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Different carbon sources influences the growth and digestive enzyme activity of grey mullet (*Mugil cephalus*) in biofloc based nursery rearing system

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Abstract

Aim: To assess the impact of different carbon sources on growth performance of grey mullet, *Mugil cephalus* fry in biofloc based culture system during nursery rearing phase.

Methodology: The experimental trial was carried out for 60 days using three carbon sources, cassava, jaggery and barley in triplicate following a Completely Randomized Design. The carbon nitrogen ratio (C:N) in the systems were maintained at 15:1. Grey mullet fry (0.91g) were stocked @ of 1 fry 10l⁻¹ and were fed with pelleted feed (35% crude protein) @ 4% of fish body weight thrice daily.

Results: Significantly higher ($P < 0.05$) growth performance, in terms of Specific Growth Rate (3.78 % day⁻¹) with lower Feed Conversion Ratio (1.35) were found in barley based biofloc group. Similarly, enhanced digestive enzyme activity in terms of amylase (8.78 Unit mg protein⁻¹ min⁻¹), lipase (2.77 Unit mg protein⁻¹ min⁻¹) and protease (4.22 Unit mg protein⁻¹ min⁻¹) were found in barley based biofloc group. Non-specific immune parameters such as lysozyme, respiratory burst activity and myeloperoxidase activity were significantly higher in barley based treatment group followed by jaggery and cassava based treatment groups. Likewise, stress indicators in serum and antioxidant status in liver were found to be lower in barley based treatment compared to cassava and jaggery based treatment groups.

Interpretation: Rearing of *Mugil cephalus* in biofloc culture system is practicable; however, the carbon source required for biofloc production could be barley, compared to cassava and jaggery, in order to achieve maximum growth and to reduce the stress.

Key words: Biofloc, Carbon source, *Mugil* sp., Nursery rearing

Different carbon source based biofloc systems enhances growth and digestive enzyme activity of grey mullet (*Mugil cephalus*) in the nursery phase

Experimental site : ICAR CIFE, Kakinada Centre, Kakinada Port, Andhra Pradesh, India

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Introduction

Exponential growth of population is an inevitable fact in the current scenario. Fish may see a surge in demand in the near future, especially farmed fish. The contribution of capture fisheries and further expansion of the same has only a limited scope. To gratify the rising demand for animal protein, extension and intensification of aquaculture is one of the significant alternative (Ahmad *et al.*, 2017; Ekasari *et al.*, 2010). However, the major limiting factors of intensive aquaculture systems is the availability of input resources (land and water) (Martínez-Córdova *et al.*, 2014). Along with enhanced production, strengthening of aquaculture practices are required to produce numerous effluents which will destruct the aquatic environment (Ahmad *et al.*, 2017). Hence, the sector requires alternative technology and substantiate sustainable production (Martínez-Córdova *et al.*, 2014). Sustainable production systems that utilize minimum input and produce maximum output (biofloc technology) are viable options to address the issues.

Biofloc technology proposes aquaculture a sustainable approach to resolve its environmental, social and economical issues concurrent with its growth (Crab *et al.*, 2012). The major principle of biofloc technology is stimulation of heterotrophic bacteria to remove harmful products, such as ammonia and other nitrogenous based wastes from the culture medium (Deb *et al.*, 2017). In general, biofloc technology can be defined as a technique of water quality management, which is based on the development and controlling heterotrophic bacteria within the culture system with zero or minimal exchange of water (Ekasari *et al.*, 2010). Microscopic organisms like bacteria and other organisms utilize carbs (sugars, starch and cellulose), as a food, to produce energy and growth that is to create proteins and new cells (Avnimelech, 1999). The paramount qualities of this system are greater stocking densities, substantial mixing, strong aeration, low to no exchange of water and high organic matter input (Browdy *et al.*, 2001).

Selection of suitable candidate species with commercial marketability as well as suitability is the primary step for establishing a successful biofloc system. Biofloc systems works best with species that can endure high solid concentration in water as well as derive nutritional benefit by consuming the developed floc (Hargreaves, 2013). Odum (1968) reported that *Mugil cephalus* consume smaller particles of 10 mm size, for instance, small inorganic and plant detrital sediment particles, which are more abundant in absorbed organic material as well as adsorbed bacteria, protozoa and other microorganisms, whereas mullet rejects coarser material. Milk fish (*Chanos chanos*), a primitive fish with a feeding regime, extremely like that of striped mullet (Odum, 1968). A study conducted by Sontakke *et al.* (2019) showed that the milk fish grown in biofloc based culture systems exhibited enhanced immune response. Similarly different mullets also showed growth and survival in biofloc based culture systems

(Rocha *et al.*, 2012; Legarda *et al.*, 2019; Borges *et al.*, 2020).

In view of the above, the present research work was formulated to evaluate the impact of different carbon source based biofloc culture systems on the growth and well being of grey mullet during the nursery rearing phase.

Materials and Methods

Experimental design: The experiment followed Completely Randomized Design (CRD) having three treatments, in triplicates (9 experimental units). Biofloc developed using different carbon sources, namely, cassava, jaggery and barley constituted three treatment groups. C: N ratio of 15:1 was maintained in the experimental tanks. The experiment was conducted for 60 days at the indoor facility of the research centre of ICAR-Central Institute of Fisheries Education at its Kakinada center, Andhra Pradesh. Circular fibre reinforced plastic tanks of 300 litre capacity was used for the experimental trial. A working volume of 150 l was maintained in the tanks. Grey mullet (*M. cephalus*) seeds were collected from Kakinada port, Andhra Pradesh, India. The fishes were acclimatised for 15 days prior to the commencement of experiment. *M. cephalus* fry (0.91 ± 0.01 g) were stocked in the experimental tanks @ 1 individual 10 l^{-1} of water (15 fish per tank).

Carbon source preparation, biofloc production and feeding:

Three easily and locally available carbon sources such as cassava (*Manihot esculenta*), sugarcane jaggery and barley (*Hordeum vulgare*) were obtained from the local market of Kakinada port, Andhra Pradesh, India. Raw tuber, cassava, was peeled and washed thoroughly, cut into small pieces and soaked in water overnight. Water was drained next morning and the pieces were kept in sunlight till they dried completely. After drying, slices were powdered in a mixer grinder, sieved through 35 μm sieves and powder stored in air-tight container. Dried barley grains was purchased and ground into a powder form directly.

Biofloc was produced following the procedure described by Avnimelech (1999). Inoculum was prepared by agitating 10 mg l^{-1} ammonium sulphate and 200 mg l^{-1} carbon source with 20 g l^{-1} pond bottom soil in 1 litre water for 24 hrs (separately for each treatment). Later, the inoculum was added in each of the treatment tanks followed by enough aeration to keep it suspended. After the development of biofloc, fishes were stocked into the treatment tanks. During 60 days of experiment, fishes were fed with commercial pelleted feed containing 35% crude protein @ 4% of body weight. The feeding ration was divided into three equal doses and applied to each tank. In order to maintain the biofloc, carbon sources was added regularly and continuous aeration was provided. Pre-weighed carbon sources was mixed with water collected from the corresponding culture tanks in a glass beaker and uniformly distributed in the biofloc treatment tanks. All the treatment tanks were maintained for 60 days without water exchange. In order to compensate the evaporation loss, water was

added as per requirement.

Monitoring of water quality: Important water quality parameters were monitored daily during the experimental period following the standard procedures (APHA, 2018). pH was measured with pH meter (HI-102; Hitech, India) by dipping the electrode into the water sample. DO was determined by Winkler's method using starch indicator. Alkalinity was measured by titrating the water sample with a 0.02N H_2SO_4 using methyl orange indicator. Ammonia-N was measured by treating the water sample in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside. The ammonia was estimated spectrophotometrically (2080N UV/Vis) by reading the absorbance at OD 640 nm against blank. For nitrite-N analysis, sulfanilamide solution and NED (N- (1-naphthyl)-ethylene diamine dihydrochloride solution) was added in water sample and the absorbance was read spectrophotometrically at 540 nm against a blank. Nitrate-N was measured by mixing the water sample with buffer (phenol solution and NaOH solution) and reducing agent (copper sulphate and hydrazine sulphate). Next, acetone and sulphanilamide solution was added. The absorbance was read on a spectrophotometer at 540 nm after adding NED solution. Floc volume was measured with Imhoff cone by 30 min settlement without disturbance.

Proximate composition of biofloc: Proximate composition analysis of biofloc was accomplished by following the standard methods (AOAC, 2005). At the end of experiment, biofloc samples were collected by filtering the tank water through a 10 μ m mesh nylon bag. The samples were then oven dried at 70°C overnight to remove the moisture content. Finally, the dried biofloc sample were used for estimating protein, lipid, ash and carbohydrate content.

Fish growth performance and survival: Fish growth performance were measured at an interval of 15 days by weighing all the fish individually (100%) from each tank after starving overnight. The following growth parameters namely, percentage weight, specific growth rate, feed conversion ratio, protein efficiency ratio and survival percentage of grey mullet fish were calculated as per Haridas *et al.* (2017).

Digestive enzyme activity: Protease, lipase and amylase activities of the liver tissue were determined by following the methods of Cherry and Crandall (1932), Rick and Stegbauer (1974) and Liu *et al.* (1991), respectively.

Blood collection and serum preparation: Blood was collected from the caudal vein of anaesthetized fish (50 mg clove oil l^{-1}) using 2 ml sterile hypodermal syringe pre-coated with anticoagulant, i.e., EDTA (2.7%). Blood samples were taken from eight fish per treatment, pooled and transferred to 1 ml EDTA coated vials for Respiratory Burst Assay. The serum sample was prepared by collecting the blood using a syringe without

anticoagulant in serological tubes. The blood was then allowed to clot and stored in a refrigerator overnight. The clot was then centrifuged at 6000 rpm for 10 min and the collected serum was stored in sterile tubes at $-20^{\circ}C$ until for analysis of non-specific immune and stress parameters.

Non-specific immune parameters: Respiratory burst activity of the phagocytes was measured by nitroblue tetrazolium (Respiratory Burst Assay) following the modified method of Anderson and Siwicki (1995). Myeloperoxidase activity of serum was determined by Quade and Roth (1999) and partially modified by Sahoo *et al.*, (2005). Lysozyme activity in serum was determined by turbidometric assay utilizing lyophilized *Micrococcus lysodeikticus* as described by Studnicka *et al.* (1986). Lysozyme activity was expressed as units ml^{-1} where one unit is defined as decrease in absorbance of 0.001 min^{-1} .

Stress parameters: Serum glucose was analyzed by glucose kit (Trans Asia Bio-Medicals, India). The detection technique was based on the Trinder's method. The result was expressed in $mg\ dl^{-1}$ of the sample. Serum cortisol levels were estimated using the Cortisol EIA Kit (Cayman Chemicals, USA).

Antioxidant enzymes: Antioxidant enzyme activities such as Superoxide Dismutase as well as Catalase in the liver tissues of experimental fishes were estimated following the methods of Misra and Fridovich (1972) and Takahara *et al.* (1960), respectively.

Statistical analyses: Statistical analyses were performed using SPSS Version 20.0 for Windows (Chicago, USA). Data were subjected to One-way Analysis of Variance (ANOVA) to test the effect of different carbon sources on the growth of fish. Duncan's Multiple Range Test (post hoc test) was used to compare means at $P < 0.05$. The results were presented as mean $\pm$ S.E.

Results and Discussion

Water quality has direct impact on the growth and health of fishes. All the water quality parameters were in the optimum range for fish culture (Bhatnagar and Devi, 2013). There were no significant differences in case of water quality parameters among the treatment groups. Water temperature in all treatment tanks throughout the experiment was $28.33 \pm 0.08^{\circ}C$. A relatively stable pH of 7.9 was recorded in all treatment groups. The dissolved oxygen level (5.8 ppm) was found similar in all treatment tanks. Alkalinity level ranged between 207.75 ppm to 209.92 ppm, respectively. Nitrogenous compound in all the treatment tanks such as ammonia-N was ranged between 0.2 to 0.4 ppm, nitrite-N 0.84 to 0.89 ppm and nitrate-N 8.90 to 9.42 ppm, respectively. This is in line with concept that the biofloc based culture systems retains the water quality parameters in desirable range for the fishes (Crab *et al.*, 2010). Eventhough there was no significant difference in the case of floc volume, higher values was observed

in barley (26.7 ml l⁻¹) followed by jaggery (25.0 ml l⁻¹) and the lowest was observed in cassava (20.0 ml l⁻¹) based on biofloc systems. Hargreaves (2013) suggested that the desirable range of floc volume to maintain finfish culture in biofloc system is 25 to 50 ml l⁻¹. Both jaggery and barley based biofloc tanks showed the floc volume in the desirable range whereas the floc volume in cassava based treatment was lower than the optimum range.

The proximate composition (% dry weight) of biofloc showed significantly higher ($p < 0.05$) amount of crude protein and crude lipid content in barley based biofloc as compared to jaggery and cassava based biofloc. Similarly, the ash content was significantly higher in barley based biofloc. The higher carbohydrate content was found in jaggery based biofloc followed by cassava based biofloc whereas the lowest carbohydrate content was found in barley based biofloc (Table 1). Differences in the proximate composition of biofloc with various carbon sources have been documented in previous studies (Li et al., 2018; Becerril-Cortés et al., 2018; Ridha et al., 2019). The nutritional composition of biofloc were found suitable for the growth of fish in previous studies (Long et al., 2015; López-Elías et al., 2015; Romano et al., 2018). In the present study, better protein and lipid content were recorded in barley based biofloc, leading to the hypothesis that the growth performance of *M. cephalus* in this group was influenced by the nutritional composition of barley based biofloc.

The maximum fish growth and better feed conversion ratio were recorded in barley based biofloc (Table 2). Final body weight of barley based treatment was significantly higher (value) than that of other biofloc treatments ($p < 0.05$). Similarly, higher percentage Weight Gain (864.56 %), Specific Growth Rate (3.78

% day⁻¹) and Protein Efficiency Ratio (2.12) with lowest Feed Conversion Ratio (1.35) was found in barley based treatment than that of other biofloc treatment ($p < 0.05$). There were no significant differences between the treatment groups in the case of survival (%) but higher survival was evident in barley based treatment (86.67%) and the lowest in cassava group (77.78). Investigation with shrimp and tilapia recommends that for every unit of growth resulting from feed, an additional 0.25 to 0.50 units of growth, i.e., 20 to 30 % of shrimp or tilapia growth are derived from microbial protein in biofloc systems (Hargreaves, 2013). The major factor that determines the quality of biofloc developed and its composition is the source of carbon and carbon nitrogen ratio. Previous studies showed that the use of different carbon sources influences the growth performance, feed conversion ratio and survival of finfishes like *Chanos chanos* (Sontakke et al., 2019), *Labeo rohita* (Ahmad et al., 2016). Similar elevated Protein Efficiency Ratio was observed in the barley-based biofloc treatment. As the fish in all treatment groups received similar dietary supplementation of proteins in the commercial diet, the higher Protein Efficiency Ratio values can only result from better protein utilization in the form of biofloc biomass, especially by the fish reared in barley based biofloc treatment. The higher Protein Efficiency Ratio in biofloc based treatment group prove that the fish can effectively convert microbial protein into body mass, thereby contributing to higher growth performance with lower Feed Conversion Ratio (Zhao et al., 2012; Xu et al., 2012; Ekasari et al., 2014). Despite, significant differences being observed among the different carbon source based treatments, all the three carbon sources used in the study such as jaggery, barley and cassava showed a positive effect on the growth performance and survival of fish.

Table 1 : Proximate composition (% dry weight) of biofloc developed from three carbon sources

Parameters	Cassava	Jaggery	Barley
Crude protein	47.27±0.72 ^a	46.05±0.25 ^a	50.05±0.38 ^b
Crude lipid	4.21±0.24 ^b	3.49±0.16 ^a	5.37±0.15 ^c
Ash	16.35±0.11 ^a	17.36±0.27 ^b	18.85±0.58 ^c
Carbohydrate	32.18±0.36 ^b	33.11±0.22 ^b	25.73±0.37 ^a

Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean ± S.E.

Table 2 : Growth, feed utilization and survival of *M. cephalus* reared in different treatment groups at the end of 60 days experimental trial.

Parameters	Cassava	Jaggery	Barley
Initial Body Weight (g)	0.91±0.01 ^a	0.91±0.01 ^a	0.91±0.01 ^a
Final Body Weight (g)	6.23±0.24 ^a	7.12±0.15 ^b	8.72±0.32 ^c
Specific Growth Rate (% day ⁻¹)	3.24±0.037 ^a	3.43±0.072 ^b	3.78±0.036 ^c
Percentage Weight Gain (%)	597.77±15.6 ^a	686.72±33.8 ^b	864.56±20.7 ^c
Feed Conversion Ratio	1.63±0.037 ^b	1.47±0.056 ^{ab}	1.35±0.049 ^a
Protein Efficiency Ratio	1.76±0.084 ^a	1.95±0.076 ^{ab}	2.12±0.079 ^b
Survival (%)	77.78±5.9	82.22±2.2	86.67±3.8

Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean ± S.E.

Table 3 : Digestive enzyme activities (Units mg protein⁻¹ min⁻¹) in the liver tissue of *M. cephalus* reared in different treatment groups at the end of 60 days experimental trial.

Parameters	Cassava	Jaggery	Barley
Amylase	6.44±0.53 ^a	6.06±0.39 ^a	8.78±0.20 ^b
Protease	3.14±0.29 ^a	3.36±0.36 ^{ab}	4.22±0.12 ^b
Lipase	1.82±0.07 ^a	1.94±0.01 ^a	2.77±0.12 ^b

Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean $\pm$ S.E.

Table 4 : Non-specific immune parameters of *M. cephalus* reared in different treatment groups at the end of 60 days experiment

Parameters	Cassava	Jaggery	Barley
Respiratory Burst activity (OD at 540 nm)	0.47±0.01 ^a	0.52±0.06 ^a	0.75±0.08 ^b
Lysozyme (Unit ml ⁻¹)	67.00±1.73 ^a	70.73±2.19 ^b	77.20±1.22 ^c
Myeloperoxidase activity (OD at 450 nm)	1.01±0.08 ^a	1.57±0.21 ^b	1.98±0.02 ^c

Values in the same rows with different superscripts differ significantly ($p < 0.05$) for each parameter. One way ANOVA was used following Duncan Multiple Range Test.

Fig. 1 : Serum cortisol (a) and Serum glucose (b) level of *M. cephalus* reared in different treatment groups at the end of 60 days experiment.

Digestive enzyme activity has a positive correlation with growth of fish (Hidalgo *et al.*, 1999). In the present study, activities of protease, amylase and lipase in the liver of fish differed significantly among the biofloc treatments (Table 3). At the end of experiment, the highest amylase (8.78 Unit mg protein⁻¹min⁻¹), lipase (2.77 Unit mg protein⁻¹min⁻¹) and protease (4.22 Unit mg protein⁻¹min⁻¹) activities were recorded in barley based treatment. Interestingly, the digestive enzyme activities may be enhanced by the polysaccharide content (Long *et al.*, 2015) or exoenzymes excreted from biofloc could be released into the digestive tract (Zhang *et al.*, 2016) and stimulate the digestion. Again, the enhanced protein assimilation by the fish reared in biofloc systems is directly associated to the rise in digestive protease activity due to the involvement of both exogenous digestive enzymes by the microbes in the biofloc and the endogenous digestive enzymes production as stimulated by the biofloc (Xu *et al.*, 2012).

The Respiratory Burst activity and Lysozyme activity were significantly higher in barley based treatment groups compared to jaggery and cassava ($p < 0.05$). Also, the Myeloperoxidase activity recorded significantly highest values in barley based biofloc unit (1.98) followed by jaggery based trial (1.57) with lowest activity observed in cassava (1.01) (Table 4). In previous studies, it has been proved that many bioactive compounds, such as chlorophylls, carotenoids, phytosterols, bromophenols, amino sugars and antibacterial compounds present in biofloc, have a positive influence on immune parameters (Ju *et al.*, 2008; Crab *et al.*, 2010). The studies have also proved that the effect of biofloc on immunostimulants is carbon source dependent (Emerenciano *et al.*, 2013; Ahmad *et al.*, 2016; Sontakke *et al.*, 2019). This is the reason behind the fish reared in biofloc using barley as a carbon source showed a higher immune status compared to fish reared in other carbon source based biofloc treatments.

Table 5 : Antioxidant enzyme activities (Units mg protein⁻¹) of *M. cephalus* reared in different treatment groups at the end of 60 days experiment

Parameters	Cassava	Jaggery	Barley
Superoxide Dismutase	12.1±0.3 ^b	11.5±0.5 ^b	9.3±0.5 ^a
Catalase	43.4±5.8 ^b	40.4±1.6 ^{ab}	28.4±1.5 ^a

Values in the same rows with different superscripts differ significantly ($p < 0.05$) for each parameter. One way ANOVA was used following Duncan Multiple Range Test.

Status of stress indicates the well being of the fishes. Glucose and Cortisol levels (indicators of stress) are directly proportional to the level of stress in the organism (Wijaya *et al.*, 2019). Cortisol and glucose showed significant differences among the biofloc treatments (Fig. 1). Significantly, the lowest values of cortisol and glucose were detected in barley based treatment and the highest values were observed in cassava at the end of the experiment. Similar to the earlier results obtained from analysis of stress parameters, the antioxidant enzymes such as Superoxide dismutase and Catalase activity in fish reared in barley showed lower levels compared to jaggery and cassava based biofloc units (Table 5). The results indicate a reduced oxidative stress of *M. cephalus* reared in barley based biofloc unit which might be due to biofloc composition (Ju *et al.*, 2008b) that perform the role of antioxidants (Xu and Pan, 2012; 2013) and might have resulted in better immunity along with reduced stress. The report of earlier studies stated the decreased level of serum cortisol and glucose levels along with reduced antioxidants in Genetically Improved Farmed Tilapia (GIFT) reared in biofloc based culture systems (Haridas *et al.*, 2017; Menaga *et al.*, 2019) helps us to conclude that the biofloc system does not induce any physiological stress. However, the reduced stress in barley based biofloc treatment compared to cassava and jaggery was supported by the study of Ahmad *et al.* (2016) who reported the influence of carbon sources on growth and well being of *Labeo rohita*. The composition of carbon sources might have influenced the composition and content of biofloc which in turn resulted in significant differences.

In conclusion, the use of *in-situ* biofloc production systems using different carbon sources (cassava, jaggery and barley) significantly improved the growth performance and survival of *M. cephalus*. Among the different carbon sources used, barley seems to be a more suitable carbon source for *M. cephalus* rearing in biofloc systems.

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Add-on Information

Author's contribution : H. Haridas: Conceptualization, writing original draft, review and editing; N.K. Chadha: Conceptualization and methodology; P.B. Sawant: Methodology, reviewing and editing; A.D. Deo: Methodology and design; M.P. Ande: Experimental trial, Data interpretation; K. Syamala: Methodology, Experimental design and samplings; R. Sontakke: Data collection, statistical analysis, writing of original draft and editing; S.S. Lingam: Data collection, statistical analysis and interpretation of results.

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