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Impact of Cyanobacterium, *Lyngbya semiplena* on Antioxidant Status of a Tropical Teleost *Oreochromis mossambicus* (Peters)

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Abstract

Biological antioxidants are compounds that protect biological system against the harmful effect of free radicals. The acetone extracts of the cyanobacterium, *Lyngbya semiplena* isolated from Cochin estuary, was found to act as an effective antioxidant in the oxidation system of emulsified linoleic acid *in vitro*. Antioxidant properties were expressed *in vivo* also. When the cyanobacterium was incorporated in the feed of ethanol-exposed *Oreochromis mossambicus*, it could protect the fish from lipid peroxidation and from subsequent tissue damage. Lipid peroxidation was assessed in terms of malondialdehyde, hydroperoxides and conjugated dienes. Antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathiones -transferase and non-enzymic antioxidant substance, glutathione in various tissues were also determined. Higher levels of lipid peroxidation were observed in the animal tissues on exposure to ethanol. However, there was a decrease in ethanol accentuated lipid peroxidation on co-treatment with cyanobacterial feed. Experimental diets could effectively bring down the requirement of defensive antioxidant enzymes in various tissues indicating that cyanobacteria could act as an antioxidant by scavenging the free radicals produced during ethanol exposure. *Lyngbya semiplena* is a food grade organism, highly nutritious and readily available from natural waters. These properties render it attractive for use in fish feed.

Introduction

Free radicals play a major role in the progression of a wide range of pathological disturbances and it can be scavenged by the addition or supplementation of antioxidants to food or to the biological system (Venkateswarlu et al. 2003). The role of dietary antioxidants and their potential benefits in health and disease have attracted great attention (Kehler & Smith 1994). The use of synthetic antioxidants has decreased due to their suspected activity as promoters of carcinogenesis (Namiki 1990). At present

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most of the researchers through out the world are interested in finding new and safe antioxidants from natural sources to prevent oxidative deterioration of food and to minimise oxidative damage to living cells (Pratt, 1992).

Cyanobacteria are sources of a wide variety of compounds with a potential of antioxidant activity. Like all photosynthesizing plants, cyanobacteria are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents (Dykens et al. 1992). The elements of photosynthetic apparatus are especially vulnerable to photodynamic damage, because polyunsaturated fatty acids are important structural components of the thylakoid membrane (Sukenik et al. 1993). The absence of such damage in cyanobacteria, in spite of the proximity of the photosynthetically produced oxygen and suitable targets within the photosynthetic apparatus, suggests that these cells have protective antioxidant compounds and mechanisms. The cyanobacterial cells possess an antioxidant defence system, which causes removal of peroxides, free radicals such as superoxide anions ($O_2^{\cdot -}$) generated during photosynthesis and other metabolic process (Karni et al. 1984). Normally this system provides the conditions required for nitrogen fixation and other metabolic events by removing peroxides (Karni et al.1984). Therefore, screening and selection of cyanobacteria with high antioxidant property for producing formulated feed offers tremendous scope in aquaculture.

The importance of cyanobacteria in aquaculture is not surprising as they are the natural food source and feed additive in the commercial rearing of many aquatic animals (Aaronson et al. 1980; De La Noue and De Pauw 1988). Cyanobacteria are usually non-pathogenic and have high nutritive value, rich in carbohydrates, proteins lipids, minerals and vitamins (Cannell 1989). They are not only important as food source, but together with bacteria, they regulate the oxygen and CO_2 balance in the aquaculture systems (Pruder 1983). They also play a role in enhancing the quality of the animal species cultured (Borowitzka 1997). Recent research in natural products of cyanobacteria has made significant advances in aquaculture and they have been shown to produce a variety of compounds and some of them have been proved to possess biological activity of potential medicinal value (Kumar et al. 2003).

Considering the untapped potential of cyanobacteria in aquaculture, the aim of the present study was aimed to determine the antioxidant activity of cyanobacteria, *Lyngbya semiplena* against ethanol induced peroxidative damage in a teleost, Tilapia, *Oreochromis mossambicus.*

Materials and methods

Determination of antioxidant activity of Lyngbya semiplena in vitro

The cyanobacterium *Lyngbya semiplena* was isolated from water samples of

Cochin estuary and cultured in the laboratory using Allen and Nelson medium (Allen and Nelson 1910). The cultures were incubated at 25^{0}C with an illumination of 2000 lux for 30 days.

The cells were harvested at their exponential phase and extracted by continuous maceration with acetone (solvent: mycelia = 100:1, v/w) for 30 min in a separatory funnel. The solvent layer was separated by passing it through Whatmann no.1 filter paper and evaporated to dryness in vacuum (Mitsuda et al. 1966).

The antioxidant activity of the crude acetone extracts in inhibiting linoleic acid peroxidation was assayed using the thiocyanate method (Yen & Chang 2003). 0.5mL methanol solution of the extract was mixed with linoleic acid emulsion (2.5 mL, 0.02M, pH 7.0) and phosphate buffer (2mL, 0.2M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.28 g of Tween-20 as emulsifier and 50ml phosphate buffer, and then the mixture was homogenised. Control containing all the above ingredients except cyanobacterial extract was also prepared. The reaction mixture was incubated at 37^{0}C to accelerate oxidation. The levels of oxidation were determined by measuring the absorbance at 500 nm after reaction with ferrous chloride and ammonium thiocyanate. The antioxidant activity was expressed as percentage of inhibition of peroxidation (IP%):

IP% = 1- (absorbance of sample at 500 nm)/(absorbance of control at 500 nm) x 100.

All tests were performed in triplicate and results averaged.

Maintenance of test organisms

Oreochromis mossambicus with an average weight of 15±3 g and an average length of 11±3 cm were collected from nearby ponds in and around Cochin, Kerala, India and from the culture ponds of Rice Research Institute, Vyttila, India. Collected fishes were immediately transported to the laboratory, using plastic carriers with the same pond water and were acclimated in dechlorinated waters in large tanks of 1000 L capacity. The water quality parameters were checked and maintained at the optimum level. Dissolved oxygen content was kept at 7.6 mg.L^{-1}; pH 7.5; temperature 26 ^{0}C and salinity at 0 ppt. The fishes were fed *ad libitum* with commercial feed from Higashimaru Pvt. Ltd. and were maintained in tanks for more than a week prior to the experiment. For experimentation the laboratory acclimated fishes were sorted into batches of six each and kept in fiber tanks of 30 L capacity. Water exchange was done daily and the fishes were maintained with adequate aeration.

Preparation of fish feed by incorporating live cyanobacteria

Experimental feed was prepared from the commercially available feed from Higashimaru Pvt. Ltd. The feed pellets were well powdered, mixed with adequate amount of water, autoclaved and then mixed thoroughly with 15% of live *Gloeocapsa*, having

high antioxidant property. Newly formulated diet was prepared in pellet form with the help of a laboratory pellet press and was allowed to air dry. Egg white, a natural binder was coated over the pellets to bind all the components of the feed together strongly.

Experimental feed named, F34, was prepared by mixing the cyanobacterial strain, *Lyngbya semiplena* (C34). Control feed was also made in the same way without incorporating cyanobacteria.

Effect of cyanobacteria in lipid peroxidation in vivo

Comparison of antioxidant status of the alcohol exposed fish fed with experimental diet and those given control diet was done by determining the level of antioxidants and antioxidant enzymes in the tissues. In order to assess long-term sub lethal toxicity of ethanol to the fish $1/10^{th}$ of the LC_{50} value was selected for treatment. A set of fishes supplied with experimental diets, but not exposed to ethanol was also tested for their antioxidant status.

Experimental design

The test organism, *O. mossambicus* were divided into four separate groups. Each group consisted of six fishes and the whole experiment was designed as follows:

Group I: Control feed (fishes fed with control diet)

Group II: Control feed + Ethanol (Ethanol treated fishes fed with control diet)

Group III: F34 (Fishes fed with *Lyngbya semiplena* incorporated diet)

Group IV: F34 + Ethanol (Ethanol treated fishes fed with *Lyngbya semiplena* incorporated diet)

The experimental animals were dosed for 21 days. Water exchange and ethanol dosage were done daily, so as to avoid any possible degradation or evaporation. They were fed on the same diet twice daily.

Preparation of tissue homogenate for biochemical analysis

The fishes were killed by pithing after the experimental period (21 days) and the tissues *viz.*, liver, gill, heart, muscle and kidney were removed from its body, wiped thoroughly using blotting paper to remove blood and other body fluids. They were washed, weighed and homogenised in ice-cold 0.1M Tris-HCl buffer of pH 7.4, using a glass tissue homogeniser. The homogenate was centrifuged at 5000 rpm for 10 minutes and supernatant was used for assessing lipid peroxidation.

Assessment of lipid peroxidation and antioxidant status of the fish

Lipid peroxidation was assessed in terms of malondialdehyde (Nihaeus & Samuelson 1968), hydroperoxides (Organisciak 1983) and conjugated dienes (Lee et al.

1982). Antioxidant enzymes such as superoxide dismutase (Kakkar et al. 1984), catalase (Machly & Chance 1955), glutathione peroxidase (Gromadzinska 1988), glutathione reductase (Bergemayer 1974) and glutathione-s-transferase (Gromadzinska 1988) and the non-enzymic antioxidant substance, glutathione (Ellman 1959) in various tissues such as heart, liver, gill, kidney and muscle were determined. The concentrations of enzymes and glutathione were estimated and expressed per milligram of protein in the corresponding tissues and therefore soluble protein content of the tissue extract was also measured by Lowry's method (Lowry et al. 1951) using bovine serum albumin as standard.

Statistical analysis

Variance analysis was done on all experimental data and statistical significance ($P<0.05$) of means of six replicates was judged by Duncan's New Multiple Range Test using SPSS (Statistical Package for Social Science) software (10.0).

Results

Lyngbya semiplena (C34), exhibited 58% inhibition of linoleic acid peroxidation (IP%), thereby suggesting its potential use as a value-added ingredient for stabilising food matrices against peroxidation reactions *in vivo*.

Superoxide dismutase (SOD) activity significantly increased ($P<0.05$) in-group II (ethanol treated) when compared to all other groups (Fig 1).

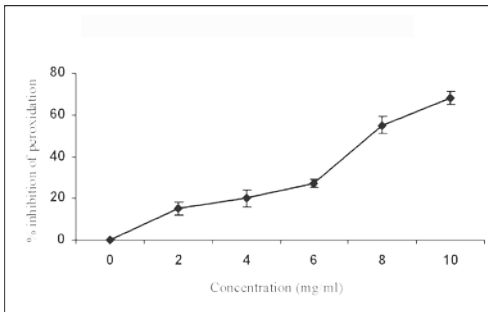

Figure 1. Antioxidant activity of *Lyngbya semiplena*

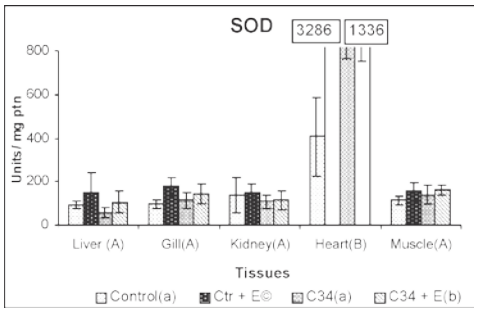

Figure 2. Activity of SOD in various tissues of the treated groups

But it was found that F34 could reduce the enhanced SOD activity due to ethanol exposure, more effectively. Similarly, ethanol exposure increased the levels of catalase, glutathione peroxidase (GPX), glutathione-s-transferase (GST), glutathione reductase (Gred), glutathione, malondialdehyde, hydroperoxides and conjugated dienes (CD). But they were found to be reduced to the normal level on treatment with experimental feed (Fig 2-10).

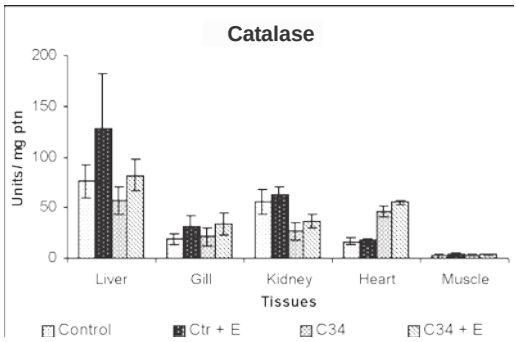

Figure 3. Activity of catalase in different tissues of the treated group

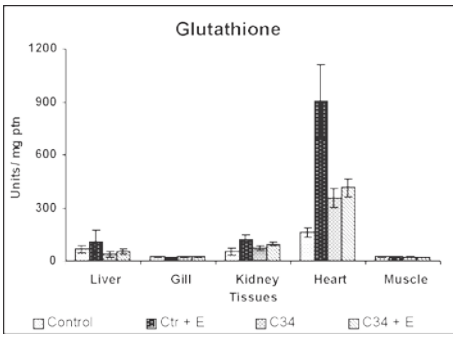

Figure 4. Level of glutathione in various tissues of the treated group

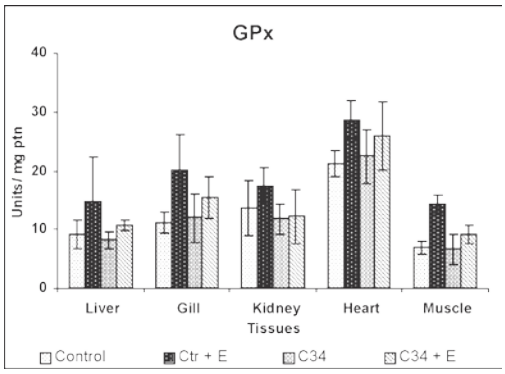

Figure 5. Concentration of Glutathione peroxidase in various tissues of the treated groups

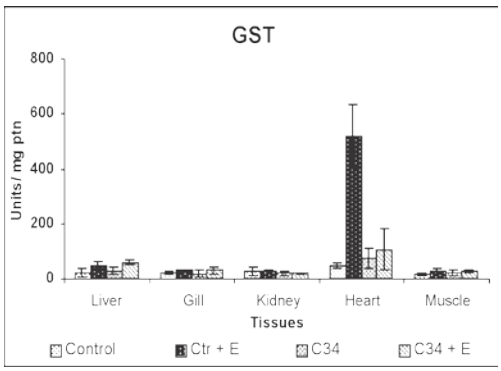

Figure 6. Concentration of Glutathione S-transferase in various tissues of the treated groups

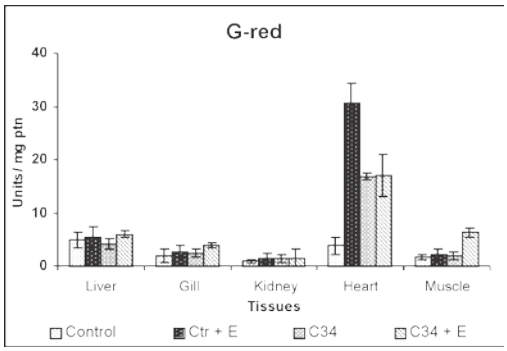

Figure 7. Concentration of Glutathione - reductase in various tissues of the treated groups

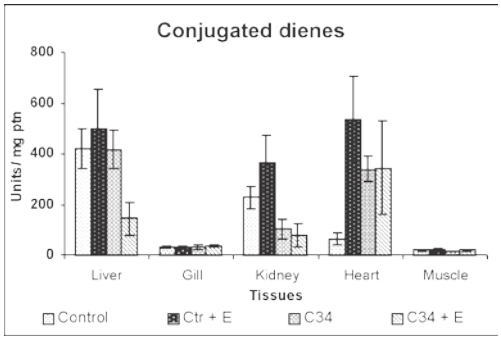

Figure 8. Concentration of conjugated dienes in various tissues of the treated groups

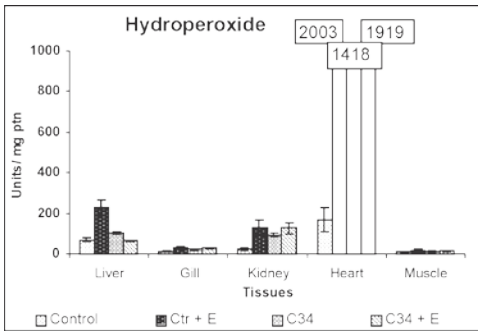

Figure 9. Level of Hydroperoxides in various tissues of the treated groups

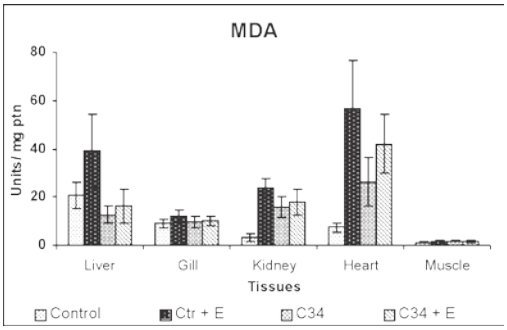

Figure 10. Level of MDA in various tissues of the treated groups

With regard to the antioxidant status of various tissues of the animal, results indicated that maximum level of SOD, glutathione, GST, Gred and hydroperoxides were observed in the heart tissue, whereas, GPX was high in both heart and gill. Catalase was maximum in liver followed by kidney. The concentration of CD was very high in both heart and liver followed by kidney. The concentrations of MDA were high in heart followed by liver and kidney. Muscle showed least activity for all the compounds studied.

Discussion

Cyanobacteria are sources of a wide variety of compounds with a potential of antioxidant activity. There are reports that β-carotene from algae could prevent cancer because of their antioxidant property (Schwartz & Shklar 1987; Fedkovic et al. 1993). It was shown that the algal extract was more effective on hamster cancer regression than by β-carotene alone suggesting a possible synergistic effect of the extract, as components, other than β-carotene, also have a decisive action in the oxidation inhibition (Schwartz & Shklar 1987). Some compounds such as vitamin C, phenols, amines and phospholipids from algae possess antioxidant activity (Tutour 1990). The levels of antioxidant compounds such as phenolic acids, tocopherols and carotenoids were determined from *Spirulina* (Miranda et al. 1998).

In the present study, a potent strain of cyanobacterium, *Lyngbya semiplena* that showed 58% of inhibition of lipid peroxidation was evaluated to test its efficacy in controlling tissue lipid peroxidation and the antioxidant status in experimental toxicity *in vivo*.

The effect of free oxygen radicals accumulation in cells under stress is lipid peroxidation via oxidation of unsaturated fattyacids leading to membrane damage and electrolyte leakage (Liu et al. 1987; Marschner 1995). Malondialdehyde, hydroperoxides and conjugated dienes are the major products of lipid peroxidation and therefore the level of these compounds in tissues can be taken as the index of lipid peroxidation. The

only mechanism which produces malondialdehyde in biological systems is lipid peroxidation.

SOD and catalase are the major antioxidant enzymes associated with scavenging the reactive oxygen species (ROS) (Marschner 1995). However, SOD detoxifies superoxide anion free radicals accompanying the formation of hydrogen peroxide (H_2O_2), which is very damaging to the nucleic acids and proteins (Fridovich 1986; Rabinowitch and Fridovich 1983) and can be eliminated by catalase and peroxidase (Marschner 1995; Scandalios 1990; Elstner and Osswald 1994). Glutathione reductase also plays a key role in oxidative stress by converting the oxidized glutathione (GSSG), to glutathione (GSH) and maintaining a high GSH/ GSSG ratio (Alscher 1989; Fadzilla et al. 1997). GSH is a major antioxidant that is known to protect cells from oxidative stress (Smith et al. 1990). Changes in processes that regulate GSH concentration and/or redox status are considered to be one of the important adaptive mechanisms of cells exposed to stressed conditions (Alscher 1989; Smith et al. 1990; Fadzilla et al. 1997).

In acute ethanol intoxication, liver microsomal metabolism of ethanol was accompanied by hydroxyl radical ($OH^{\cdot}$) generation by cytochrome p450 system. Hydroxyl radicals are responsible for the conversion of ethanol to acetaldehyde. The alcoholic liver injury appears to be generated by the effects of ethanol metabolism and the toxic effects of acetaldehyde, which may be mediated by acetaldehyde altered proteins (Ishak et al. 1991). There is no tissue storage of ethanol, and it reaches all organs of the body. In chronic lipid accumulation the liver cells become fibrotic and leads to impaired liver function. Ethanol increases triglycerides and cholesterol levels thus inducing imbalance in lipid metabolism in liver, heart, kidney and other organs and this could explain the reason for the increase in lipid peroxidation in these organs. Recently free radical induced lipid peroxidation has gained much importance because of its involvement in several pathologies (Salin and McCord 1975; Rowley and Halliwell 1983). Protection of cell membrane from lipid peroxidation has become a necessity to prevent, cure or delay of the aforesaid diseases.

In the present study, all antioxidant enzymes were stimulated on exposure to sublethal concentration of ethanol to *O.mossambicus*. Khan et al. 1997 and Balasubramanian et al. 2003 reported a similar observation of significant increase in lipid peroxidation in the tissues of mice that received ethanol. This may be a general adaptive defence response of the animal to toxic alcoholic environments (Karakoc et al. 1997; Sachin et al. 1997). The alcohol induced lipid peroxidation increased with experimental time. However, cyanobacterial diet (F34) significantly reduced the activities of antioxidant enzymes and the concentration of GSH in various tissues of Tilapia coincided with a decrease in concentration of MDA and a decrease in the formation of hydroperoxide and conjugated diene as well, suggesting that oxidative damage induced by alcohol be alleviated by the supplementation of cyanobacterial feed. The antioxidant

components of the feed restored the lipid peroxidation level to nearly those observed in control organisms.

The ability of cyanobacteria to protect the animal from ethanol-induced damage might be attributed to its direct antiperoxidative effect or may be due to its ability to restore the activity of antioxidants, superoxide dismutase and glutathione. *In vivo* experiment has proved that *Lyngbya semiplena* (C34) could act as a very good antioxidant in ethanol-induced Tilapia. The antioxidant effect and resultant protective ability of cyanobacteria may be attributed to the presence of natural compounds such as flavanoids, phenolic acids, vitamin A, vitamin E, vitamin C, phycocyanin, β-carotene and other carotenoid molecules (Miki 1991; Miranda et al. 1998; Bhat and Madyasta 2000) as they can reduce the damage caused by the free radicals. The specific component of the feed is able to reduce the levels of lipid peroxidation and restore the antioxidant status by enhancing acetaldehyde elimination and thus prevent the binding of acetaldehyde to cellular proteins and thereby exerts a protective effect in the animal.

It appears from our studies that the cyanobacteria exhibit its antioxidant role either directly by scavenging the oxidative species or indirectly by modulating the antioxidant levels. In addition, the chemical composition of the *Lyngbya semiplena* indicated that they have high nutritional value due to the presence of high contents of carbohydrates, proteins, lipids and pigments. The species is readily available from natural waters. Therefore, it will be profitable if this species could be cultured commercially for use as natural food source or feed additives in aquaculture and also as source of valuable chemicals such as antioxidant compounds.

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