Influence of raw polysaccharide extract from mushroom stalk waste on growth and pH perturbation induced-stress in Nile tilapia, *Oreochromis niloticus*

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**Abstract**

Fish contributes to half of the total world animal protein produced. In vivo ionic imbalance caused by aqua-environmental pH perturbation makes fish more susceptible to oxidative stress and hence its growth and yield are compromised. Enhancing the antioxidant defense status by supplementing with natural antioxidant rich source is the most convenient alternative to protect fish from stress. Disposal of undesirable mushroom part (mushroom stalk waste, MSW) increases exponentially along with its production. As mushroom is a rich source of polysaccharides and antioxidants, we extracted raw polysaccharide (RP) from MSW for 2 h (RP2) and 5 h (RP5) respectively and supplemented to fish feed to evaluate the protective effect on stress caused by pH fluctuation. RP5 contained significantly higher amount of beta glucan (P = 0.0023) and crude protein (P = 0.0000). Phenolic content for the RP5 was 70.67 mg GAE/g extract exhibiting significantly better IC50 values for DPPH radical scavenging, metal chelating activity and inhibition of LPO, with values of 0.821 (P = 0.0000), 4.48 (P = 0.0000) and 1.052 (P = 0.0001) mg/mL. The experimental range of supplementation (5 and 10 g RP/kg feed) showed zero mortality for Nile tilapia. Higher amount of SGR was observed in case of diet RP 5 g/kg supplementation, however better WG and HSI were found for RP 10 g/kg. In in vivo pre and post stress condition, diet RP 5 g/kg feed has significantly improved the activity of SOD (P = 0.0039 and P = 0.0352, respectively for low and high pH) and CAT (P = 0.0006, P = 0.0006 and P = 0.0074, respectively for pH 5.5, pH 8.5 and pH 10.5). Pearson correlation analysis showed TPC and beta glucan has strong correlation respectively with DPPH (1.00 and 0.942), LPO (0.998 and 0.958) and metal chelating activity (0.988 and 0.980). CAT and SOD has shown strong correlation with SGR (0.960 and 0.935), moderate correlation with WG (0.461 and 0.170), respectively. In the principle component analysis, SGR and WG were observed clustered together with supplemented diet both in pre and post stress condition. In summary, the results suggest that RP5 is a promising antioxidant agent and supplementation of RP5 in fish feed showed the capacity of mitigating pH induced stress in fish, which enhances the growth rate in stress condition.

**Statement of relevance:** This experiment was conducted to improve the growth performance and to enhance the oxidative stress protection capacity of Nile tilapia. Raw polysaccharide of mushroom stalk waste (MSW) was used as supplement in diet which was available, cheap and rich in beta glucan content. This is first report on utilization of MSW as supplement of fish feed to increase the oxidative stress protection in aquatic organism. Nile tilapia, second largest aquatic fish, was used as experimental model fish. Enhancing the antioxidant defense status by supplementing with natural antioxidant rich source is the most convenient alternative to protect fish from stress. Disposal of undesirable mushroom part (mushroom stalk waste, MSW) increases exponentially along with its production. As mushroom is a rich source of polysaccharides and antioxidants, we extracted raw polysaccharide (RP) from MSW for 2 h (RP2) and 5 h (RP5) respectively and supplemented to fish feed to evaluate the protective effect on stress caused by pH fluctuation.

We believe our findings will be of particular interest to the scientific community and very useful in practical to fish feed as supplement.

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**Keywords:** Physiological stress, Mushroom waste, Oxidative stress, Antioxidants, Beta-glucan, Nile tilapia

1. **Introduction**

Fish provides more than half of animal protein required by the world’s population. Unfortunately every year, fish farming industry...
Nile tilapia as the most produced and widely distributed fish since 2004 and 72% are raised in Asia. The total production of Nile tilapia (3.33 MT) positioned second after carp and it is the most desirable among 200 species of fish which need to grow by being fed with exogenous feed (supply feed/formulated feed) (FAO, 2012). This experiment was carried out to evaluate the effect of feeding raw polysaccharide (RP) supplemented diets on growth the immunity of fish against pH stress in Nile tilapia.

2. Materials and methods

2.1. Raw polysaccharide (RP) from MSW and feed preparation

MSW (collected from Vita Agrotech. Sdn. Berhad, Tanjung Sepat, Selangor, Malaysia, 02°40’31″N, 101°34’22.3″E) were cleaned, cleaved into single stalk before oven dry. Oven-dried MSW was ground to fine powder in a Waring blendor (Torrington, USA). Before extraction procedure MSW powder were soaked in 80% ethanol overnight with continuous stirring. The mixture was filtered through Whatman No. 1 filter paper and the residual ethanol was evaporated at room temperature.

Glass-distilled water (1:20 w/v) was used in the extraction procedure with separate boiling times of 2 h or 5 h. The boiled samples were then cooled at room temperature, filtered twice through linen cloth, and centrifuged at 10,000 × g for 10 min. The supernatant was collected and freeze dried, and the RP were stored at −20 °C until further use. Logically available ingredients (local fish meal, soybean meal, rice bran as a protein source, and palm oil as a lipid source) were used to prepare the basal diet (approximately 34% crude protein and 6% crude lipid, Winfeed284, Winfeed (UK) Limited, Cambridge, UK). All the ingredients were powdered separately, mixed together and made stiff dough by gradually adding water. Next, the dough was passed through fish feed extruder (16 mm die) separately and gently broken to make pellets (fish feed extrusion facilities of Freshwater Fisheries Research Division (BTAP) were used). The diets were then oven dried (45 °C, 48 h), cool down at room temperature, packed separately in labeled polypropylene bags, sealed and stored at 4 °C for future feeding trial. Available commercial feed was used as the control diet. The basal diet was considered as the 0-supplemented. The basal diet was then supplemented with either 0.5 g/kg or 10 g/kg RP5, where the experimental feed would contain 0.09–0.18% beta-glucan since the RP 5 h contained 16.91% beta-glucan.

2.2. Determination of chemical composition

Following the official methods of analysis of AOAC (Association of Official Analytical Chemists) (AOAC, 2005), dry matter (method 934.01), crude protein (method 968.06), crude fat (method 945.18), ash (method 942.05), and crude fiber (method 962.09) of RP were analyzed. The carbohydrate amount was determined using the formula as follows:

\[
\text{Carbohydrate (g/kg)} = 1000 - (\text{crude protein} + \text{crude fat} + \text{ash} + \text{crude fiber}) \text{in g/kg}
\]

Mushroom and yeast β-glucan assay kit (Megazyme International, Ireland, Ireland) was used to determine the β-glucan content of RP. Total glucan and α-glucan were determined by enzymatic hydrolysis, and β-glucan (% dry weight) was measured by calculating the difference between total glucan and α-glucan.

2.3. Measurement of antioxidant properties of RP in vitro

To evaluate in vitro antioxidant properties of RP, total phenolic content (TPC) (Slinkard, Singleton, 1977), metal chelating activity (MCA) (Jayakumar et al., 2009), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Niki et al., 1994) and inhibition of lipid

suffers great economic loss worldwide owing to the severe deterioration of its natural habitat. The U.S. Food and Drug Administration (FDA) have approved some vaccine and immune booster to increase fish immunity against stress. The limited application or failed or unrealistic use of these in broad range opens the opportunity to find alternative sustainable solution (Darwish, 2007; Ming et al., 2012). The feed and feeding rate were found to be a significant protector against heat stress in rainbow trout (Cara et al., 2005) and white sturgeon (Deng et al., 2009).

Water pH is a crucial factor for the development, growth and survival of fish. The surrounding aqua-environmental habitat of fish makes them more susceptible to oxidative stress compared to the terrestrial vertebrate other than mammals. Fluctuations of pH in water causes ionic imbalance in fish and could lead to death. Both alkaline (pH 8.5–10) and acidic (pH below 6.0) water causes acute physiological disturbance in fish, affecting the normal growth rate and finally becoming a potential lethal factor for aquatic biota including fish (Holland et al., 2014; Laurent et al., 2000; Lear et al., 2009; Saha et al., 2002). Elevation of pH was found to be lethal for Tambaqui production (de Croux et al., 2004), hence, maintaining the optimum pH level is necessary for the normal growth of the fish or cultured fish.

It is also reported that, adding NaCl to diet reduces acidic water effect on the growth of silver catfish (Copatti et al., 2011). Carboxylated and DNA damage in aquatic organism (Abalone) causes environmental stress (thermal fluctuation or oxygen demand-uptake mismatch) and that can be reduced or reversed by primary antioxidant defense system (Vosloo et al., 2013b). The adult Abalone shows more sensitivity to environmental stress than young one (juvenile) because of the stronger antioxidant defense system and supplementation of amino acid (γ-proline) to fish feed minimizes the sensitivity of the adult (Vosloo et al., 2013a). Supplementation of vitamin C and E was also reported to reduce oxidative stress to the fish, Japanese flounder (Gao et al., 2014). Fish feed supplemented with natural antioxidant rich source is an alternative to increase antioxidative status of fish (Ferreira et al., 2013).

In line with the exponential increase in mushroom consumption, the volume of the undesirable parts of mushroom (mushroom stalk waste, MSW) disposed of by mushroom industries is increasing drastically every year along with spent mushroom substrate (SMS) (Agrawal et al., 2010; Filipa et al., 2012; Randive, 2012; Sarker et al., 2007a). Two types of waste, namely MSW and SMS, are produced in oyster mushroom industries. In our previous work, we found that 165 to 502 g of MSW need to be discarded to get 1 kg commercial mushroom from biological yield (primary yield) (Ahmed et al., 2013). Chou et al. (2013) mentioned that MSW cut off is 25 to 33% of total production. Thus, an enormous output of MSW piled on open environment every day can lead to environmental pollution. MSW takes a huge cut off of profit margin of the mushroom growers every year. Though many uses of SMS were evaluated, no work has yet been published on alternative uses of MSW. Recently, finding ways to safely dispose of MSW becomes a burning issue to the producers of mushroom industry as well as to waste management authority.

Previously, the effect of oyster mushroom and/or the beta glucan extracted on growth performance, immune properties, histological characters or disease resistance were evaluated for different types of fish. Some of them used as prebiotics (Jabir et al., 2012) for red tilapia, some replaced mushroom for rice bran to prepare feed for Nile tilapia (Muin et al., 2014) or catfish (Muin et al., 2013). Beta glucan of mushroom was evaluated as immunostimulant in common carp (Dobskova et al., 2013). Mostly, the in vivo antioxidant properties of mushroom were conducted using animal other than fish, like poultry (Giannenas et al., 2011) and rats (Kanagasabapathy et al., 2013). All of these experiments were evaluated using mushroom fruiting body. Thus, limited or no work has been reported on the utilization of MSW in fish feed to protect against stress. As mushrooms are rich source of protein, long chain polysaccharide (beta glucan) and immune-stimulant (pleuron), MSW (being part of mushroom fruiting body) may also be a good source of antioxidant.
peroxidation (LPO) activity (Abdullah et al., 2011) assays were carried out. IC$_{50}$ (concentration of the extract where it shows the half maximal activity or inhibition) values of the extract were calculated for MCA, DPH and LPO.

2.4. Experimental procedures

Healthy Nile tilapia (O. niloticus) was obtained from BPTAP, Department of Fisheries, Malaysia. The juveniles were acclimatized for 1 week in continuous aerated fiberglass tank (26 ± 1 °C, pH 7.5–8, 6.2 mg L$^{-1}$ dissolved oxygen, <0.2 mg L$^{-1}$ total ammonium level and nitrite level were below 0.05 mg L$^{-1}$ with natural photoperiod in dechlorinated tap water) at the Aquatic Laboratory, University of Malaya. During acclimatization, juveniles were fed with commercial feed (Kristal Prima fish feed, Kristal Prima Resources Corporation Sdn. Bhd., Malaysia). A total of 900 fish were maintained for the following experiment, toxicity evaluation and growth performance assay. Throughout both experiments, the daily water exchange rate was 30% of the total volume. Continuous si-phoning (water pump H6350, Shanda Aquarium, China, connected to filter box) of fecal material and recirculation of water and the air supply (Sonic air pump P85, China) were maintained throughout the experiment. Toxicity of the RP supplemented feed was evaluated prior to the experiment. For the feed toxicity or fish mortality, feeds with different levels of supplementation (0–100 g/kg) of RP were prepared (0, 5, 10, 50, and 100 g RP/kg). Three replicates and 20 juveniles for each replicate were fed for 20 days with RP-supplemented feed. After 20 days of feeding, fish mortality was calculated to determine the feed toxicity.

Four experimental diets were used including one commercial diet. Three hundred fish were evaluated for the toxicity test of RP supplementation, and the other 600 were equally distributed into 12 fiberglass tanks (500-L water holding capacity). The initial mean weight of the fish was 18.59 ± 1.67 g/fish. All the fish were fed 3% of their body weight twice a day (morning and evening) until the end of the experiment.

To evaluate the potential of the RP supplemented feed against pH stress in tilapia, fish from the 1 month trial were exposed for 1 week to different levels of pH, acidity to alkalinity (pH 5.5, pH 8.5 and pH 10.5), separately. Ten fish from each feed group were sampled. The pH was measured 3 times a day and adjusted by NaOH and HCl if necessary.

Growth performance was calculated using the formulae: specific growth rate (SGR) = ($W_f - W_i$)/$W_i$ × 100/TA; weight gain (WG) = $W_f - W_i$; hepatosomatic index (HSI) = ($W_i/W_2$)100, where $W_1$ and $W_2$ are the initial and final weights of the fish, respectively. $W_i$ is the weight of the liver, and TA is the number of days between T1 and T2.

2.5. In vivo measurement of antioxidant properties and the pH challenge

In vivo antioxidant properties of the supplemented feed in Nile tilapia were evaluated before and after exposure to pH challenge. The activity of two antioxidant enzymes (SOD and CAT) was evaluated in the liver, kidney, and plasma collected from the experimental fish. Liver and kidney tissues were rinsed with phosphate-buffered saline (pH 7.4) to remove blood cells or clots. Tissues were homogenized (1:5–10, w/v) separately in cold HEPES buffer (20 mM, pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. After centrifugation ($1500 \times g$; 5 min) at 4 °C, the supernatant was collected and stored for further analysis. Plasma was sampled by collecting blood from the vena caudalis with anticoagulant and centrifuged at 1000 × g for 10 min at 4 °C. The top yellow plasma was separated without disturbing the white buffy layer and stored at −80 °C until analysis. To determine the SOD activity in plasma and homogenates of the liver and kidney, an SOD assay kit (Cayan Chemical Company, USA) was used. In this assay, superoxide radicals produced by xanthine oxidase and hypoxanthine were determined with tetrazolium salt. All three iso-enzymes of SOD (Cu/Zn, Mn, and Fe SOD) were measured. CAT activity was evaluated using a catalase assay kit (Cayan Chemical Company). The principle of the assay is based on the peroxide function of CAT. In this assay, the enzyme reacts with methanol in the presence of an optimal concentration of H$_2$O$_2$. The formaldehyde produced was measured colorimetrically using Purpald as a chromagen. Purpald with aldehyde forms heterobicyclics, changes from colorless to purple upon oxidation.

2.6. Statistical analysis

All in vitro experiments were performed in triplicate. For the in vivo enzymatic activity, blood and tissue samples (liver and kidney) were collected from nine fish from each experimental tank, and samples from three fish were pooled separately. Data were expressed as the means with pooled SD for each group. The Shapiro–Wilks test and Bartlett's test were used to determine the normality and homogeneity of variance, respectively. To compare the means, two-way analysis of variance was performed using the statistical package tool STAR (Version 1.1.2013, Biometrics and Breeding Informatics, PBGB Division, International Rice Research Institute, Los Baños, Laguna). A post-hoc Duncan’s multiple range test ($P < 0.05$) was used to determine significant differences between the means. Principal component analysis and Pearson correlation regression were performed using Microsoft excel version 2010 using XLSTAT2015 (Addinsoft, New York, United States).

3. Results and discussion

3.1. Proximate composition and beta glucan content of RP of MSW

Chemical composition and beta content of the both RP2 and RP5 were determined prior to extrusion of feed. Table 1 shows the chemical composition and beta glucan content (g/100 g) on dry weight basis. Overall, RP5 extract contained higher amount of dry matter, crude protein, total ash and beta glucan and RP2 contained higher amount of moisture, crude lipid, carbohydrate and crude fiber. To our knowledge, no work has been published on nutritional composition of MSW of oyster mushroom. On average, ground P. pulmonarius (previously known as P. sajor-caju) consist of 90, 10, 25.50, 4.0, 7.23, 38, 25.20% of moisture, dry matter, crude protein, crude lipid, total ash and crude fiber, respectively (Alam et al., 2007) on dry weight basis except moisture (moisture was calculated on fresh weight). This proximate composition was determined for whole fruit bodies. MSW is a part of mushroom fruit bodies but this waste product consists of the lower hard portion and growing medium residue (agricultural byproduct). Although MSW is discarded

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MSW of P. pulmonarius</th>
<th>Pooled SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content</td>
<td>6.613$^b$</td>
<td>9.17$^a$</td>
<td>0.53</td>
</tr>
<tr>
<td>Dry matter</td>
<td>93.39$^a$</td>
<td>90.83$^b$</td>
<td>0.53</td>
</tr>
<tr>
<td>Crude protein</td>
<td>22.86$^a$</td>
<td>20.04$^b$</td>
<td>0.91</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>1.98$^a$</td>
<td>2.94$^a$</td>
<td>0.06</td>
</tr>
<tr>
<td>Carbohydrate$^c$</td>
<td>45.57$^b$</td>
<td>55.29$^b$</td>
<td>4.05</td>
</tr>
<tr>
<td>Total ash</td>
<td>27.96$^a$</td>
<td>20.33$^b$</td>
<td>1.13</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>3.41$^a$</td>
<td>4.05$^a$</td>
<td>2.03</td>
</tr>
<tr>
<td>Beta glucan$^d$</td>
<td>16.91$^a$</td>
<td>11.21$^b$</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± standard deviation of three replicates. Means with different letters within a row are significantly different ($P < 0.05$).

$^a$ Proximate compositions of extracts were determined by using AOAC 2000 protocols.

$^b$ RP was prepared by boiling 5 h (RPs) and 2 h (RP2) separately MSW of P. pulmonarius with deionized water (1:20 w/v) after removed the low molecular weight compounds from MSW (soaking overnight, 80% ethanol, 1:10 w/v).

$^c$ Amount of carbohydrate (CHO) was determined by calculation.

CHO (g/kg) = 1000 – (crude protein + fat + ash + crude fiber).

$^d$ Beta glucan content was determined by manufacturer protocols, Megazyme Interna-

Table 1

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3.2. Antioxidant properties of RP in vitro

Before supplementing RP to fish feed as antioxidant, in vitro antioxidant activity of RP2 and RP5 were examined based on reducing capacity, free radical scavenging ability (DPPH scavenging activity), chelating activity of metal ion (ferric/ferrous ion chelation) and inhibition of lipid peroxidation potential.

The phenolic content has a very strong correlation with antioxidant properties. Higher amount of TPC in mushroom or plant materials showed better antioxidant properties. The results of this assay are shown in Table 2. The higher amount of TPC was found in RP5 (P < 0.05). Abdullah et al. (2011) reported a value of 17.77 mg GAE/g sample of TPC for the fruiting body of grey oyster mushroom, which leads to the total phenolic content of RP. RP5 possesses significantly higher amount of β-glucan than RP2. Synytsya et al. (2008) reported the content of β-glucan ranging between 27.4 and 39.2% and 35.0–50.0% in pilei and stipes, respectively in Pleurotus spp. They extracted pure β-glucan from different mushroom parts. In this experiment, lower amount of β-glucan was obtained which might be due to different extraction methods used.

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (mg/mL)</th>
<th>TPC (mg GAE/g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH</td>
<td>Metal chelating</td>
</tr>
<tr>
<td>RP2</td>
<td>1.67x</td>
<td>1.50x</td>
</tr>
<tr>
<td>RP5</td>
<td>0.82x</td>
<td>0.43x</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.43x</td>
<td>0.19x</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.82x</td>
<td>0.43x</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.040</td>
<td>0.058</td>
</tr>
<tr>
<td>Statistics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Mean values of three replicates. Different lowercase letters after the value in column are significantly different (P < 0.05). IC_{50}, concentration of the extract at which inhibit by 50%; lower value indicate higher activity; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging ability; LPO, inhibition of lipid peroxidation; TPC, total phenolic content; GAE, Gallic acid equivalent; EDTA, Pooled SD, pooled standard deviation; sample, effect of extract and positive control; concentration, effect of different concentration level of sample; S × T, interaction between sample and extract.

3.3. Evaluation of the toxicity of RP supplementation on Nile tilapia, O. niloticus

The toxicity of MSW supplemented feed on O. niloticus was evaluated first, before the stress analyses was carried out. For this, 0 to 100 g of RP was supplemented with per kg basal diet separately (35% protein, 6% lipid). The juveniles were allowed to feed for 20 days ad libitum with different supplemented diet and the mortality was recorded to evaluate the toxicity (Table 3). RP supplementation at experimental range (5 & 10 g/kg of supplementation) shows zero mortality. Fivefold supplementation of different range shows no toxicity in first day of feeding for Nile tilapia juveniles but after 5 days of feeding, this range shows minimum toxicity (3% of mortality), which is not significantly different. Significant toxicity is found on increasing feeding time for 5 and 10 folds supplementation, where the toxicity is detected at day 14. Significant difference in toxicity level could also be observed between 5 times and 10 times supplemented diets in the same period of feeding time except on day 10. Different mushroom and their spent substrate were evaluated as fish feed or as supplementation of fish diet previously, mostly spent mushroom substrate (Hasiyati et al., 2013; Williams et al., 2001). Range of β-glucan in supplemented diet may explain the toxicity of supplementation fold relay with feeding time. Supplementing >1% dietary β-glucan was reported to be toxic for the fish, large yellow croaker (Qinghui et al., 2007). Thus, supplementing with higher percentage of RP in this study may increase the total amount of β-glucan in the supplemented diet, which leads to the toxicity.

### Table 3

<table>
<thead>
<tr>
<th>Evaluation time</th>
<th>Supplementation of RP (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Day-1</td>
<td>0.00</td>
</tr>
<tr>
<td>Day-5</td>
<td>0.00</td>
</tr>
<tr>
<td>Day-10</td>
<td>0.00</td>
</tr>
<tr>
<td>Day-14</td>
<td>0.00</td>
</tr>
<tr>
<td>P value</td>
<td></td>
</tr>
<tr>
<td>Supplementation</td>
<td>0.000</td>
</tr>
<tr>
<td>Time</td>
<td>0.000</td>
</tr>
<tr>
<td>S × T</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Data are mean of three replicates for each supplementation (60 fish). Different small and capital letters in same column after the toxicity value indicate significant differences in group and within groups of supplementation respectively (P < 0.05, ANOVA performing combined analysis with CBD). Supplemented feed were prepared by mixing mentioned percentile of RP with basal diet (see Table 2 for formulation of basal diet). RP was prepared from waste mushroom stalk of P. pulmonarius.
3.4. Effect of RP supplemented diet on growth performance of Nile tilapia, *O. niloticus*

Once non-toxicity of experimental range of RP concentration was confirmed, observation on the effect of RP supplementation on the visible outcomes (growing parameters) of Nile tilapia was carried out. The juveniles were fed for 1 month with the experimental diets to satisfy the queries, 1. “Does RP of MSW disturb the normal growing of tilapia?” 2. “Does RP supplementation can ameliorate pH induced stress on tilapia?” Results analyzed in Table 4 answer the query 1 by comparing with readily available commercial diet (CD) and prepared basal diet (BD).

Supplementation of RP and feeding time showed significant effect on SGR (P < 0.05) when compared to that of BD (Table 4). Regarding HSI, significant results were recorded for both feeding time and diet (P < 0.05). Although value of HSI (1.58) was highest at the higher level of supplementation (RP 10 g/kg) followed by CD (1.51), however, it was significantly similar to the results for RP 5 g/kg (1.49). The lowest HSI results were recorded for BD (1.35). HSI is defined as the ratio of liver weight to body weight. It indicates reserve energy status of animal. Both metabolism and excretion of xenobiotics is immensely depended on the condition of liver, hence determination of HSI is important to know the health stature of fish (Sadekarpawar, Parikh, 2013). The liver index of fish is depended on many factors like, sex, breed, condition of the habitat and most importantly the feed and feeding behavior of the fish (Caballero et al., 2002; Leatherland et al., 1979). Variation on the value of HSI for different diets could be the effect of nutrient composition of RP. Mushroom is well known as the source of good quality protein, lipid and beta glucan. Tibbetts et al. (2005) found a correlation of dietary protein and lipid with HSI of juvenile haddock. Replacing corn protein, lipid and beta glucan with mushroom stalk of *Pleurotus* spp. because the *Pleurotus* spp. from fungus and other plant sources is a good growth promoter for fish (Qinghui et al., 2007).

### Table 4

<table>
<thead>
<tr>
<th>Variables</th>
<th>Diet</th>
<th>Pooled SD</th>
<th>P value</th>
<th>Diet</th>
<th>Time</th>
<th>D × T</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.54</td>
<td>0.0069</td>
</tr>
<tr>
<td>WGR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.91</td>
<td>0.0526</td>
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<tr>
<td>HSI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.49&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.14</td>
<td>0.0036</td>
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</tbody>
</table>

Data were shown as mean of three replications for each diet (150 fish). Different small letter in same raw after the mean value indicate significant differences within diet group (P < 0.05, ANOVA performing combined analysis with RCBD). Supplemented feed were prepared by mixing mentioned percentage of RP with basal diet (BD) Base diet were formulated containing 35% of crude protein and 6% of crude lipid. RP was prepared from waste mushroom stalk of *Pleurotus* sp. Performance of supplemented feed was compared with the commercial feed (CD) and BD. **A:** SGR, specific growth rate = (lnW2 – lnW1)/100×T; W<sub>1</sub> & W<sub>2</sub> are initial and final weight of fish at number of days between T<sub>1</sub> & T<sub>2</sub>. **B:** HSI, hepatosomatic index = (W<sub>r</sub>/W<sub>0</sub>)100; W<sub>r</sub> and W<sub>0</sub> are weight of liver and final weight of fish respectively. **C:** W<sub>g</sub>, weight gain = W<sub>2</sub> – W<sub>1</sub>; W<sub>1</sub> & W<sub>2</sub> are initial and final weight of fish.

Living organisms maintain a steady state of reactive oxygen species (ROS) in normal condition by a continuous production and elimination of ROS. The imbalance between production and elimination (enzymatic and non-enzymatic antioxidant defense system) of ROS creates stress condition in organisms, known as oxidative stress. The enhanced production of ROS may be a common event or caused by oxidative stress (Lushchak, 2011b). In modern aquaculture system, the genetic potentiality of fish and the in situ environmental condition are considered as the most influential factors to maximize the growth performance. The substandard water quality, physical perturbation and individual social domination to others are acted as persuasive environmental stresses (Pickering, 1993). The environmental stresses control the expression of growth regulated genes. Living organisms possess both enzymatic and non-enzymatic defense system to protect themselves from this stress. However, adverse environmental condition, physiological state (ageing), diseases, pathogenic, toxic products and many anthropogenic factors fasten ROS production into the living body (Holland et al., 2014; Lear et al., 2009; Lushchak, 2011b; Sakai, 1999) which may disrupt the auto defense system. It may result in growth disturbance of the biota and sometimes may cause death. Naturally fish expends some extra energy to respond to the stress and this additional energy mostly comes from the feed (nutrient) that could be utilized for maintaining growth (Martins et al., 2011). Allowing balance nutrition, maintain proper culture environment and changes in endocrine system could improve the growth of the fish by returning the stressed condition to normal (Nakano et al., 2014). When the auto defense system of the animal is disrupted, some foreign products are required to boost that defense system to keep it normal. Rodriguez et al. (2009) observed that injected beta glucan in Zebra fish increased ability of kidney cell to kill the bacteria, *Aeromonas hydrophila*. Djordjevic et al. (2009) reported that beta glucan from mushroom *Leninula edodes* modulate the immune response of Rainbow trout. In aquatic animal, superoxide dismutase (SOD) and catalase (CAT) are widely found/report antioxidant enzyme with notable range of glutathione peroxidase and free radical scavenger (Livingstone, 2003). SOD is the first enzyme response to stresses and enhanced expression of SOD could neutralize the bad effects of superoxide in stressed environment (Liu et al., 2011; Lushchak, 2011a). SOD converted superoxide to hydrogen peroxide and CAT shows catalytic activity, which neutralizes the hydrogen peroxide to oxygen and water. A clear interaction was reported on the activity of SOD and CAT (Ferreira et al., 2013).

### 3.5.1. Activity of SOD in pre and post stress condition

Pre and post stress SOD activity in Nile tilapia after one month of feeding the experimental diets are shown in Figs. 1 and 2, respectively. The BD and the CD were fed to determine the antioxidant status of the Nile tilapia and this was compared to that of RP 5 g supplemented feed. Supplementation shows significant improvement (P = 0.0000) of SOD activity in pre-stress (one month of feeding) experiment and also positively ameliorated (P = 0.0000) of SOD activity in post pH stress (moderate, low and high) condition in Nile tilapia. The highest SOD activity (U/mL) is found in samples from fish fed with RP supplemented feed compared with the CD and BD for Nile tilapia (Fig. 1) and the lowest SOD activity found in all three samples of blood, liver, and kidney for the BD fed fish. The different samples (liver, blood and...
kidney) of the fish in each diet fed (pre-stress) show different significant SOD activity (P < 0.05) whereas liver shows the best activity. At higher (pH 10.5) and lower (pH 5.5) pH environments, the improved SOD activity is found in different sample (P = 0.0039 and P = 0.0352, respectively for low and high pH), whereas it was not significant at 95% of probability in moderate pH (pH 8.5, P = 0.6937).

In cellular antioxidant mechanism, the dismutation of superoxide is a crucial factor which is catalyzed by metaloenzyme, superoxide dismutase. Qinghui et al. (2007) reported that high supplementation with β-glucan in feed is toxic to fish and reduces their innate immunity. Different SOD activities in differently supplemented and BD may be a consequence of β-glucan supplementation. Previous investigations of β-glucan-mediated protection against oxidative stress showed that its activity may be partially due to its free-radical scavenging activity (Kogan et al., 2005; Saluk-Juszczak et al., 2010). The effect of β-glucan on the increase in SOD activity is most likely because it counteracts harmful effects of reactive oxygen species or because of an increase in the number of neutrophils and/or activation of phagocytes in response to free radicals (Dalmo, Bogwald, 2008). β-Glucan plays an important role in stimulating macrophages and activating anti-oxidative enzyme-related proteins and genes (Selvaraj et al., 2005; Wang et al., 2014).

3.5.2. Activity of CAT in pre and post stress condition

Similar to the SOD activity of Nile tilapia, CAT activity exhibited feed (supplementation) and samples (tissues and blood) depended pattern (Fig. 1). Higher CAT activity is observed in fish fed with supplemented diet as compared to control diets (CD and BD). Kidney of RP supplemented diet fed fish shows the highest CAT activities than others in all cases (control diets and or samples). After one month of feeding, significant effect of feed (P = 0.0000), sample (P = 0.0000) and combination of both factors (P = 0.0000) on CAT activities are recorded (Fig. 3). In stress tolerance experiment (exposing the fish pH perturbation, low and high pH), the most significant changes of CAT activities occur in RP supplemented fed fish during both stress condition (low and high pH). Though no significant CAT activities are found in different samples at different pH habituate (P = 0.1143 for pH 5.5, P = 0.1580 for pH 8.5 and P = 0.0537 for pH 10.5) like pre-stress experiment, the supplementation shows significant effect in this enzyme activity of fish in all pH conditions (P = 0.0000). Lower supplementation of RP shows that better stress arsenal than higher dose. Combined effect of feed and sample (liver, blood and kidney) is significant for all pH environments (P = 0.0006, P = 0.0006 and P = 0.0074, respectively for pH 5.5, pH 8.5 and pH 10.5).

CAT protects living organism from oxidative stress caused for ROS by decomposing hydrogen peroxide to water and oxygen (Winston and Di Giulio, 1991). The CAT activity is consistent in living organism in normal environmental condition. In stress condition, the acid-base disturbance and the metabolic consequences in animal produce more ROS (Chapman et al., 2011). In the stress experiment, CAT activity was higher (in both low and high pH) in the samples from the fish fed control diets (CD and BD). More CAT is required to decompose more ROS. In contrast, the range of CAT activities in the fish fed supplemented diet at both lower and higher pH was consistently similar to the CAT activity in fish at moderate pH. RP contained β-glucan, higher amount of protein and phenolic compounds. Supplementation of β-glucan (Wang et al., 2014).
and dietary phenolic compounds (Kishimoto et al., 2013) helps to maximize stress arsenal of organisms by means of increasing anti-oxidative status.

3.6. RP supplementation minimize the negative effect of pH perturbation on growth performance of fish

Table 5 shows the mortality and growth performance of Nile tilapia affected by pH stress. A lower SGR and WG of the fish are observed in stress condition than pre-stress condition. Supplementation or feed shows significant effect on SGR (P = 0.0000) and WG (P = 0.0001) in stress condition. Although both RP supplementations show similar significant SGR in moderate pH, the highest SGR is recorded in higher supplementation in low and high pH stress. The BD shows lower effect on SGR of fish. In the case of WG, higher supplementation shows the results in low and high pH. Despite oxidative stress, gill injury and food intake have also been reported as factors affecting growth and survival of the fish (Zhu et al., 2008). Supplementation of RP with 5 and 10 g/kg feed shows no toxicity in pre-stress experiment and the RP supplemented feed fasten the growth of the fish comparing BD with the highest level of antioxidant enzymes. This explains that the RP supplemented feed intake might not have adverse effect on fish. Increasing rate of SGR and WG along with decreasing SOD and CAT activity of the fish in RP supplemented feed at stress experiment might also explain mitigating pH stress, whereas, decreased SGR and WG was found in BD and CD fed fish with increasing SOD and CAT. This result showed that CAT and SOD had the ground to deal with stress caused by pH. The results of supplementation of RP on CAT and SOD activity in fish on pH stress experiment exhibit the ability of RP against stress. Polysaccharide of different edible and medicinal mushroom has reported before for character to improve antioxidant enzyme activity (Jia et al., 2009; Klaus et al., 2011).

3.7. Correlation effect on antioxidant activity and growth performance of fish in related with RP and supplementation

Pearson regression correlation (Table 6) and PCA biplot analysis (Figs. 4–6) was performed among TPC and beta glucan with antioxidant activity and growth performance of fish for the extract and supplemented diet both in pre and post stress status. In brief, TPC, crude protein and beta glucan have strong correlation respectively with DPPH (r = 1.00, 0.984 and 0.942), LPO (r = 0.998, 0.992 and 0.958) and MCA (r = 0.988, 0.999 and 0.980). This findings are in agreement with Reddy et al. (2012) and Hatami et al. (2014) which reported that phenolic compounds naturally has a better correlation with DPPH radical scavenging.
activity, MCA and ferric ion reducing activity. On the contrary, they did not find any correlation of TPC with copper ion reducing activity. This explains the effect of TPC on antioxidant properties relates with source and or type of it. A very strong significant correlation of TPC and antioxidant properties was found in this experiment. A similar correlation was also reported previously for Lentinus squarrosulus mushroom (Abdullah et al., 2015). The eigenvalues of two components exceeded 1 in PCA of the antioxidant results, which revealed 100% of total variance, whereas the contribution of the component 1 and component two are 88.14% and 11.86% respectively (Fig. 4). The interpretation of the two components shows consistent regression correlation of Table 6 in Varimax rotation. In PCA biplot protein, beta glucan and TPC were clustered together, whereas DPPH were grouped with TPC and LPO and MCA were grouped with beta glucan and protein. In general antioxidant mechanisms are involved in the two groups, electron transfer reactions and others based on hydrogen transfer reactions (Ndhlala et al., 2010), which were explained in biplot (Fig. 6).

![Fig. 3. Activity of catalase (CAT) in different stress pH perturbation in liver, kidney and blood of Nile tilapia Oreochromis niloticus in vivo. Data are mean value of three replicates of each diet fed fish in each pH stress. ANOVA was performed in combined analysis using complete block design. Different uppercase and lowercase letter on bar is significantly different among group of diets (D) (P < 0.05). CD, commercial diet and BD, basal diet.](image-url)

### Table 5
Growth performance of Nile tilapia Oreochromis niloticus under different pH level post fed raw polysaccharide (RP) supplemented feed.

<table>
<thead>
<tr>
<th>Feed</th>
<th>SGR^a</th>
<th>WG^b</th>
<th>pH 5.5</th>
<th>pH 8.5</th>
<th>pH 10.5</th>
<th>pH 5.5</th>
<th>pH 8.5</th>
<th>pH 10.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP 10 g/kg</td>
<td>1.42^a</td>
<td>1.69^a</td>
<td>1.13^d</td>
<td>8.31^d</td>
<td>9.98^d</td>
<td>6.55^c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP 5 g/kg</td>
<td>1.25^b</td>
<td>1.70^a</td>
<td>0.95^a</td>
<td>6.84^b</td>
<td>9.51^b</td>
<td>5.17^b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>1.20^a</td>
<td>1.62^a</td>
<td>0.88^b</td>
<td>8.25^a</td>
<td>11.26^a</td>
<td>5.98^a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>0.64^c</td>
<td>1.56^c</td>
<td>0.43^c</td>
<td>2.85^c</td>
<td>7.18^c</td>
<td>1.91^c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistics**
- Pooled SD: 0.09, 0.25, 0.12, 0.60, 1.60, 0.77
- P value (P < 0.05): 0.0001
- Stress: 0.0000
- Feed x stress: 0.0034

Data were shown as mean of three replications for each stress condition (10 fish/replicate). Different small letter in same column after the value indicate significant differences within diet group (P < 0.05, ANOVA performing combined analysis with CBD). Supplemented feed were prepared by mixing mentioned percentile of RP with basal diet (BD). Basal diet was formulated containing 35% of crude protein and 6% of crude lipid. RP was prepared from mushroom stalk waste of P. pulmonarius. Performance of supplemented feed was compared with the commercial feed (CD) and BD.

^a SGR, specific growth rate = (lnW2 − lnW1)/100/ΔT, W1 & W2 are initial and final weight of fish ΔT is Number of days between T1 & T2.

^b WG, weight gain = W3 − W1, W1 & W2 are initial and final weight of fish.

### Table 6
Interrelationship of in vitro and in vivo antioxidant activities, growth performance and TPC, crude protein, beta glucan and antioxidant enzyme.

<table>
<thead>
<tr>
<th>Variables</th>
<th>DPPH</th>
<th>LPO</th>
<th>MCA</th>
<th>SGR</th>
<th>CAT</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>0.984</td>
<td>0.998</td>
<td>0.988</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta glucan</td>
<td>0.942</td>
<td>0.938</td>
<td>0.980</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td></td>
<td></td>
<td></td>
<td>0.960</td>
<td>1.00</td>
<td>0.897</td>
</tr>
<tr>
<td>SOD</td>
<td></td>
<td></td>
<td></td>
<td>0.935</td>
<td>0.897</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Values in bold are different from 0 with a significance level alpha = 0.05. TPC, total phenolic content, DPPH, scavenging 1,1-diphenyl-2-picrylhydrazyl radical, LPO, inhibition of lipid peroxidation, MCA, metal chelating ability, SGR, specific growth rate, CAT, activities of catalase and SOD, activities of superoxide dismutase.
During the pre-stress period, antioxidant enzyme CAT ($r = 0.960$) and SOD ($r = 0.935$) exhibits a very good correlation ($P < 0.05$) with growth performance (SGR) of the fish, Nile tilapia (Table 6). The PCA biplot relates with antioxidant enzyme and growth performance for the supplemented diet show 98.65% of total variance, whereas 77.82% and 20.83% are the contributions of the component 1 and component 2, respectively (Fig. 5). The eigenvalues of one component is higher than 1 (2.594) and the other component is near to 1 (0.759). The Varimax rotation has supported the regression correlation. The SGR, WG, CAT and SOD are clustered tightly in a group with RP supplemented diet along CD, whereas HSI and BD were in same group. This grouping explained the effect of supplementation of RP on the growth performance of Nile tilapia which was discussed previously (Section 3.4).

The post stress experimental results are subjected to Pearson regression correlation and PCA biplot (Fig. 6). The eigenvalues of the two components are 2.583 and 0.805, respectively for component 1 and component 2. The PCA biplot for the post stress condition revealed 84.71% of the total variance and the contribution of the component 1 and component 2 are 64.57% and 20.13%, respectively. In the PCA biplot, both RP supplementations are clustered in a group with CAT and pH 5.5 and pH 10.5, whereas BD and CD are grouped with SOD. RP supplemented diets are also closed together with SGR and WG. The position of the factors and variables in this plot also supported the explanation of the effect of supplementation on antioxidant enzyme and growth of Nile tilapia stated previously (Section 3.5).

4. Conclusion

In summary, polysaccharide (beta glucan) and phenolic compounds present in RP and the panoply of this on antioxidative array in vitro would promulgate the MSW as new era of vista on mushroom waste. The RP supplemented diet fed fish to enhance the activities of antioxidative enzyme in vivo which led fish to mitigate aqua-environmental pH stress and proclaim normal growth. These uncovering, novel uses of MSW, would impart in upcoming global hunger challenge and sustainable mushroom production.

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