide variety of potential pathogens throughout its lifetime, resulting in reduced production. Sheath blight has been the most economically significant disease of rice. Sheath blight, caused by Rhizoctonia solani Kühn (Gangopadhyay & Chakrabarti, 1982; Ou, 1985), is the most important rice (Oryza sativa L.) disease (Groth & Lee, 2003; Lee & Rush, 1983; Rush & Lee, 1992). The disease is favored by dense stands with a heavily developed canopy, warm temperature and high humidity. The fungus survives between crops as structures called sclerotia or as hyphae in plant debris. Sclerotia or plant debris floating on the surface of irrigation water serves as sources of inoculum that attack and infect lower sheaths of rice plants at the waterline. Sheath blight is characterized by large oval spots on the leaf sheaths on stems (Fig. 1) and irregular spots on leaf blades (Fig. 2). To control the fungal disease, Hexaconazole (Contaf) fungicide has been used conatantly by farmers. Agrochemicals may be hazardous to the environment, because of their persistence, bioaccumulation and toxicity (Kristoff, Cacciatore, Verrengia & Cochón, 2011). Pesticides are used to kill a number of harmful insect pests in order to increase the food grain production to overcome the food requirement of increasing population. Studies concerning the effect of pesticides on fishes have their own importance in relation with the factors increasing ecological problems and toxicity in organisms. The pesticides applied on land eventually find their way to the aquatic environment, thus contaminating soil and water for several years and subsequently get accumulated in aquatic organisms (Mathivanan, 2004). Acording to Kumari, Sinha, Gopal & Prasad, (2001) the aquatic organisms like fish are able to accumulate several fold higher concentration of pesticide residues than the surrounding water.

"Figure 1 and Figure 2 about here"

Fish have been proposed as indicators for monitoring land-based pollution because they may concentrate indicative pollutants in their tissue, directly from water through respiration and also through their diet. Fish are frequently subjected to pro-oxidant effects of different pollutants often present in the aquatic environment. Channa punctatus constitute an excellent model to understand the oxidative stress in aquatic ecosystems. Indian catfish and Indian major carps are of great commercial importance and the most common fresh water fishes widely consumed. They can serve as good model to study responses to various environmental contaminants. Fish are relatively sensitive to changes in their surrounding environment, including an increase in pollution. Fish health may as a result reflect, and give a good indication of the health status of

the aquatic ecosystem in which the fish occurs.

Several investigations have been concerned with the characterization of tissue-and organ-specific isoenzyme patterns (Mo, Young and Gracy, 1975; Fisher & Whitt, 1978; Leslie & Pontier, 1980; Berg & Buth, 1984; Holt & Leibel, 1987; Basaglia, 1991; Xia, Wu, and Wang, 1992; Seimiya, Kusakabe and Suzuki, 1997) among which little were concerned with fishes. LDH and MDH isoenzymes are major system found in fishes. They are classified in different groups on the basis of their possession in different tissues or cell organelles (M. Seimiya et. al., 1997; Goward & Nicholls 1994). Chaudhuri & Krishna (1998) studied the tissue specificity and the degree of polymorphism of five enzyme systems in Labeo rohita from Yamuna namely in liver, muscle, heart and brain tissues. Peroxidase enzyme (H2O2 donor and consumer) contains many isoforms which partake in a variety of metabolic functions. In animals, per-

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oxidase enzymes are involved in phagocytosis and immune cell function (Rodriguez, Esteban and Meseguer, 2003; I. M. Soares-da-Silva, Ribeiro, Valongo, Pinto, Vilanova, Bleher, and Machad, 2002), cell adhesion (Holmblad & Soderhall 1999), antioxidant function (Gamble, Goldfarb, Porte, and Living-stone, 1995; T. S. Galloway and M. H. Depledge 2001) and the oxidative polymerization of hydroquinones to melanin (D'Ischa, 1991).

Materials and Methods

Sample collection

The samples were collected from a local market of length 30±5 cm and weight 52±5g. Prior to the experimentation the normal uninfected healthy fish were selected for experiment. The fish were properly washed in tap water and treated with 0.02% KMnO4 and 0.004% formalin solution to remove external infection of fungi, algae, etc. The samples were acclimatized for 15 days to laboratory conditions and kept in aquariums containing 35 litre of water and regularly fed. The changes in physico-chemical characteristics of water, such as temperature, pH, DO (dissolved oxygen), hardness and total alkalinity of experimental water were recorded throughout the experimental period (Table 1).

"Table 1 about here"

Experimental design

10 samples of each Channa puntatus were randomly selected from the stock and were exposed to ten different concentrations of fungicides Contaf for 96 hr to determine mean lethal concentration (LC_{50}). Different concentrations of Contaf were ranged from 5-50 ppm (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 ppm). LC_{50} for Contaf was found to be 25.003 ppm. For chronic sub-lethal study $1/4^{th\ of}$ LC_{50} value (6.25 ppm) was chosen for Channa puntatus. Fish were exposed to sub-lethal concentration for 90 days. A control group was maintained simultaneously. All these experiments were performed in triplicates.

All analyses were performed using the Statistical Package for Social Sciences (SPSS) software Vs. 16 in a PC-compatible computer and the confidence level was 95%.

Sample Preparation and Isoenzymatic study

Channa puntatus were colleted after 90 days of exposure to Hexaconazole and rinsed properly under tap water, autopsized. The target organs were gill, heart, liver, kidney, muscle and spleen. The organs were washed in 70% ethanol and stored in Bouin's solution in -70□C until use. For better results, isoenzymatic tests were done on the same day.

For sample preparation 100mg of tissue (gill, heart, liver, kidney, muscle and spleen) were menced in 500µl (1:5) of 0.1M Tris-HCl buffer (pH 7.4). The lysate was centrifused at 14000 rpm for 40 min at 4°C and supernatent was preserved for enzyme analysis.

Native PAGE (10% native polyacrylamide gel) was performed for LDH, MDH and Peroxidase in gill, heart, liver, kidney and spleen. For electrophoresis, 30 µL of the extract was mixed with 10 µL of treatment buffer and 35 µL of this mixture was applied to the well. Isoenzymes were electrophorsed as described by Stegemann, Afify, and Hussein, (1985). After electrophoresis, the gels were stained according to their enzyme system with the appropriate substrate and chemical solutions then incubated at room temperature in dark for complete staining. In most cases the incubation for about 1 to 2 hours is enough.

In gels staining, protocols of Jonathan & Wendell (1990) for MDH and LDH, Heldt, (1997) for Pox were used. MDH staining was done by incubating the gel in 100 mL of 0.05 M Tris HCl pH 8.5 containing 25 mg NBT, 25 mg EDTA, 25 mg NAD, 10 mg malic acid and 3 mg PMS for 15-30 min in dark. For LDH the gel was soaked in 100 mL of 0.05 M Tris HCl pH 8.5 containing 25 mg NBT, 25 mg EDTA, 25 mg NAD, 1ml lactic acid and 3 mg PMS for 15-30 min in dark. Bands of Peroxidase were visible upon incubation in 100ml of Tris-HCl buffer+50mg O-dianisidine (dissolved in few drops of acetic acid) + 1ml of H2O2 for 15-30 min in dark.

Gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours; and photographed. After the appearance of the enzyme bands, the reaction was stopped by washing the gel two or three times with tap water. This was followed by adding the fixative solution, which consists of ethanol and 20% glacial acetic acid (9:11 v/v). The gel was kept in the fixative solution for 24 hours and then was photographed.

For isoenzymes, the bands of enzyme activity were designated using the known system of nomenclature (Allendorff & Utter, 1978). An abbreviation which corresponds to the name of the enzyme designated each locus. When multiple loci were involved, the fastest anodal protein band was designated as locus one, the next as locus two and so on.

Results

The electrophoretic behaviors for the studied isoenzymes are shown in Figures 3-8 (MDH, LDH and Peroxidase control and toxicated respectively). Three, three and six isomorphic bands have been recorded respectively for the three isoenzymes in different studied tissues. In all zymograms, the origin of the electrophoretic patterns is at the top and the anode is at the bottom.

The isoenzyme malate dehydrogenase in toxicated sample showed three fractions in the electrophoretic pattern (Fig. 4). The three fractions (MDH 1, MDH 2 and MDH 3) were recorded in all tissues only with the exception of kidney. The isoforms MDH 2 and MDH 3 were not observed in kidney. All the three isoforms which occurred in different tissues of Channa punctatus did not show much variation between heart and liver followed by muscle and gill. Malate dehydrogenase enzyme activity was recorded highest in spleen than any of the rest tissues. MDH isoforms were expressed significantly in spleen followed by kidney. The stress related enzyme shows an overall increase in activity in toxicated Channa punctatus after 90 days as compared to control (Fig. 3). The presence and absence of bands for malate dehydrogenase in native gel was shown in Table 2.

"Figure 3 and Figure 4 about here"

"Table 2 about here"

Figure 6 reveals expression of lactate dehydrogenase with three isoforms LDH 1, LDH 2 and LDH 3 in Channa punctatus when exposed to contaf for 90 days. According to table 3, two fractions LDH 2 was present in gill, heart, liver, kidney and muscle whereas LDH 3 were significantly observed in all tissues. Only one isomorphic band, LDH 1 was expressed in muscle. The activity was recorded highest in liver followed by spleen, kidney, gill heart and muscle. There was no significant difference between spleen and kidney. Same was observed in case of heart and mus-

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cle too. The activity was higher in gill, liver and muscle than any other tissue in control (Fig. 5).

"Figure 5 and Figure 6 about here"
"Table 3 about here"
Regarding peroxidase isozyme, six isomorphic bands were recorded in different tissues (Fig. 8). This enzyme could also be considered as a good indicator for tissue specificity as it was expressed as two fractions, Pox 4 only in heart and Pox 5 in liver and kidney. Pox 1 was expressed in all tissue without an exception whereas isoform Pox 2 was absent in kidney and muscle as recorded in table 4. Similarly, Pox 3 and Pox 6 was not observed in case of gill and spleen respectively. Exposure of channa punctatus for 90 days to fungicide hexaconazole caused highest overall activity of peroxidase in gill and heart as compared to any other tissue. Kidney, muscle and spleen did not show much variation in case of control (Fig. 7).

"Figure 7 and Figure 8 about here"

"Table 4 about here"

Discussion
Because of their constant and direct contact with the aquatic environment fishes are ideal indicator for behavioral assays of various stressors toxic chemicals exposure (Schlenk & Benson 2001; Srivastava & Singh 2013). They retain or assimilate the pollutants via active or passive processes. Sub-lethal concentration of pesticides cause structural and functional changes in non-target organisms and that is more common than mortality.

The changes in the enzymatic activity of the TCA cycles (MDH and LDH) evidenced that this crucial metabolic pathway appears to be an important supplier of the precursor that plays an important role in the adaptation to fungicide stress. MDH using oxalacetate as a substrate and MDH using NADH as a substrate differed in normoxia and hypoxia may reflect its dual role in both aerobic and anaerobic energy metabolism at low temperature, as pointed out by Hochachka & Somero (1984). MDH is involved in reversible conversion of L-malate and oxaloacetate. In the present study, its activity in the highly toxicated sample (after 90 days of sub-lethal contaf exposure) was found to be higher than in control. This increased activity could be used to consume the product/substrate (oxaloacetate) for production of more energy (ATP) which may be utilized for other physiological activities (Kumar, Sahu, Pal, Kumar, Kumar, Ranjan and Baruah 2010) during stress. Moreover, considering that this enzyme is involved in the respiratory process, such increase in activity during the exposure to sublethal concentration of fungicide would allow the organism bearing higher energy availability for cellular metabolic processes. Similar results were observed in case of plants also. Enhanced level of MDH has been reported for plant cells under dark chilling (Heerden, Villiers, Staden and Krüger, 2003) and heavy metal stress (Duressa, Soliman, Tylor and Senwo, 2011). It is suggested that up-regulation of individual TCA enzymes, including MDH is involved in stress tolerance.

Application of pesticide causes depletion in oxygen supply in water body which influences many organisms to switch from aerobic metabolism to anaerobic metabolism in order to maintain their metabolism. Lactate and Pyruvate contents constitute the inter convertible products of glycolysis their inter conversion is mediated by the enzyme LDH which requires NAD+/ NADH+ as coenzymes. The NAD+ - dependent LDH catalyze the conversion of lactate. Because LDH is an enzyme used under anaerobic conditions, we expected that the LDH activity in spot would increase after being exposed to hypoxic conditions. Brinson, Huffman, David, Shaver, Matthew, Cooper, Rebecca and Lisa (2011) suggested that hypoxic conditions increase anaerobic respiration in spot, hence the antioxidant enzyme lactate dehydrogenase gets elavated. They further observed Regarding LDH activity, enzyme activity appeared to increase initially in muscle tissue, but it did not remain elevated over time. In gill tissue, LDH activity decreased over time, possibly due to a shut-off of metabolic activity after low oxygen exposure. ATPase activity also showed an increase in activity in liver tissue, but like with LDH activity in muscle, this did not maintain over time. Overall, Brinson et. al., (2011) succeeded in conducting experiment for studying the effects of hypoxia on enzyme activity in spot. They strongly supported Hypoxia-tolerant organisms typically down regulate their major ATP consuming pathways when presence of oxygen is low. Na^+/K^+ ATPase uses a large amount of ATP, and it was expected that spot would down regulate this pathway after being exposed to hypoxia. Our result is in consistent with their view that the increase, which occurred with the LDH activity in muscle, may be the result of an increase in enzyme activity after being exposed to hypoxia.

Present observation also agrees with Palanisamy, Mallikaraj, Sasikala and Natarajan, (2011) who exposed Channa striata to cleistanthus collinus suicidal plant extract. They said lactate dehydrogenase is an anaerobic enzyme involved in the conversion of pyruvate to lactate in the Embden Meyerhoff pathway. The increase LDH activity is attributed to the conversion of accumulated pyruvate (Reichl, Khan and Sleet, 1987) into lactate (Anuradha & Raju 1996; Mary Chandravarthy, 1990) which is transported through blood to liver and reconverted glucose and glycogen to meet energy need under physiological stress. Increased malate dehydrogenase and lactate dehydrogenase activities in the crab Oziotelphusa senex exposed to sumithion, an organophosphate insecticide (Reddy, Anandkumar, Reddy and Reddy, 1983). Similar increase in LDH and alkaline phosphatase activities were observed in the English sole Parophrys vetulus treated with carbon tetrachloride (Casillas & Ames, 1986). Also, cadmium was reported to have elicited increased muscular LDH activity in Fiddler crab, Uca pugilator (Devi, Reddy and Fingerman, 1993) and in the brook trout, Salvelinus foninalis (Christensen, Munt and Fiandt, 1977). On the other hand, some agrochemicals and heavy metals inhibit tissue enzymes. Depression of SDH and elevation of LDH strongly indicate favoring of anaerobic metabolism in plant extract stressed fish to meet the energetic demands. Recent researchers performed an extended research for determination of the behavior of antioxidant isozymes in non-target organisms under the influence of pesticides. Tripathy et. al. (2013) also found that when exposed to progressive hypoxia up to experimental hypoxia level without access to air, the serum lactate levels of C. batrachus were significantly increased as compared to normoxic conditions. Thus it can be assumed that the oxygen uptake from the air was not sufficient to sustain complete aerobic metabolism at this aquatic oxygen tensions and that this fish was metabolically in a hypoxic condition and anaerobic glycolysis was activated. Simultaneously, at this stage, serum glucose as well as LDH activity in oxidative tissues, liver and gills, at 12 hr at experimental hypoxia level, were found to be significantly increased.

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In animals, peroxidase enzymes are involved in phagocytosis and immune cell function, cell adhesion, antioxidant function and the oxidative polymerization of hydroquinones to melanin. It can be considered as good indicator of tissue specificity where it expressed as six isoforms (Pox 1-6), with higher activity in gill and heart. According to Dyrynda, Pipe, Burt, and Ratcliffe, (1998), in marine invertebrates, changes in peroxidase levels can signify immunomodulation due to contaminants and other environmental stressors. The present finding agrees with Mydlarz and Harvel (2007) who also suggested that the enzyme processes an importance in the defenses of many different organisms and the level gets elevated upon exposure to toxic environment. Early roles of peroxidase in induction of resistance may include the oxidation of substrates to produce cytotoxic molecules (Nappi & Christensen, 2005), production of reactive oxygen as cytotoxic molecules (Bolwell, Davies, Gerrish, Auh, & Murphy, 1998) and regulation of the oxidative state of the tissue (Gamble et. al., 1998). Nagaraju, Rathnamma, Venkata, Karra and Somaiah, (2013) also found that GPx levels were increased in various tissues of freshwater fish Labeo rohita (Hamilton) on exposure to Chlorantraniliprole. They said that Glutathione peroxidase (GPx) plays an important role in the prevention of cell damage induced by oxidants. GPx being an antioxidant enzyme removes precursors of free oxygen radicals and is necessary for the conversion of hydrogen peroxide to molecular oxygen and water for detoxification. GPx reduces reactive oxygen species (ROS) and intervene in hydrogen peroxide detoxification, leading to GPx formation of their corresponding alcohols or water. Moreover, Oost, Beyer and Vermeulen, (2003) suggested that GPx is considered to play an important role in protecting membranes from damage due to lipid peroxidation.

The hidden strategies of the organism to respond against pesticide induced hypoxia lie in that its high affinity for lactate dehydrogenase, malate dehydrogenase and peroxidase (stress enzymes) in acute toxic environment.

Conclusion

In conclusion, on the basis of the results presented here, it was found that the Channa puntatus a freshwater, carnivorous fish upon exposure to sub-lethal concentration of hexaconazole fungicide (contaf) which induced hypoxia in aquatic environment influenced the fish for adjustment of oxygen carrying capacity, metabolic depression and antioxidant defense system. These physiological alterations might be correlated with its capacity to tolerate hypoxic conditions. In addition, pesticidal role in evoking immune response geneating reactive oxygen which is a potent toxin and capable of oxidizing most cellular components (for example, nucleic acids, proteins, membranes, and lipids), resulting in significant damage, disruption of enzyme activity, and reduction of cellular integrity (Li, Zlabek, Vladmir, Grabic, Roman, Li, Ping, Machova, Jana, Velisek, Josef, Randak and Tomas, 2010). The brain, with its high density of lipid-rich neural tissue, is particularly susceptible to lipid peroxidative damage compared to other organs (Li et. al., 2010), indicating oxidative stress could lead to behavioral changes. Thus, oxidative stress, as evaluated by reactive oxygen generation, lipid damage, and (or) behavioral change, could be an important sublethal endpoint for fungicide toxicity studies. Present research work indicates that liver and kidney being the detoxifying organs, the stress response is greater in these organs. Also, Gill, heart, liver, kidney, muscle and spleen tissues are better applied in studying the isoenzymatic profiles for fish physiology, toxicology and may also be used as clue for taxonomical studies. Although these broad outlines of adaptation for hypoxic survival are recognized through this study, understanding of signals involved in these interrelated processes needs to be further explored.

Fig. 1: Early lesions on rice plant

Fig. 2: Irregular lesions on leaf sheaths of

caused by R. solani
rice plants

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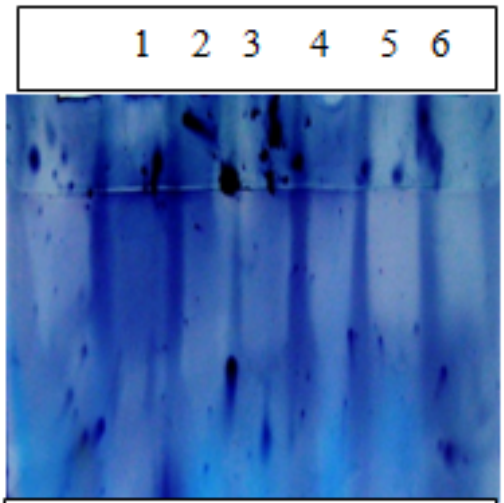

Fig.3: MDH CONTROL:
Lane 1-6: Gill, heart, liver, Kidney, muscle, spleen

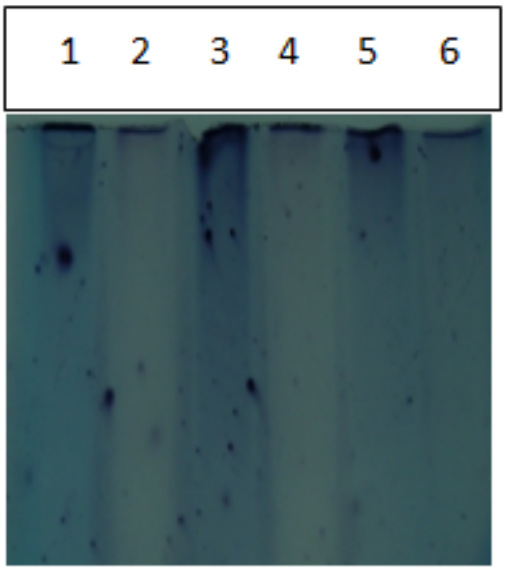

Fig. 5: LDH CONTOL

Lane 1-6: Gill, heart, liver, Kidney, muscle, spleen

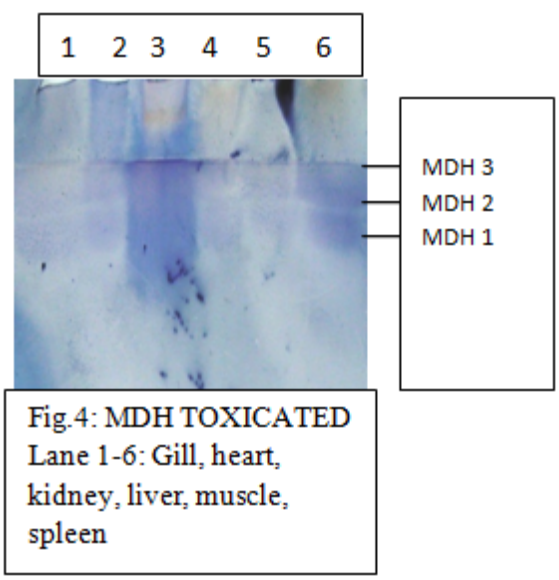

Fig.4: MDH TOXICATED
Lane 1-6: Gill, heart, kidney, liver, muscle, spleen

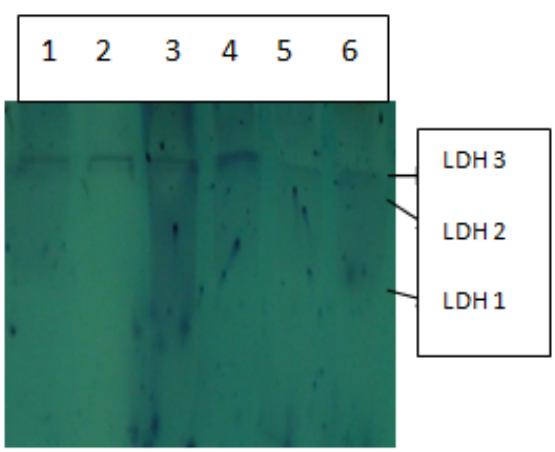

Fig. 6: LDH-TOXICATED

Lane 1-6: Gill, heart, liver, Kidney, spleen, muscle

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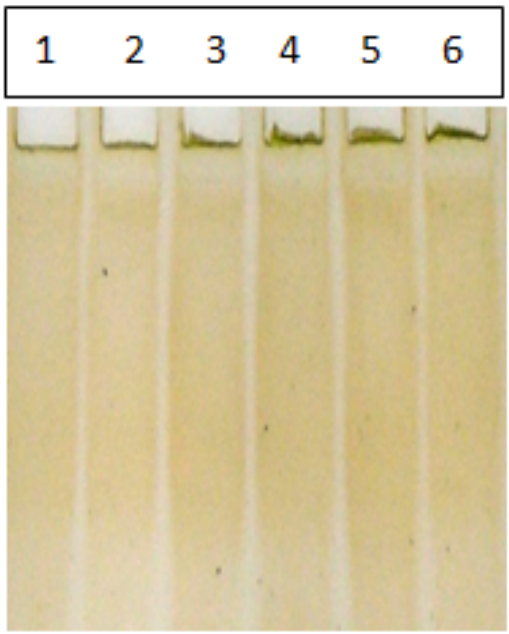

Fig. 7: Peroxidase CONTROL
Lane 1-6: Gill, heart, liver, Kidney, muscle, spleen

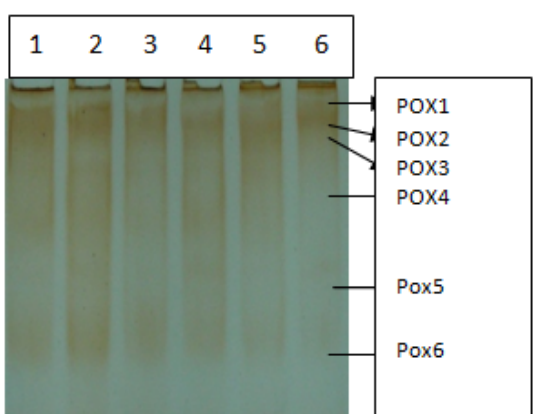

Fig. 8: Peroxidase TOXICATED
Lane 1-6: Gill, heart, liver, Kidney, muscle, spleen

TABLES

Table 1: Table 1. Hydrographical condition of control tank and testing tank (90 days)

Sl. No.	Parameters	Concentrations Control tank Test tank
1.	Temperature °C	27±2°C 29±2°C
2.	pH	7.4 8.1
3.	Dissolved oxygen (mg/l)	8.0 3.8
4.	Hardness (mg/l)	116 864
5.	Total alkinity (mg/l)	27 118

Table 2: Description of isoforms of MDH in different tissues of Channa punctatus

LOCUS	RF	Gill	Heart	Liver	Kidney	Muscle	Spleen
MDH 1	0.066	+	+	+	+	+	+
MDH 2	0.133	+	+	+	-	+	+
MDH 3	0.166	+	+	+	-	+	+

Band present: +, Band absent: -

Table 3: Description of isoforms of LDH in different tissues of Channa punctatus

LOCUS	RF	Gill	Heart	Liver	Kidney	Muscle	Spleen
LDH 1	0.083	-	-	-	-	+	-
LDH 2	0.094	+	+	+	+	+	-
LDH 3	0.061	+	+	+	+	+	+

Band present: +, Band absent: -

Table 4: Description of isoforms of Peroxidase in different tissues of Channa punctatus

LOCUS	RF	Gill	Heart	Liver	Kidney	Muscle	Spleen
Pox 1	0.023	+	+	+	+	+	+
Pox 2	0.027	+	+	+	+	+	+
Pox 3	0.031	+	+	+	+	+	+
Pox 4	0.042	+	+	-	+	+	-
Pox 5	0.053	-	-	-	+	-	+
Pox 6	0.072	+	+	+	+	+	-

Band present: +, Band absent: -

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