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Study of Glucose-6 Phosphatase activity in *Clarias batrachus* (Linn.) after feeding the probiotic fish feed

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Abstract

Glucose-6 Phosphatase activity was studied in the air breathing fresh water catfish (*Clarias batrachus)* by giving the probiotic feed. The experiment was conducted in laboratory condition with glass jar aquarium. Bacteriological and different biochemical tests like cytochrome oxidase, fermentative and catalase test were analysed along with certain physiological parameters of water samples. *Clarias batrachus* was fed by prepared four diets, F1, F2, F3 and F4. The diets were prepared using fishmeal, soyabean meal, groundnut oil cake, rice bran, vitamin mineral mixture and different concentration of probiotics. The *Clarias batrachus* in experimental treatments were fed by the prepared diets upto six weeks. The significant amount of Lactic acid bacteria (LAB) was found in the gut microflora of the experimental fish. Glucose 6- phophatase activity was decreased in liver with increase in probiotic concentration in feed. But the enzyme activity in kidney, gill and stomach did not follow any trend. The water quality parameters like pH, CO_2, DO, alkalinity, ammonia concentrations of the experimental tanks were varied significantly with treatment time.

Keywords: *Clarias batrachus,* glucose-6 phosphatase, probiotic, lactic acid bacteria (LAB)

Introduction

The worldwide demand for fish protein is expected to increase, because the harvest of wild fish populations has already reached (and in some cases exceeded) the carrying capacity. The need for aquaculture production in 2025 has been estimated to become 63 million tones, whereas the production in 1997 was 27 million tons (FAO, 1998). Unless the fish supply is increased through successful aquaculture programs, fish protein will become a scarce and costly commodity. High fish prices will result in increased incentives for over fishing of wild stocks and reduced food security for a large number of poor consumers that depend on fish as a source of animal protein (Olsen et al., 1992).

Retention of dietary protein and energy in fish farming is approximately twice than in chicken and swine production, with correspondingly lower waste production (Asgard and Austreng, 1995). Air breathing catfish, *Clarias batrachus* was widely known as Magur has immense prospects for developing trade in India. Due to prolonged freshness out of water, high nutritive and therapeutic values particularly to their high protein and low fat physiologically available iron, delicious taste of the tender meat having less intramuscular

spines, the fish commands high price in the market than carp and other freshwater fishes and to certain extent in the neighboring countries of the Indian subcontinent for its high sale price (Mishra, 1994).

Feed is the important source of nutrient loading in aquaculture production, clear understanding of its impact is essential for sustainable development, either intensive or semi intensive. Use of feed additives to enhance fish and shellfish production has acquired considerable importance in the recent years. It has been observed that supplementation of fed additives enhance the efficiency of feed utilization (Viola and Arieli, 1987). In recent a variety of substances, which have growth promoting effect, are being added in aqua-feeds. The drugs and hormones are used in the feed to improve the growth of an animal can be generally called as growth promoters or feed additives. The important feed additives are hormones, antibiotics, microbial cell wall preparations, enzyme extracts and many other non-hormonal substances (Murthy and Naik, 2002).

Probiotic is a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, et al., 1989). In aquaculture, addition of live bacteria to tanks, ponds or through feed was practiced in some instances. The health of the animal is improved by the elimination of pathogens or at least minimizing the effects of pathogens and improving the water quality. Hence probiotics are used in aquaculture not only as a feed additive but also as a water additive (Abraham et al., 2000). They are the effective means of enhancing the enzyme activity, growth rate, feed conversion, assimilation, enhance the immunity under stressful environmental conditions, and hence improving the nutritional value of aquatic animals (Mohapathra et al., 2012; Panigrahi, 2007; Taoka et al., 2006 Lipton, 1998).

Material and Methods

Collection of raw materials and preparation of test diets

Four practical diets were formulated to contain about 35% crude protein. The raw materials (rice bran, soyabean cake, groundnut oil cake, fish meal) used for preparation of diets were collected from the local market of Bhubaneswar, Odisha. All the ingredients were dried at 60^0C for 48 h in a hot air oven. The dried ingredients were ground with a mechanical grinder and stained through a 200 mesh size sieve. The proportions of dry ingredient required for 500 g diet were then weighed and mixed thoroughly. The vitamin mineral premix was added to all types of diets. Probiotic was added @ 0.0, 1.0, 2.0 and 3% in the diets and blended thoroughly. The feeds were designated as F1, F2, F3 and F4 respectively. The lukewarm water was then added to the dry ingredients to mixed and kneaded to produce dough. 2 mm diameter die pellet was prepared for feeding. The extruded pelleted diets were dried over night at 60^0C, packed in plastic airtight jars and stored in a freeze at 4^0C until used.

Test animals and feeding rate

Clarias batrachus of average body weight of (7.19±0.23g) were obtained from the local fish market of Bhubaneswar and acclimated in laboratory condition for 2 weeks in glass aquaria with properly stored tap water. Rice bran and groundnut oil cake mixture in 1:1 proportion was offered as sole feed for 15 days (twice a day) and the feed was accepted well. Prior to start of experiment the fish were divided and stocked in 8 glass aquarium (20 lit water), each with 4 *Clarias batrachus* of mixed sexes and 2 tanks used as replicates per each treatment. The treatment were designated as T1, T2, T3 and T4 and fed on F1, F2, F3 and F4 diets respectively. Small pieces of perforated PVC pipes were placed in each tank to provide shelter for the fish. Diets were provided @ 3% of body weight following a rigid schedule (at 10

A. M). This ensured minimum wastage of diet. The waste feed was siphoned out every day with minimal disturbance to the fish. Tank water was replenished by carefully siphoning about two third volume of water. Always stored water was used to prevent any deleterious effect due to chlorine used as disinfectant in water supply. Besides water change, daily observation was done on general health of the fish including injury, symptoms of distress, feeding behavior etc. The feeding trial was continued for 6 weeks.

Proximate composition of feed

Proximate composition of feed such as moisture content, total ash content, crude protein by the Kjeldahl digestion method and lipid content performed following the method by Bligh and Dyer, 1959.

Water quality parameters

Certain physiological parameters of tank water sample viz., pH, dissolved oxygen, free carbon dioxide, total alkalinity, ammonia nitrogen were recorded once in 7 days while temperature was recorded every day. The study was carried out by following the APHA (1989).

Bacteriological study

Clarias batrachus post larva was collected from the cistern and cleaned with alcohol to remove any external bacterial contamination. The gut was removed from the post-larva and then introduced into Brain Heart Infusion (BHI) broth. This broth was kept for 24 hours incubation at 37^0C. The BHI agar plates were prepared and inculcated by streaking from the broth. Bacterial growth was found grown on BHI agar plates after 24 hours of incubation at 37^0C. Different biochemical tests like agar plates after hrs of incubation at 37^0C. Different biochemical tests like cytochrome oxidase, oxidative, fermentative test and catalase test were conducted on the bacterial isolated gut of *Clarias* as stated by Sharpe (1962).

Probiotic bacterial strain

The bacterial strains used were *L. sporogenes* procured from the medical stores. The bacterial strains were obtained in the form of tablets. Each tablet *L. sporogenes* contain 6 x 10^7 million spores. These strains were checked for its viability by inoculating it in test tubes containing sterilized skim milk. The inoculated skim milk was incubated at 34^0C for 24 hrs and checked for growth of *L. sporogenes* in tones of appearance of curdling.

Biochemical test

Cytochrome Oxidase Test

Solution a-1% aqueous P-amino dimethyl aniline

Solution B-1% x-Naphtal solution in ethanol

A few drops of both solutions are introduced into the agar plates containing the bacterial growth. Colour reaction should takes place within 10 second.

Oxidase positive colonies develop a pink colour which is successively dark red purple and black within 10 seconds.

Catalase test

A test used to determine ether or not a given bacterial strain produces catalase. One drop of H_2O_2 is placed in a bacterial colony or added to an emulsion of bacteria. A positive test (presence of catalase) is indicated by the appearance of bubbles of gas (O_2) either immediately or within a few seconds.

Lactose fermentation test: (O-F Test Hugh and Leifson Test)

A test used to indicate whether a given carbohydrate (usually glucose) is utilized oxidatively or fermentatively by a given strain of bacterium. The peptone agar medium includes sodium chloride, K_2HPO_4, and the given carbohydrates (% W/V) and incorporates of pH indicator (usually bromothymol blue). The medium is green if bromothymol blue is used and has the pH-7. During the test each of the two tubes of the above medium is stab inculcated with the test organisms in one of the tubes the medium is

immediately 1 cm. Both tubes are then incubated at a temperature appropriate to the test organism and subsequently examined for evidence of carbohydrate utilization (acid production) bromothymol blue becomes yellow in each tube. Yellowing of indicator in both tubes indicates that the test organisms can attack the carbohydrate fermentatively. An acid reaction only in the medium not covered by paraffin indicates that the carbohydrate is attacked oxidatively. No reaction in either tube indicates that particular carbohydrates aren't attacked by the test organisms.

Enzymes activity

Sample preparation

Fishes were starved for 24 hours before dissecting on ice plates. Gut and digestive glands were removed washed externally with chilled 0.85 % sterilized normal saline solution. Gut was longitudinally cut opened and washed with 0.89 % sterilized normal saline solutions 2-3 times. Gut tissue and digestive glands were homogenized with 10 ml of 0.85% normal saline solution. Homogenate prepared was centrifuged at 5000 rpm for 15 minutes in refrigerated centrifuge. The supernatant to be used as enzyme source was collected in separate eppendorf tubes for further analysis.

Assay of glucose-6-phosphatase enzyme

The glucose-6-phosphatase activity was measured following the method of David T. Plummer (1998). The assay mixture consisted of 1:2 ml of carbohydrate buffer (7, 0.2 ml Ethylene diamine tetra acetic acid (EDTA) and 0.4 ml Glucase-6-phaspahte containing 0.2 ml of diluted tissue fraction. A blank without the substrate were also run simultaneously all the tubes were incubated for 20 minutes at 39^0C which gave an optimum activity. After incubation the reaction were stopped by adding 1 ml of ice-cold TCA (10%). After centrifuge the supernatant were used for estimation of phosphate was done and calculation done as macromolecules of Glucose-6-phosphate hydrolysed per minutes.

Water stability of different diets

The water stability of different diets were determined by the wet durability method (Jayaram and Shetty, 1981) with modified periods of durability test. About 1-2 g of feed was taken in 100 ml glass beaker and tested triplicate. At the end of 1 hr water from each beaker was filtered through bolting silk cloth and the residue was taken in a pertidish and kept in a hot air oven at 60^0C for during till the constant weight. The experiment was repeated for different time intervals (1-4 hr) and the corresponding values were recorded. The water stability of the feed was calculated by taking the percentage mean difference of dry matter between pre immersion and post during of different diets separately.

Sinking rate of experimental diets

The sinking rate of experimental diets was found out after their prepared 10 glass beaker (250 ml capacity) with 10cm depth of water was chosen for the study. Equal size experimental pellets of different diets were gently released at the surface of water in aquarium and time taken by the pellet to reach the bottom was recorded by using a stop watch. The average time taken by each type of diet to reach the bottom of the aquarium was recorded separately and the mean value of sinking rate was calculated and expressed as a sec.

Results

Physio-chemical parameters of tank water

The mean values of the physicochemical parameters such as temperature, dissolved oxygen, free carbon dioxide, total alkalinity, ammonia concentration recorded during the experimental period were presented in table 1. The temperature of the different groups ranged from 31-37^0C.There was not much variation in pH value during the experimental period. The dissolve oxygen values did not show any marked variation

Table 1. Physico-chemical properties of the experimental tanks.

Diets				
Parameters	**F1**	**F2**	**F3**	**F4**
Temperature (^{0}C)	35-37	32-33	33-35	31-33
pH	7.6-7.7	7.6-8.8	7.6-7.8	7.6-8.8
Dissolved oxygen (mg/l)	3.0	3.2	4.9	6.1
Free CO_2 (mg/l)	36.85	26.4	17.6	16.13
Alkalinity (mg/l)	80-102	80-102	80-102	80-102
Total ammonia(mg/l)	1.001-1.017	0.90-1.01	0.213-0.226	0.104-0.109

Table 2. Biochemical tests for the Lactic acid bacteria (LAB) in the gut of *Clarias batrachus.*

Tests	Before feeding	After feeding
Gram test	-	-ve
Cytochrome oxydase	+ve	-ve
Catalase	+ve	-ve
Lactose fermentation	-ve	+ve

Table 3. Viability test of probiotics at different time intervals in experimental diets.

Experimental days						
Diet code	**7**	**14**	**21**	**28**	**35**	**42**
F1	-	-	-	-	-	-
F2	+	+	+	+	+	+
F3	+	+	+	+	+	+
F4	+	+	+	+	+	+
'+' Curdling of sterilized skim milk; '-' No curdling						

Table 4. Proximate composition of experimental diets.

Diets				
Proximate composition	**F1**	**F2**	**F3**	**F4**
Dry matter (%)	96.76	96.71	96.66	96.69
Organic matter (%)	85.19	85.54	85.25	85.19
Ash (%)	11.57	11.17	11.41	11.50
Protein (%)	35.01	34.99	35.04	34.89
Lipid (%)	6.89	6.81	6.86	6.88
Total carbohydrate (%)	43.24	43.74	43.35	43.42

Table 5. Water stability of experimental diets.

Period of immersion (h)				
Diet code	**1**	**2**	**3**	**4**
F1	90.68±0.32	0.89±0.40	85.5±0.45	79.89±0.50
F2	91.19±0.55	89.1±0.57	84.69±0.49	79.99±0.56
F3	92.0±0.75	89.21±0.51	83.89±0.55	78.1±0.54
F4	91.21±0.62	88.87±0.60	86.1±0.58	80.12±0.55

Table 6. Average sinking rate of different experimental diets.

Diet code	Average sinking rate cm/sec
F1	4.9 ± 0.21
F2	5.1 ± 0.33
F3	5.11 ± 0.34
F4	5.2 ± 0.19

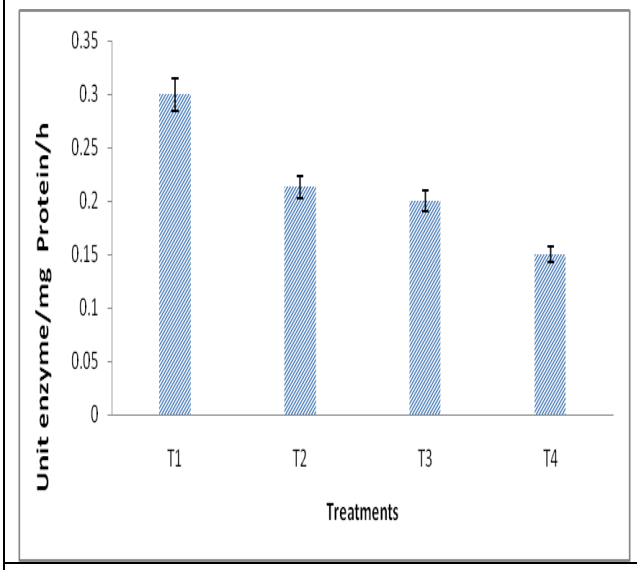

Fig.1. Glucose 6-phosphatase activity in liver of *Clarias batrachus* fed probiotic enriched different experimental diets.

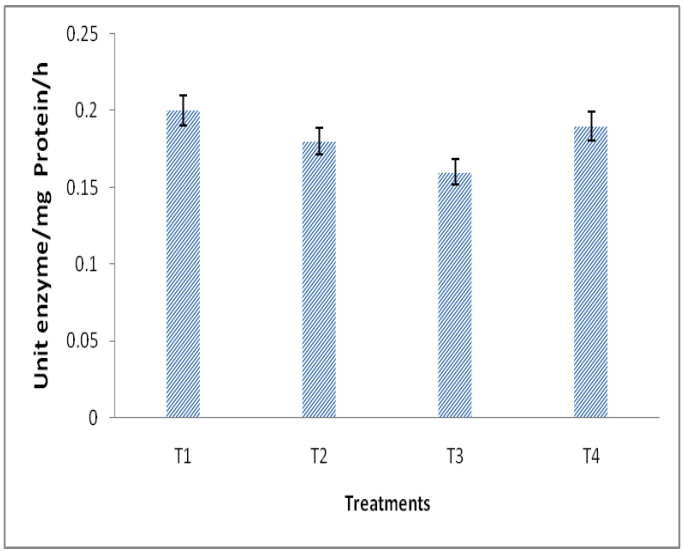

Fig.2. Glucose 6-phosphatase activity in kidney of *Clarias batrachus* fed probiotic enriched different experimental diets.

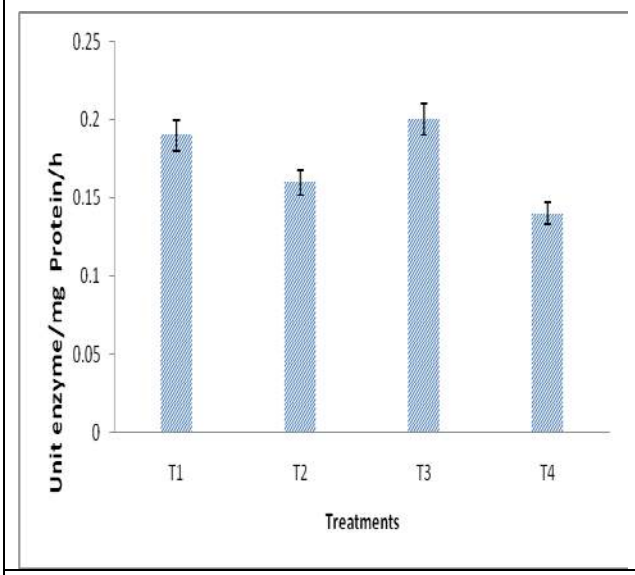

Fig.3. Glucose 6-phosphatase activity in stomach of *Clarias batrachus* fed probiotic enriched different experimental diets.

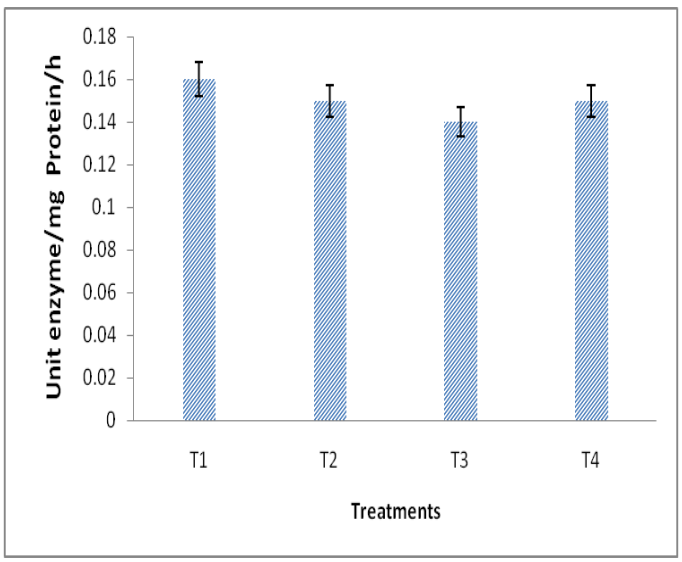

Fig.4. Glucose 6-phosphatase activity in gill of *Clarias batrachus* fed probiotic enriched different experimental diets.

the range for all the tanks. Little higher values recorded in the tanks treated with diets F3 and F4 diets while comparatively lower values were observed in tanks treated with diets F1 and F2. In case of free carbon dioxide highest concentration was observed in T1 treatment fed on F1 diet and lowest in T4 treatment fed on F4 diets. There was not much more wide variation of alkalinity. The load of ammonium-nitrogen in the tanks during successive sampling varied widely. Higher values were recorded in every sampling in the tank of T1 treatment and lowest in T3 and T4.

Isolation and characterization of gut microflora of *Clarias batrachus*

Different bacteria were isolated from the gut microflora of *Clarias batrachus* for finding the presence of lactic acid bacteria (LAB) during before and after feeding. Different biochemical tests were conducted for confirmatory test for as shown in table 2. The bacterial isolated were found to be gram –ve. They were found cytochrome oxidase positive and catalase positive. Test conducted for lactose fermenting showed –ve result.

Viability test of probiotics

The viability test of the probiotics was presented in table 3. The positive results indicated the presence/survival of lactic acid bacteria (LAB) in the feed.

Isolation of Lactic acid bacteria (LAB) from gut microflora of *Clarias batrachus*

After experimental period of 6 weeks, the *Clarias batrachus* from different experimental groups were scarified to check the presence of lactic acid bacteria (LAB). The gut microflora streaked on De Man Rogosa and Sharpe (MRS) agar showed a negative response to cytochrome oxides and catalase (Table 2).

Proximate composition of experimental diets

The proximate composition of the experimental diets was presented in the table 4. The protein content of the diets ranged from 34.89 to 35.04%. The lipid content ranged from 6.81 to 8.89 %. As the diets were variable inclusion level of probiotics, the chemical composition of the different diets did not vary much.

Water stability of experimental diets

Data on water stability of experimental diets are given in table 5. As all the ingredients were in same quantity expect probiotic the water stability of different diets did not vary much. The water stability progressively decreased in all the pellets with time.

Sinking rate of the pelleted diets

Average sinking rate of pellets was given in table 6. The sinking rate of the pellets in different diets was also did not varying much as that of water stability pelleted diet.

Glucose 6-phosphatase activity

Glucose 6-phosphatase enzyme activity was decreased in liver with increase in probiotic concentration in feed. But the enzyme activity in kidney, gill and stomach did not follow any trend (Figure 1-4).

Discussion

The lower values of the free carbon dioxide 16-26 mg/l in all the tanks except the tanks in T1 might be attributed due to the high level of oxygen, in the rearing system. Probiotic applied in the present study revealed their usefulness in maintaining water quality which is similar with the findings of Prabhu et al., (1999). The alkalinity level of 80-102 mg/l could be considered moderate and observed in highly productive waters (Alikunhi, 1957). The above levels of alkalinity may be due to ponds. The ammonium nitrogen level of 0.104-0.23 mg/l was found in T3 and T4 treatments was low and considered suitable for rearing of fingerling and grow out stages of fish. The above optimal levels of NH_4-N may be attributable to the better utilization of diet in T3 and T4 tanks than T1 and T2.

Bacterial isolates were analyzed for the presence of Lactic acid bacteria (LAB) in the gut micro flora of *Clarias batrachus* before and after feeding the probiotics. From the biochemical tests it revealed that all the before feeding bacterial isolates were cytochrome oxidase +ve, catalase +ve and oxidative fermentative –ve ruled out any possibility of presence of LAB in the gut of *Clarias* and isolates of after feeding gut microflora confirm the presence of LAB. LAB are characterized as gram -ve, non-motile, non-spore forming (except *L. sporogenes*) rods that occurs singly or chains, other characteristics of LAB includes, lack of cytochrome oxidase, catalase, Sunders et al., (1980). Askarian et al., 2012 reported that the LABs have the ability to produce digestive enzymes such as amylase, lipase and protease.

Water stability of formulated diets is essential as the diet should be made available to fish without loss of nutrients, especially for slow feeding fishes like carps, which may require an hour or more to ingest the feed (Hastings, 1976). Among the four experimental diets used in the present experiment showed more or less same stability as all the ingredients were same expect the concentration of probiotic. Generally different factors such as feed composition, nature of raw material, processing methods and moisture content affect the water stability of formulated diets (Hastings, 1977). Factors such as feed composition, nature of raw material, processing methods and moisture content affect the water stability of formulated diets (Hastings & Mitchell, 1971). After 4 h the stability ranges from 78-80%. However, very high stability of pellets is not desirable as the nutrients may get tied up so tightly that they become unavailable to fishes (Balazs et al., 1973). All the formulated feeds used in the present study had sufficient water stability as carp feed.

The microsomal enzyme glucose-6-phosphatase catalyses the hydrolyses of glucose-6-phospahte to produce glucose and phosphate. The glucose-6-phoshate is a marker enzyme in the process of gluconcogenesis. When the body synthesis glucose from non-carbohydrate source then pyruvate is formed and from pyruvate through the process of gluconegenesis the enzyme Glucose-6-phospatase releases glucose from Glucose-6-phospahte by expelling inorganic phosphate group. In 2015 Abareethan and Amsath found that feeds the adherence of isolated probiotic feed in the intestine of *Labeo rohita*, alter the enzymes, microbial metabolism and improve the weight gain and survival rate. Probiotics can also produce inhibitory substances against pathogens, competition for essential nutrients and adhesion sites. In addition, they supply essential nutrients and enzymes resulting in enhanced nutrition in the host (Ringø and Gatesoupe, 1998). Glucose level maintenance is the autonomous function of the master organs of body. When there is a scarcity of glucose in blood the alternate way of formation of glucose in body is the process through gluconeogenesis. The enzyme glucose-6-phosphatease is required to synthesize glucose from pyruvate. By increasing concentration of probiotics the amount of Glucose-6-phospate decreased which shows the use of normal glucose in body. The present study revealed that the Glucose 6- phophatase activity was decreased in liver with increase in probiotic concentration in feed, but the enzyme activity in kidney, gill and stomach did not follow any trend. This concluded that in the presence of probiotics the gluconegenesis process was down regulated.

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