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ORIGINAL ARTICLE

Dietary protein requirement of white shrimp, *Penaeus vannamei* (Boone, 1931) juveniles, reared in inland ground water of medium salinity

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Abstract

A 60-day feeding trial was conducted to evaluate the effect of dietary protein level on growth, digestive enzymes and haemato-biochemical responses of *Penaeus vannamei* juveniles in inland ground saline water (IGSW) of 15 ppt salinity. The acclimated shrimp (avge. wt., 4.03 ± 0.05 g) were randomly distributed into seven groups, viz. TCP_{20}, TCP_{25}, TCP_{30}, TCP_{35}, TCP_{40}, TCP_{45} and TCP_{50}, in triplicate with the stocking density of 15 shrimp per tank (275 L). Seven semi-purified hetero-nitrogenous (200–500 g crude protein/kg), iso-caloric (396 Kcal DE/100 g) and iso-lipidic (60 g/kg) diets were prepared for feeding the shrimp of respective group four times daily on satiation basis. Results indicated that the highest ($p < 0.05$) WG and SGR, and the lowest FCR were observed in TCP_{40} group. But PER and ANPU values decreased significantly ($p < 0.05$) with increasing dietary protein. Whole-body protein and ash contents varied significantly ($p < 0.05$) with an inverse relationship. Shrimps of TCP_{40} group had the highest ($p < 0.05$) haemocyanin and serum total protein, while TCP_{20} group showed the highest ($p < 0.05$) serum glucose. Serum cholesterol and triglyceride increased significantly ($p < 0.05$) with increasing dietary protein level up to 400 g protein/kg and beyond that these decreased gradually. Protease activity increased with increasing dietary protein level, but amylase activity showed an inverse trend. Second-order polynomial regression analysis in relation to WG, SGR and FCR indicated that 393.0–397.90 g protein/kg diet could be optimum for culture of *P. vannamei* juveniles in IGSW of 15 ppt salinity.

KEYWORDS
growth, inland ground saline water, nutrient utilization, *Penaeus vannamei*, protein requirement

1 | INTRODUCTION

Estimated global aquaculture production target is 109 MMT by 2030 (FAO, 2018) to meet the food and nutritional security of the ever-growing human population. This huge estimated target can only be achieved by vertical expansion of aquaculture practices, and conversion of unused and under-utilized land and water resources for aquaculture operation with technological interventions. Absolutely unutilized salt-affected land areas are more than 480 million hectares throughout the globe, and it is likely to be increased 50% more by 2050 (Jamil et al., 2011; Partridge et al., 2008). This huge areas and inland ground saline water (IGSW) are neither suitable for agricultural production nor fit for drinking purpose. This can only be utilized for aquaculture purpose to achieve the targeted aquaculture production. But, in contrast to brackish and marine water, low K^+ and inconstant Ca^{2+} and Mg^{2+} concentrations make the IGSW highly imbalanced (Jana et al., 2004; Saoud et al., 2003; Singha et al., 2020; Talukdar et al., 2020a; Antony et al., 2020) and not suitable for commercial culture of aquatic organisms (Aklakur, 2017). However, after fortification of water and dietary supplementation with K^+ through feed, successful culture of shrimp has been reported in IGSW of different salinities (McNevin et al., 2004; Ur-Rahman et al., 2005; Roy et al., 2007; Saoud et al., 2007; Liu et al., 2014; Raizada et al., 2015; Jahan et al., 2018; Talukdar et al., 2020b).

Penaeus vannamei is a versatile euryhaline species of shrimp which exhibits higher growth rate, disease tolerance and survival with better economic return than the other penaeid species (Cuzon et al., 2004; Lim, 1996). Moreover, *P. vannamei* generally requires comparatively lower amount of dietary crude protein and it can easily withstand with a wide range of salinity (1 to 50 ppt) (Pante, 1990; Moss et al., 2007; Lightner et al., 2009; Martínez-Rocha et al., 2013), but 15–30 ppt was found to be ideal for *P. vannamei* in relation to survival and growth (Huang et al., 2004; Li et al., 2007). However, too high or low ambient salinity creates osmoregulatory stress to shrimp with higher energy demand leading to growth retardation and poor survival (Chen et al., 2014; Walker et al., 2009). During last one decade, *P. vannamei* has become a widespread species for commercial aquaculture practices and it alone contributes 53% of the total shrimp production (4.1 mmt) with annual growth rate of 5.8% (FAO, 2018). Since the past decade, the coastal shrimp culture has been shifting to IGSW to take advantage of unutilized land areas, disease-free clean environment and socio-economic development of the region (Davis et al., 2002). In the present Indian scenario, the domestication and culture of shrimp, mainly white shrimp, *P. vannamei*, in IGSW, have become popular among the farmers and is continuously expanding throughout the world as well (Cheng et al., 2006; Roy et al., 2009). The salinity of IGSW in India usually ranges from 3 to 25 ppt depending on the geographical location and climatic conditions (Aklakur, 2017). Owing to its promising economic returns and euryhaline in nature, *P. vannamei* has become a preferable and profitable candidate species for the IGSW aquaculture. Though *P. vannamei* can adapt to the salinity changes through osmoregulatory process involving regulation of cell volume, ion-transport enzymes and amino acid pools (Huong et al., 2010), changes in the salinity of the ambient water significantly influence the survival, growth performance, physiological status and disease resistance of *P. vannamei* (Diaz et al., 2001; Li et al., 2008), because a large portion of the energy is utilized to accomplish high energy demand for physiological adaptation and that eventually affects growth and survival of shrimp (Tseng and Hwang 2008). Therefore, providing optimum dietary nutrients and energy would be a practical approach to improve growth performance and physiological adaptations of *P. vannamei* in low saline water (Fierro-Sañudo et al., 2018; Wang et al., 2014; Xu et al., 2016). From the perspective of cost-effective aquaculture, in-depth knowledge on optimum dietary protein requirement and P:E ratio (protein to energy ratio) is crucial to formulate nutritionally balanced, economically feasible and eco-friendly shrimp feed. According to previous reports, optimum dietary protein (Gao et al., 2016; Li et al., 2011; Perez-Velazquez et al., 2007; Xu et al., 2012), lipid (Chen et al., 2015), carbohydrate (Wang et al., 2015) and astaxanthin (Flores et al., 2007) could improve the growth performance and nutrient utilization of *P. vannamei* cultured in low ambient saline water condition.

Among these, dietary crude protein is the most crucial and costliest nutrient in aqua feed and essential factor affecting the growth of *P. vannamei* (Aranyakananda & Lawrence, 1993). Though the main function of protein is to support growth, it can also supply energy to shrimp. It is well known that like vertebrates, shrimp feeds on energy satiation, and unlike mammals, shrimp can more efficiently utilize dietary protein for energy production (Shahkar et al., 2014). However, provision of high dietary protein with less quantity of non-protein energy sources, that is lipid and carbohydrate, lead to breakdown of more amino acids for energy production with the excretion of more ammonia to pollute the environment leading to growth retardation of shrimp (Talukdar et al., 2020b). Thus, the higher level of crude protein than the optimum requirement not only increases the cost of feed but also causes environmental pollution in terms of nitrogenous wastes without giving extra benefit in relation to growth of shrimp. Therefore, optimization of dietary protein is very much essential to make the shrimp culture operation profitable and sustainable (Li et al., 2017).

Several studies have been reported on dietary protein requirement of *P. vannamei* by different authors at different salinities (Kureshy & Davis, 2002; Huang et al., 2003; Liu et al., 2005; Xia et al., 2010; Wang et al., 2015; Lee & Lee, 2018; Gil-Núñez et al., 2020). The requirement of dietary crude protein of *P. vannamei* for optimum growth in brackish water or seawater condition is reported to be within the range of 300–360 g/kg (Kureshy & Davis, 2002). Huang et al. (2003) observed that requirement of dietary protein of *P. vannamei* is lower (260 g/kg dietary protein) in low saline water (2 ppt salinity) and higher (330 g/kg dietary protein) in high saline water (28 ppt salinity) culture conditions. Wang et al. (2015) observed better growth of *P. vannamei* with the optimum dietary protein of 340 g/kg rearing at 2 ppt saline water, whereas Liu et al. (2005) and Li et al. (2008) found that *P. vannamei* requires higher level of dietary protein (400–440 g/kg dietary protein)

TABLE 1 Formulation and proximate composition of the experimental diets

Composition Ingredients (g/kg)	Diets[a]						
	TCP_{20}	TCP_{25}	TCP_{30}	TCP_{35}	TCP_{40}	TCP_{45}	TCP_{50}
Fishmeal	200	200	200	200	200	200	200
Soya bean meal	100	100	100	100	100	100	100
Casein	16	64	112	160	208	256	306
Gelatin	4	16	28	40	52	64	76.5
Starch	397.5	347.5	297.5	247.5	197.5	147.5	95.0
Dextrin	150	140	130	120	110	100	90
Cellulose	19.5	19.5	19.5	19.5	19.5	19.5	19.5
Fish oil	60	60	60	60	60	60	60
CMC[b]	25	25	25	25	25	25	25
Vit. min. mix[c]	15	15	15	15	15	15	15
Soya lecithin	5	5	5	5	5	5	5
Cholesterol	2	2	2	2	2	2	2
BHT[d]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Betaine	5	5	5	5	5	5	5
Choline chloride	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Stay C 35[e]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Proximate composition (g/kg, on dry matter basis)							
Dry matter	915.4	914.1	916.9	917.1	916.1	914.7	915.2
Crude protein	202.5	251.7	301.1	350.5	400.3	450.2	501.3
Ether extract	83.2	82.7	83.5	82.1	82.2	81.9	82.8
Crude fibre	29.5	28.9	29.9	29.0	29.5	30.3	29.4
Total ash	80.3	80.9	81.5	83.1	81.7	80.8	82.1
Nitrogen-free extract	604.5	555.8	504.0	455.3	406.3	356.8	304.5
GE[f] (Kcal/ 100 g)	433.10	437.14	441.90	446.91	450.95	452.86	459.05
DE (Kcal/100 g)[g]	397.62	397.38	397.12	396.18	396.43	396.45	396.63
P/E (mg CP/Kcal DE)[h]	50.93	63.34	75.82	88.47	100.98	113.56	126.39

[a]TCP_{20} (200 g/kg dietary protein), TCP_{25} (250 g/kg dietary protein), TCP_{30} (300 g/kg dietary protein), TCP_{35} (350 g/kg dietary protein), TCP_{40} (400 g/kg dietary protein), TCP_{45} (450 g/kg dietary protein) and TCP_{50} (500 g/kg dietary protein).

[b]CMC, carboxymethyl cellulose

[c]Composition of vitamin–mineral mix (PRE-EMIX PLUS) (quantity/kg): vitamin A, 5,500,000 IU; vitamin D3, 1,100,000 IU; vitamin B2, 2,000 mg; vitamin E, 750 mg; vitamin K, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 6 mg; calcium pantothenate, 2,500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 l-lysine, 10 g; dl-methionine, 10 g; selenium, 125 mg; and vitamin C,2,500 mg.

[d]BHT, butylated hydroxytoluene.

[e]Stay C 35, protected vitamin C

[f]GE, gross energy.

[g]DE, digestible energy (Kcal/100 g) =4 × CP (g/100 g) +9 × EE (g/100 g) +4 × NFE (g/100 g) (Halver, 1976).

[h]P/E, protein to energy ratio (mg CP/kcal DE) = (CP% ×1000)/DE.

while rearing at 3 ppt salinity. Thus, the dietary protein needs to be optimized in *P. vannamei* according to the salinity level of culture condition (Li et al., 2017). However, so far our knowledge goes, reports on dietary protein requirement of *P. vannamei* reared in IGSW are scarce. With this background, the present study was conducted to optimize the dietary crude protein requirement of white leg shrimp, *P. vannamei* juveniles in IGSW rearing condition of 15 ppt salinity.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The research undertaken complies with the current animal welfare laws in India, and the use of animals in this study followed the guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment &

Forests (Animal Welfare Division), Govt. of India on care and use of animals in scientific research.

2.2 | Procurement and acclimatization of shrimp

One thousand two hundred healthy *P. vannamei* juveniles were procured from the Mahem shrimp farm of Haryana, India, and were safely transported to Shrimp Laboratory of ICAR-Central Institute of Fisheries Education, Rohtak centre, Haryana, India, using airtight plastic bags filled with oxygenated water of 15 ppt salinity. The juvenile shrimps were then transferred to a circular tank (10.05 m^2 × 0.89 m, 770 L capacity) containing IGSW of 15 ppt salinity with round the clock aeration facility and acclimated for 15 days. During acclimatization of the experimental shrimp, a commercial diet containing 380 g CP/kg and 40 g EE/kg fed to satiation level thrice a day. During this period, 30% water was exchanged in an alternate days.

2.3 | Experimental diets formulation and preparation

Seven semi-purified hetero-nitrogenous (200 to 500 g/kg dietary crude protein), iso-caloric (around 396 Kcal DE/100 g) and iso-lipidic (60 g/kg) experimental diets such as TCP_{20} (200 g CP/kg), TCP_{25} (250 g CP/kg), TCP_{30} (300 g CP/kg), TCP_{35} (350 g CP/kg), TCP_{40} (400 g CP/kg), TCP_{45} (450 g CP/kg) and TCP_{50} (500 g CP/kg) were formulated (Table 1). As per the formulation, the finely ground ingredients were weighed accurately and kept properly in the containers. The ingredients (except additives and oils) and required volume of water were homogenously mixed to prepare dough. Then, the dough was steam cooked in a pressure cooker for 20 min followed by cooling at room temperature. Then, oil and additives (BHT, vitamins and minerals mixture, soya lecithin, betaine hydrochloride and stay C) were uniformly mixed with the cooked dough and dough was re-made and pressed through a mechanical pelletizer (Uniextrude-single screw extruder; S. B. Panchal & Co) to get uniform pellet strands (diameter 1.5 mm) which were then subjected to air-drying at room temperature followed by oven drying at 40^0C until achieving around the 10% moisture content. Then, the dried pellets were broken to 4 to 6 mm length, packed into airtight polyethylene zipper bags, sealed and labelled according to dietary groups and stored in 4^0C till further use.

2.4 | Inland ground saline water collection and storage

Inland ground saline water (IGSW) of 15 ppt salinity was pumped out from a bore well and filtered through the filter bags (mesh size 100 μm) to remove any unwanted dirt and debris followed by filling into rectangular cemented tanks (3 × 2 × 1.5 m^3, 9000 L capacity). After ten days, IGSW was transferred to circular tanks (10.05 m^2 × 0.89 m, 935 L capacity) provided with continuous aeration. The 15 ppt salinity level was maintained in storage water which was used for the experiment as and when necessary.

2.5 | Experimental design, set-up and feeding trial

Twenty one circular FRP (fibre glass reinforced plastic) tanks (8.85 m^2 × 53 m, 325 L capacity, 275 L water volume) were washed thoroughly for setting up of the experiment. Then, potassium permanganate solution (4 mg/L) was poured in all the tanks and kept overnight. On the very next day, all the tanks were flushed out and were properly washed with clean water to remove all the traces of potassium permanganate and dried well under bright sunlight. The tanks were then filled with muriate of potash (KCl) fortified IGSW from storage tank and provided with round the clock aeration. The IGSW (15 ppt salinity) in storage tank was routinely fortified with muriate of potash (KCl) and supplied to each experimental tank as and when needed throughout the experimental period. According to Davis et al. (2005), the requirement of K^+ for IGSW fortification = (10.7 × desired salinity) − available K^+ in IGSW. Before commencement of the experiment, diet was withheld overnight and weight of shrimp was carefully recorded. Then, healthy and well-acclimatized three hundred and fifteen juveniles of *P. vannamei* (avge. wt., 4.03 ± 0.05 g) were distributed randomly in triplicates in seven distinct treatment groups following a completely randomized design (CRD) with the stocking density of 15 shrimps per tank. Shrimps of each experimental group were fed to satiation level four times a day (06.00, 11.00, 18.00 and 23.00 h) for 60 days under natural photoperiod conditions provided with continuous aeration. The excreta of shrimp in each tank were syphoned out daily morning with addition of equal volume of syphoned water. Around 30% water of each experimental tank was replaced by IGSW of storage tank at 3-day interval. Every fortnight, all the experimental shrimps were carefully weighed from each tank for assessing the satiation feeding level and the total quantity of diet used for feeding was recorded properly for calculation of dry matter intake. During the entire experimental period, the number of deceased shrimps was recorded from each experimental tank for calculation of percentage survival of shrimp.

2.6 | Physico-chemical parameters of water

At every seven-day interval, all the physico-chemical parameters of water were analysed by following the standard protocol of APHA (1998). Potassium concentration in water was estimated using a flame photometer (Microprocessor flame photometer, Model 1382, ESICO). A blank solution and potassium standards of 25, 50, 100 and 200 mg per litre potassium were prepared. The instrument was set at zero using blank solution. Then, standards followed by samples were measured at 766.5 nm, and using the calibration curve

of standard, the concentration of potassium in water samples was calculated.

2.7 | Sampling protocol

At the beginning and end of the feeding trial, diets were withheld overnight followed by weight measurement of shrimp using an electronic balance (Shimadzu, C054-E032S) and finally average body weight of shrimp was calculated. Initially, twenty-five shrimps, and at the end of the feeding trial, three shrimps from each tank (9 from each group) were used for analysis of initial and final whole-body proximate composition. At the end of trial, six shrimps of inter moult stage were carefully taken out from each tank, anaesthetized in ice-cold chilled water. For serum collection, haemolymph (300–500 µl) was collected by piercing the needle (1.0 ml BD 26 Tuberculin Syringe, DISPO VAN; Hindustan Syringes, India) into abdominal cavity (joining of cephalothorax and abdomen) of the three shrimps and pooled (Antony et al., 2015). Then for clotting, the collected haemolymph was immediately poured into Eppendorf tubes (1.5 ml; Tarsons Products Pvt. Ltd.) and kept in a refrigerator (4^0C, 15 min). To obtain serum, the clot was instantly crushed by a flexible rod and was centrifuged in a cooling centrifuge (Thermo Scientific) at 3000 g for 15 min (Tantulo & Fotedar, 2006). Serum samples were collected in sample vials (Tarsons Products Pvt. Ltd.) and stored at −40^0C until used for the analysis of the total protein, glucose, cholesterol, triglyceride, osmolality and osmoregulatory capacity (OC). For haemocyanin estimation, haemolymph was collected from three shrimps in the same manner and mixed in the Eppendorf tubes which were previously filled with cold shrimp anticoagulant (SIC-EDTA) at 1:2 ratio (Vargas-Albores et al., 1993). Then from these six shrimps of each replicate, hepatopancreas were carefully dissected out and weighed accurately for the calculation of hepatopancreas-somatic index (HPSI). Then, immediately the hepatopancreas was subjected to homogenization with ice-cold phosphate buffer (0.025 M KH_2PO_4, 0.025 M $Na_2PO_4.12H_2O$, pH 7.5) in a glass tube by using a mechanical tissue homogenizer coated with Teflon (D-9 MICCRA; ART Prozess and Labortechnik, Germany). Then, the tissue homogenate was subjected to centrifugation in a cooling centrifuge at 10,000 g for 10 min and the supernatants were collected in sample vials and stored at −40^0C till further use. For estimation of the activity of hepatopancreatic digestive enzymes, a 5% tissue homogenate was prepared (Talukdar et al., 2020b). For estimation of branchial Na^+K^+-ATPase enzyme activity, gills were carefully dissected out and immediately homogenized in ice-cold chilled SEI buffer (250 mmol/L sucrose, 10 mmol/L EDTA, 50 mmol/L imidazole, pH 7.3) in a glass tube by using a mechanical tissue homogenizer coated with teflon and serum samples were used for the analysis of the total protein, haemocyanin, glucose, cholesterol and triglyceride. Then, the gill homogenates were centrifuged in a cooling centrifuge at 4^0C, 5,000 g for 10 min. Then, the supernatant was carefully transferred to sample vials and stored at −40^0C freezer till further use. In the present study, for estimation of activity of branchial Na^+K^+-ATPase enzyme a 10% tissue homogenate was prepared (Saraswathy et al., 2020).

2.8 | Analysis of proximate composition

Proximate composition of experimental diets and whole body of shrimp was analysed according to the standard procedures of AOAC (1995). Moisture content (%) of the samples was determined by drying in a hot air oven at 105^0C till to achieve a constant weight. Some samples were dried in hot air oven at 80^0C until achieving the constant weight and used for estimation of other component of proximate composition on per cent dry matter basis. Crude protein of the experimental diets and shrimp samples was estimated by micro-kjeldahl method (Kelplus; PELICAN Instruments, India). Ether extract (lipid) of diet and crude fat of shrimp samples were estimated by solvent extraction method (SOCSplus, 08, SAS-AS; PELICAN Instruments, India). Crude fibre of the experimental diets was estimated by acid and alkali digestion of samples in Fibretec (Tulin Equipments, India), and total ash of samples was determined by burning the samples in a muffle furnace at 550^0C for 6 h. Nitrogen-free extract of the diets was calculated by subtraction methods as follows:

Nitrogen-free extract (NFE, g/kg) =1000 - (g CP /kg +g EE /kg +g CF /kg +g TA /kg).

An automated bomb calorimeter (5E-AC/PL; Changsha Kaiyuan Instruments Corporation Pvt. Ltd., China) was used for measuring the gross energy content of the experimental diets according to standard protocol provided by the manufacturer. Gross energy content of the experimental diets was expressed in Kcal/100 g.

Finally, the whole-body proximate composition of the experimental shrimp was represented on %wet weight basis.

2.9 | Growth performance, protein utilization, survival rate and body indices

The following formulas were used for the calculation of the growth performance, nutrient utilization and body indices parameters:

$$\text{Weight gain (WG, g)} = \text{Final wet weight (g)} - \text{Initial wet weight (g)}$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{\text{lnof final wet weght} - \text{lnof initial wet weight}}{\text{Duration of feeding trial in days}} \text{x100}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed intake (dry weight in g)}}{\text{Wet weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Wet weight gain (g)}}{\text{Protein intake (dry weight in g)}}$$

$$\text{Apparent net protein utilization (ANPU, \%)} = \frac{\text{Final body protein on wet weight basis} - \text{Initial body protein on wet weight basis}}{\text{Protein intake(dry weight in g)}} \text{x100}$$

$$\text{Survival}\,(\%) = \frac{\text{Total number of shrimp harvested at the end of feeing trial}}{\text{Total number of shrimps stocked at the commencement of feeding trial}} \text{x100}$$

$$\text{Hepatopancreas somatic index (HPSI, \%)} = \frac{\text{Wet weight of the hepatopancreas in g}}{\text{Wet weight of the shrimp in g}} \text{x100}$$

2.10 | Haemato-biochemical parameters

For estimation of haemocyanin (Hc) content of shrimp, instantly 10 µl haemolymph was well mixed with 990 µl de-ionized water in a 1 cm quartz cuvette and reading was taken at 335 nm using a double-beam spectrophotometer (Shimadzu 1800; Shimadzu Corporation, Pvt. Ltd., China). For calculation of the final concentration of haemocyanin (Hc), an extinction coefficient of E = 17.26 was used based on the 74 KDa functional subunit of shrimp (Chen & Cheng, 1993a; Chen & Cheng, 1993b).

An automated cryoscopic osmometer (Osmomat® 030; GEnotech GmbH Corporation Pvt. Ltd., Berlin, Germany) was used for determination of osmolality in serum of shrimp and experimental IGSW. For calculation of osmoregulatory capacity (OC), water osmolality was subtracted from the estimated serum osmolality of the shrimp in different dietary groups (Lignot et al., 2000). Osmoregulatory capacity (OC) of shrimp *P. vannamei* is expressed as mOsmol/kg.

Osmoregulatory capacity (OC) = The mean serum osmolality of the shrimp − The mean osmolality of the rearing media (water).

Commercial assay kits namely total protein kit (BLT00054), glucose kit (BLT12235), total cholesterol kit (BLT00034) and triglyceride kit (XSYS0041), respectively, were procured from Erba® Mannheim, Transasia Diagnostic Bio-Medicals Pvt. Ltd., Himachal Pradesh, India, and performed as per the manufacturer's instruction provided.

2.11 | Enzyme assays

2.11.1 | Estimation of total protein in the tissue homogenates

Estimation of total of different tissue samples was done by Bradford method (Bradford, 1976). 20 µl samples were mixed well with 180 µl of distilled water followed by addition of 250 µl 1 N NaOH and 5 ml of Bradford reagent. After five minutes, blank and sample absorbance was measured at 595 nm using a double-beam spectrophotometer (Shimadzu 1800, Shimadzu Corporation Pvt. Ltd., China). Protein content of different tissue samples was represented in mg/g of wet tissue and subsequently used for the estimation of enzyme activities.

2.11.2 | Branchial Na^+K^+-ATPase enzyme activity

Branchial Na^+K^+-ATPase activity was estimated as described by McCormick (1993). Two assay solutions were prepared. Solution A containing 5U pyruvate kinase (PK)/ml, 4U lactate dehydrogenase (LDH)/ml, 50 mmol imidazole, 0.7 mmol ATP, 0.22 mmol NADH and 2.8 mmol phosphoenolpyruvate (PEP), pH 7.5, was prepared and placed in a refrigerator (4^0C) for 3–4 days. Subsequently, solution B was prepared (composition of solution A along with 0.5 mmol ouabain). Then, a salt solution was prepared by mixing 10.5 mmol $MgCl_2$, 189 mmol NaCl, 50 mmol imidazole and 42 mmol KCL (pH 7.5). Then, separately two assay solutions (solutions A and B) and the salt solutions were mixed in 3:1 ratio and kept over ice. Just prior to analysis, the assay mixture was placed in a water bath (25^0C). The slope of the standard curve should be −0.019 to −0.020 OD unit mmol/ADP. Then in a quartz cuvette (1 cm path length), 50 µl sample was mixed uniformly with 1 ml of solution A and salt mixture and absorbance was measured at 240 nm for 2–10 min in a double-beam spectrophotometer (Shimadzu 1800; Shimadzu Corporation Pvt. Ltd., China) against blank. For calculation of branchial Na^+K^+-ATPase enzyme activities, the differences in ATP breakdown in the presence and absence of ouabain was measured and unit expressed in micromoles of ADP released per hour per milligram of protein at 37^0C.

2.11.3 | Hepatopancreatic digestive enzyme activities

The hepatopancreatic protease activity was estimated as described by Drapeau (1974) and unit represented in millimole of tyrosine released/min/mg protein. The activity of the hepatopancreatic amylases was determined as described by Rick & Stegbauer (1974). Carbohydrates in presence of di-nitro salicylic acid (DNS) react with gluco-amylase and α-amylase to form reducing sugars. Activity of the hepatopancreatic amylases was expressed in micromole of maltose release/min/mg protein. The hepatopancreatic lipase activity was estimated as described by Cherry & Crandall (1932). In glass tubes, phosphate-buffered solution (pH 7.0), distilled water, olive oil emulsion and sample were homogenously mixed and placed in an incubator (37^0C). After 24 h, titration was done with 0.05 N NaOH to get an end point of pink colour. The activity of lipase specific enzyme was measured as the milliequivalent volume of alkali used up in the titration reaction and expressed as units/min/mg protein.

2.12 | Statistical analysis

The data were statistically analysed by using Statistical Package SPSS version 22.0. for Windows. One-way ANOVA and Duncan's multiple range tests (DMRT) were used to determine the significant difference ($p < 0.05$) among the means of different experimental groups in the experiment, and the significant difference was observed at 5% probability level ($p < 0.05$). All the data were represented as mean±SE. Second-order polynomial regression analysis was performed in relation to weight gain, SGR and FCR to estimate the optimum requirement of dietary crude protein for juvenile white leg shrimp, *P. vannamei*, in IGSW rearing condition of 15 ppt ambient salinity.

TABLE 2 Water quality parameters of different experimental groups during experimental period of 60 days

Parameters	Diets							*p*-value
	TCP_{20}	TCP_{25}	TCP_{30}	TCP_{35}	TCP_{40}	TCP_{45}	TCP_{50}	
Temperature (°C)	31.80 ± 1.29	31.52 ± 1.48	31.76 ± 1.74	31.25 ± 0.94	31.58 ± 0.98	31.64 ± 1.34	31.92 ± 1.37	0.863
pH	8.35 ± 0.37	8.30 ± 0.31	8.45 ± 0.28	8.28 ± 0.29	8.42 ± 0.33	8.32 ± 0.20	8.37 ± 0.24	0.576
Salinity (g/L)	15.25 ± 0.08	15.05 ± 0.17	15.21 ± 0.14	15.11 ± 0.12	15.29 ± 0.34	15.15 ± 0.21	15.26 ± 0.26	0.672
DO (mg/L)	6.48 ± 0.52	6.72 ± 0.37	6.25 ± 0.67	6.38 ± 0.74	6.45 ± 0.29	6.57 ± 0.33	6.64 ± 0.28	0.892
Free CO_2 (mg/L)	Nil	Nil	Nil	Nil	Nil	Nil	Nil	-
Alkalinity (mg/L)	292.37 ± 5.28	294.72 ± 3.45	293.98 ± 3.76	292.67 ± 4.54	295.89 ± 3.96	298.32 ± 2.22	294.86 ± 4.29	0.980
Hardness (mg/L)	2893.50 ± 10.55	2896.00 ± 8.98	2902.50 ± 13.56	2898.00 ± 5.68	2892.00 ± 7.50	2896.50 ± 12.34	2891.50 ± 13.92	0.820
TA-N (mg/L)	Nil	Nil	0.09^{a}±0.02	0.21^{b}±0.03	0.36^{c}±0.06	0.53^{d}±0.09	0.75^{e}±0.10	0.003
NO_2-N (mg/L)	Nil	Nil	0.11 ± 0.04	0.16 ± 0.05	0.18 ± 0.03	0.20 ± 0.04	0.24 ± 0.06	0.762
NO_3-N (mg/L)	Nil	Nil	0.32 ± 0.07	0.35 ± 0.05	0.37 ± 0.04	0.38 ± 0.05	0.41 ± 0.11	0.640
Ca^{2+} (mg/L)	238.78 ± 2.43	239.31 ± 1.42	241.56 ± 3.10	239.97 ± 2.67	238.13 ± 1.46	239.05 ± 1.87	238.89 ± 2.46	0.568
Mg^{2+} (mg/L)	525.54 ± 2.46	526.87 ± 2.17	527.85 ± 2.69	527.28 ± 2.78	524.58 ± 1.72	527.02 ± 2.31	524.37 ± 1.78	0.746
K^{+} (mg/L)	100.79 ± 1.02	100.12 ± 0.82	100.56 ± 1.25	100.22 ± 1.33	100.76 ± 1.28	100.43 ± 1.37	100.34 ± 0.85	0.782
Osmolality (mOsmol/kg)	244.33 ± 1.20	246.00 ± 1.78	243.66 ± 1.70	247.33 ± 3.88	246.00 ± 2.89	244.66 ± 2.00	247.00 ± 3.05	0.590

Data are presented as mean ±SE (n = 3); values with different superscripts in the same row differ significantly ($p < 0.05$).

Abbreviations: Ca^{2+}, calcium ion; CO_2, carbon di-oxide; DO, dissolved oxygen; K^{+}, potassium ion; Mg^{2+}, magnesium ion; NO_2-N, nitrite nitrogen; NO_3-N, nitrate nitrogen; TA-N, total ammonia nitrogen.

3 | RESULTS

3.1 | Physico-chemical parameters of water

Estimated average physico-chemical parameters of IGSW such as temperature, pH, salinity, DO, alkalinity and hardness ranged between 31.25 ± 0.94 and 31.92 ± 1.37^{0}C, 8.28 ± 0.29 and 8.45 ± 0.28, 15.05 ± 0.17 and 15.29 ± 0.34 g/L, 6.25 ± 0.67 and 6.72 ± 0.37 mg/L, 292.37 ± 5.28 and 298.32 ± 2.22 mg/L, 2891.50 ± 13.92 and 2902.50 ± 13.56 mg/L respectively. Free CO_2 was not detected throughout the experimental period. The concentrations of Ca^{2+}, Mg^{2+} and K^+ ions ranged from 238.13 ± 1.46 to 241.56 ± 3.10 mg/L, 524.37 ± 1.78 to 527.85 ± 2.69 mg/L and 100.12 ± 0.82 to 100.79 ± 1.02 mg/L respectively. The concentration of nitrogenous compounds such as TA-N, NO_3-N and NO_2-N ranged from nil to 0.75 ± 0.10 mg/L, nil to 0.24 ± 0.06 mg/L and nil to 0.41 ± 0.11 mg/L respectively. Water osmolality of the experimental IGSW ranged between 243.66 ± 1.70 and 247.33 ± 3.88 mOsmol/kg (Table 2).

3.2 | Growth performance, protein utilization, survivability and body indices

Growth performance, nutrient and protein utilization, survivability and body indices like HPSI of *P. vannamei* juveniles significantly ($p < 0.05$) influenced due to feeding of graded level of crude protein in the experimental diets (Table 3). Highest WG and SGR ($p < 0.05$) were observed in group fed with 400 g/kg (TCP_{40}) dietary crude protein level followed by TCP_{35} and TCP_{45}, while the lowest were recorded in 200 g/kg protein-fed group (TCP_{20}). The lowest and the highest FCR values were also observed in TCP_{20} and TCP_{40} groups, respectively, whereas PER and ANPU decreased ($p < 0.05$) significantly among the experimental groups in relation to increase in protein level in the diets. However, survival rate of the experimental animals did not vary significantly ($p > 0.05$) among the groups. According to second-order polynomial regression analysis based on WG, SGR and FCR, the optimum dietary protein requirement of *P. vannamei* juveniles was found to be 390.30 (Figure 1), 397.90 (Figure 2) and 392.60 g/kg diet (Figure 3) respectively.

3.3 | Whole-body proximate composition

Whole-body proximate composition analysis revealed that moisture and ash content decreased significantly ($p > 0.05$) due to feeding of graded level of dietary protein. Lowest whole-body moisture content was observed in group fed with 500 g CP/kg diet (TCP_{50}), but whole-body moisture content did not varied significantly ($p > 0.05$) among the groups fed with 400, 450 and 500 g crude protein/kg diet (Table 4). Whole-body total ash content also showed similar fashion in relation to increase in dietary protein level, but it was found non-significant ($p > 0.05$) among TCP_{30}, TCP_{35} and TCP_{40} groups. Whole-body protein and lipid content showed a significantly ($p < 0.05$) increasing trend with the increase in the level of dietary protein. However, the highest ($p < 0.05$) body protein content was found in TCP_{50} group (500 g protein /kg) which did not show significant variation ($p > 0.05$) with TCP_{45} group (450 g protein /kg), while TCP_{40} group exhibited significantly ($p < 0.05$) the lowest whole-body lipid content.

3.4 | Serum osmolality, osmoregulatory capacity and branchial Na^+K^+-ATPase activity

Serum osmolality, osmoregulatory capacity and branchial Na^+K^+-ATPase activity in the experimental animal did not differ significantly ($p > 0.05$) among the treatment groups (Table 5).

TABLE 3 Growth, nutrient utilization, survivability and hepatopancreas-somatic index (HPSI) of *Penaeus vannamei* juveniles fed diets with graded level of dietary protein for the experimental period of 60 days

	Parameters						
Diets	WG (g)	SGR	FCR	PER	ANPU	Survival rate (%)	HPSI (%)
TCP_{20}	6.38^a±0.12	1.58^a±0.02	2.02^e±0.02	2.46 ^{g} ± 0.03	40.15^f±0.48	88.63 ± 1.26	1.57 ± 0.04
TCP_{25}	10.13^b±0.12	2.09^b±0.01	1.71^d±0.01	2.32^f±0.02	38.60^e±0.35	89.66 ± 2.08	1.54 ± 0.08
TCP_{30}	12.18^c±0.11	2.33^c±0.02	1.51^c±0.03	2.20^e±0.01	36.27^d±0.27	88.54 ± 2.58	1.47 ± 0.04
TCP_{35}	14.97^d±0.15	2.58^d±0.02	1.38^b±0.01	2.07^d±0.02	33.79^c±0.29	90.11 ± 1.47	1.51 ± 0.05
TCP_{40}	18.18^e±0.17	2.83^e±0.03	1.25^a±0.01	1.99^c±0.02	30.28^{bc}±0.22	90.55 ± 1.36	1.48 ± 0.07
TCP_{45}	14.65^d±0.07	2.56^d±0.01	1.40^b±0.02	1.58^b±0.01	28.30^b±0.34	89.16 ± 3.05	1.55 ± 0.08
TCP_{50}	12.08^c±0.21	2.30^c±0.03	1.55^c±0.01	1.29^a±0.01	24.58^a±0.19	88.14 ± 2.38	1.47 ± 0.04
p-value	0.001	0.001	0.001	0.001	0.002	0.780	0.840

Data are presented as mean ±SE ($n = 3$); values with different superscripts in the same column differ significantly ($p < 0.05$).

Abbreviations: ANPU, apparent net protein utilization; FCR, feed conversion ratio; HPSI, hepatopancreas-somatic index; PER, protein efficiency ratio; SGR, specific growth rate; WG, weight gain.

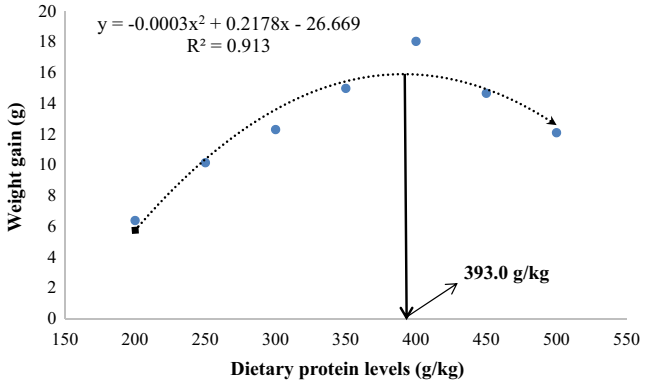

FIGURE 1 Second-order polynomial regression analysis between weight gain (g) and dietary protein levels (g/kg) for *Penaeus vannamei* juveniles reared in inland ground saline water for the experimental period of 60 days. [Colour figure can be viewed at wileyonlinelibrary.com]

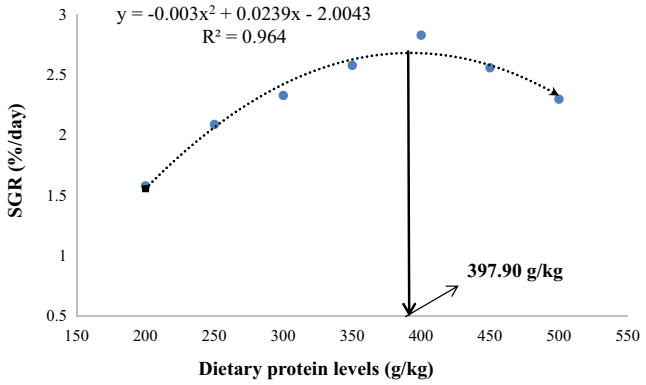

FIGURE 2 Second-order polynomial regression analysis between SGR (%/day) and dietary protein levels (g/kg) for *Penaeus vannamei* juveniles reared in inland ground saline water for the experimental period of 60 days. [Colour figure can be viewed at wileyonlinelibrary.com]

3.5 | Serum total protein, haemocyanin (Hc), glucose, cholesterol and triglyceride levels

The total protein, haemocyanin, glucose, cholesterol and triglyceride levels in the serum differ significantly ($p < 0.05$) among the groups fed with varying level of dietary protein in the experimental diet (Table 6). Serum total protein and haemocyanin level increased significantly ($p < 0.05$) with an increase in crude protein level in the diet up to 400 g/kg (TCP_{40}) and beyond that these parameters gradually decreases ($p < 0.05$). On the other hand, feeding graded dietary protein level to shrimp significantly decreases ($p < 0.05$) serum glucose concentration up to 500 g/kg dietary protein (TCP_{50})-fed group. But serum cholesterol and triglyceride levels were exhibited with a significantly ($p < 0.05$) inverse relationship in contrast to glucose level in the serum of shrimp.

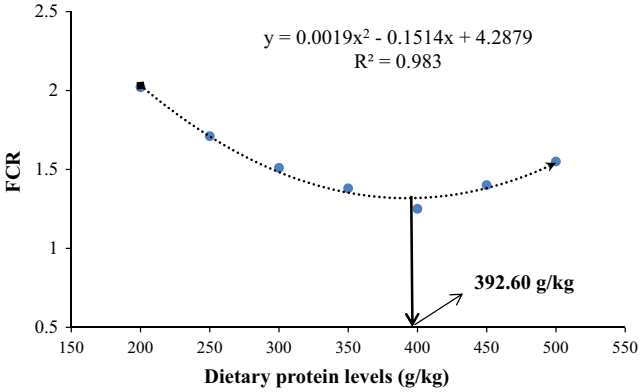

FIGURE 3 Second-order polynomial regression analysis between FCR and dietary protein levels (g/kg) for *Penaeus vannamei* juveniles reared in inland ground saline water for the experimental period of 60 days. [Colour figure can be viewed at wileyonlinelibrary.com]

3.6 | Digestive enzyme activities

Hepatopancreatic protease and amylase activities significantly affected ($p < 0.05$) by feeding graded level dietary crude protein in the experimental diet (Table 7). However, activity of hepatopancreatic lipase did not vary significantly ($p > 0.05$) among the experimental groups. The activities of hepatopancreatic proteases and amylases differ significantly ($p < 0.05$) among the dietary groups in an increased and decreased fashion, respectively, by feeding graded level of dietary protein up to 350 g/kg (TCP_{35}); however, above 350 g CP/kg in diet could not cause the further significant change in these enzyme activities.

4 | DISCUSSION

In our study, all the physico-chemical parameters of water quality except total ammonia nitrogen (TA-N) in all the experimental groups were within the acceptable limits as recommended by Van Wyk & Scarpa (1999) and Talukdar et al. (2020b) for optimum growth and health of *P. vannamei* juveniles. The TA-N concentration was nil in the experimental tanks of groups fed with 200 g/kg (TCP_{20}) and 250 g/kg dietary protein (TCP_{25}) level, but beyond that it increases significantly ($p < 0.05$) by increasing the dietary crude protein level in the experimental diet. Excess amount of ammonia nitrogen in the ambient water is toxic to aquatic organisms (Burford & Lorenzen, 2004). Excess level of dietary protein increases discharge of nitrogenous wastes in terms of ammonia in the water (Boonyaratpalin, 1996; Carbajal-Hernández et al., 2012; Mishra et al., 2008, Carbajal-Hernández et al., 2013) causing stress to the aquatic organisms resulting in poor growth, increase susceptibility to diseases and eventually mortality of the cultured animal (Kathyayani et al., 2019). Generally, shrimps are highly sensitive to variations in TA-N (total ammonia nitrogen), especially the unionized ammonia. The toxicity of ammonia is highly dependent on the pH of the water; as the

TABLE 4 Whole-body proximate composition (on % wet weight basis) of *Penaeus vannamei* juveniles fed diets with graded level of dietary protein for the experimental period of 60 days

Diets	Proximate composition			
	Moisture	CP	EE	TA
Initial	76.11^{d}±0.18	18.69^{c}±0.06	1.13^{c}±0.05	2.87^{e}±0.03
TCP_{20}	77.62^{f}±0.21	17.30^{a}±0.04	1.41^{f}±0.04	2.99 ^{g} ± 0.04
TCP_{25}	77.37^{e}±0.19	17.55^{b}±0.06	1.39^{f}±0.03	2.96 ^{fg}±0.005
TCP_{30}	76.14^{d}±0.23	18.68^{c}±0.09	1.15^{c}±0.03	2.86^{e}±0.03
TCP_{35}	76.68^{c}±0.17	18.46^{d}±0.05	1.09^{b}±0.02	2.70 ^{cd}±0.07
TCP_{40}	75.38^{b}±0.19	19.55^{e}±0.04	1.02^{a}±0.03	2.75^{c}±0.06
TCP_{45}	75.44^{ab}±0.12	19.59^{f}±0.12	1.27^{d}±0.05	2.61^{b}±0.03
TCP_{50}	75.49^{a}±0.15	19.57^{f}±0.09	1.30^{de}±0.06	2.47^{a}±0.05
p-value	0.002	0.003	0.001	0.001

Data are presented as mean ±SE (n = 3); values with different superscripts in the same column differ significantly ($p < 0.05$).

Abbreivations: CP, crude protein; EE, ether extract or crude fat; TA, total ash.

TABLE 5 Serum osmolality, osmoregulatory capacity (OC) and branchial Na^+K^+-ATPase activity of *Penaeus vannamei* juveniles fed diets with graded level of dietary protein for the experimental period of 60 days

Diets	Parameters		
	Serum osmolality[a]	Osmoregulatory capacity[b]	Na^+K^+-ATPase activity[c]
TCP_{20}	645.66 ± 2.53	400.66 ± 2.21	11.78 ± 0.79
TCP_{25}	650.33 ± 2.12	404.33 ± 2.32	12.35 ± 0.87
TCP_{30}	648.66 ± 3.98	404.66 ± 1.86	12.48 ± 0.35
TCP_{35}	653.66 ± 6.35	402.33 ± 1.44	12.76 ± 0.32
TCP_{40}	649.33 ± 6.45	403.33 ± 1.52	13.25 ± 0.68
TCP_{45}	644.33 ± 4.34	398.66 ± 3.12	12.69 ± 0.56
TCP_{50}	651.00 ± 3.89	403.66 ± 2.88	11.97 ± 0.37
p-value	0.780	0.860	0.720

Data are presented as Mean ±SE (n = 3).

[a, b]Serum osmolality and osmoregulatory capacity are expressed in mOsmol/kg.

[c]Branchial Na^+K^+-ATPase activity is expressed in micromoles of ADP released/hour/mg protein at 37°C.

pH increases, the proportion of unionized ammonia in the TA-N will also increase (Thurston et al., 1981). The ammonia toxicity depends upon other factors such as size, temperature, species, dissolved oxygen level, salinity concentration and duration of exposure also (Kathyayani et al., 2019; Magallón Barajas et al., 2006; Straus et al., 1991). However, nitrate and nitrite are less toxic than TA-N to shrimp. However, Van Rijn et al. (2006) reported that a minimum of 100 mg/L concentration of nitrate is lethal to shrimps.

Researchers have conducted several studies to determine the dietary optimum protein requirement of *P. vannamei* which varies under different conditions like species, body weight, stocking density, culture system, temperature, salinity, non-protein energy and biological value of different protein sources (Bautista, 1986; Brito et al., 2001; Hajra et al., 1988; Huang et al., 2003; Rosas et al., 2001; Smith et al., 1985). Kureshy & Davis (2002) reported that more than 320 g/kg protein in the diet gives higher growth performance of *P. vannamei* but better feed efficiency achieved with feeding of 480 g/kg crude protein in the diet. In our study, WG and SGR increased significantly ($p < 0.05$) due to feeding of graded dietary protein level up to 400 g/kg (TCP_{40}), beyond that these values significantly decreased (Hu et al., 2008). Results of our study showed that FCR decreased significantly ($p < 0.05$) with increasing level of crude protein in the experimental diet up to 400 g/kg (TCP_{40}) but further rise in the level of dietary protein caused significantly increased FCR value. This result clearly indicates that feeding with 400 g/kg crude protein could be efficiently utilized by the white leg shrimp for maximum growth with the best FCR, which is also corroborated by other researchers (Huang et al., 2003; Xia et al., 2010). PER and ANPU exhibited with a significant ($p < 0.05$) decreasing trend in relation to level of crude protein in the experimental diet. This finding of our study suggests that shrimp can efficiently utilize dietary protein low level for maintenance; however, low dietary protein probably could be insufficient to support growth. On the other hand, amino acids derived from surplus protein beyond optimum instead of synthesis and deposition as body protein lead to preferential catabolism for energy supply probably through gluconeogenesis (Rosas et al., 2001) concomitant with excess excretion of nitrogenous wastes leading to environmental pollution (Shiau & Peng, 1992). Thus, maximum growth only could be observed at optimum level of dietary protein with optimum P: E value. Our observations corroborated with the findings of Huang et al. (2003), Liu et al. (2005), and Hu et al. (2008). Second-order polynomial regression analysis of WG and dietary protein level suggested that optimum requirement of dietary protein will be 393 g/kg for *P. vannamei* juveniles under IGSW rearing condition of 15 ppt ambient salinity. In support of our findings, Liu et al. (2005) reported that 400 g/kg dietary protein could be optimum and attributed to maximum growth and protein utilization in white leg shrimp, *P. vannamei*. The dietary requirement of crude protein of *P. vannamei* under IGSW

TABLE 6 Haemolymph haemocyanin and serum biochemistry of *Penaeus vannamei* juveniles fed diets with graded level of dietary protein for the experimental period of 60 days

	Parameters				
Diets	Haemocyanin (Hc) (mmol/L)	Total protein (g/dl)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
TCP_{20}	1.46^{a}±0.03	17.04^{a}±0.53	99.56^{f}±2.07	129.93^{a}±2.54	136.74^{a}±3.35
TCP_{25}	1.55^{b}±0.02	18.92^{b}±0.32	94.45^{e}±1.12	138.34^{b}±1.65	153.99^{b}±2.87
TCP_{30}	1.62^{bc}±0.03	21.96^{c}±0.35	83.87^{d}±2.68	152.67^{c}±3.06	178.76^{c}±3.43
TCP_{35}	1.76^{d}±0.04	25.65^{e}±0.43	77.26^{cd}±1.95	165.97^{d}±1.86	207.42^{d}±1.52
TCP_{40}	1.89^{e}±0.03	26.64^{f}±0.16	75.54^{c}±2.56	197.34^{e}±1.45	240.21^{e}±2.65
TCP_{45}	1.65^{bc}±0.02	24.76^{d}±0.25	64.98^{b}±2.99	223.28^{f}±0.98	288.78^{f}±2.92
TCP_{50}	1.56^{b}±0.04	22.06^{c}±0.27	56.93^{a}±2.78	245.94^{g} ± 2.65	317.37^{g} ± 3.08
p-value	0.00	0.001	0.001	0.001	0.001

Data are presented as mean ±SE (n = 3); values with different superscripts in the same column differ significantly ($p < 0.05$).

TABLE 7 Digestive enzyme activities in the hepatopancreas of *Penaeus vannamei* juveniles fed diets with graded level of dietary protein for the experimental period of 60 days

	Parameters		
Diets	Protease[1]	Amylase[2]	Lipase[3]
TCP_{20}	54.08^{a}±0.45	1.86^{f}±0.03	6.12 ± 0.56
TCP_{25}	60.42^{b}±0.84	1.74^{e}±0.05	6.22 ± 0.68
TCP_{30}	67.74^{c}±0.73	1.56^{d}±0.08	6.35 ± 0.47
TCP_{35}	71.83^{d}±0.67	1.39^{c}±0.13	6.72 ± 0.79
TCP_{40}	76.67^{e}±0.98	1.15^{ab}±0.09	7.21 ± 0.87
TCP_{45}	77.04^{e}±1.54	0.98^{ab}±0.12	7.67 ± 0.92
TCP_{50}	77.19^{e}±1.78	0.87^{a}±0.15	7.88 ± 1.34
p-value	0.001	0.001	0.790

Data are presented as mean ±SE (n = 3); values with different superscripts in the same column differ significantly ($p < 0.05$).

[1]Protease activity is expressed in millimole of tyrosine released/min/mg protein.

[2]Amylase activity is expressed in micromole maltose released/min/mg protein.

[3]Lipase activity is expressed in units/min/mg protein.

rearing condition could be higher because dietary protein derived amino acids could be utilized for osmoregulation and energy supply for improving the physiological adaptation and growth of *P. vannamei* (Li et al., 2011). Castille & Lawrence (1981) reported that any deviation from the isosmotic point (20–24.7 ppt), nutrients including protein satisfy the high energy demand for osmoregulation of aquatic organisms. Therefore, 393 g/kg dietary protein level with corresponding P:E of 99.21 mg protein/kcal DE could be optimum for growth and to satisfy the energy demand of *P. vannamei* juveniles reared in IGSW. In corroboration of our findings, Yun et al. (2016) also reported that diet containing 400 g/kg crude protein level with corresponding P:E ratio of 2.45 (g protein/KJ DE) could be optimum for *P. vannamei* juveniles. However, Gauquelin et al. (2007) reported that *Litopeneaus stylorostris* (Stimpson, 1874) juveniles could grow well by feeding diet with 250–580 g CP/kg with corresponding P:E ratio of 2–3 (g protein/KJ DE). In our present study, survival percentage of shrimp varied between 88.14 and 90.55% which was found similar ($p > 0.05$) among the dietary groups suggesting that dietary CP level have no influence on survival of the shrimps (Wang et al., 2015).

Result of whole-body proximate of shrimp revealed that whole-body protein content increased significantly ($p < 0.05$) whereas whole-body moisture and ash contents significantly decreases with an increase in the dietary crude protein level; however, whole-body crude fat content decreased significantly ($p < 0.05$) feeding with graded level of dietary protein up to 400 g/kg (TCP_{40}), beyond that it significantly increases. This finding of our study suggested that diet with 400 g/kg crude protein with corresponding P:E ratio of 100.98 mg protein/kcal DE could meet the protein and energy requirement of shrimp with more protein retention at the given salinity of 15 ppt. Because white shrimp needs more energy for osmoregulation at high salinity, thus, optimum dietary protein with sufficient carbohydrate and lipid could possibly supply this energy to maximize growth of shrimp due to protein sparing action of non-protein energy-producing nutrients (Li et al., 2011). In accordance with our finding, Shahkar et al. (2014) found similar trend of body fat in *P. vannamei* juveniles and Goda (2008) in freshwater prawn, *Macrobrachium rosenbergii* post-larvae respectively. However, in contrast to our observation, Lee & Lee (2018), Gao et al. (2016), Wang et al. (2015) and Hu et al. (2008) also concluded that feeding varying levels of dietary protein to shrimp could not produce significant effect on the whole-body crude fat and ash content of *P. vannamei* juveniles.

The *P. vannamei* is popularly known for its high salinity tolerance ranges from 1 to 50 ppt as compared to other penaeid species (Pante, 1990; Moss et al., 2007; Lightner et al., 2009). However, culturing of shrimp at higher and lower than optimum salinity induces physiological adaptations at the cost of growth where considerable quantity of dietary protein takes part in energy production for maintaining osmoregulation, making the culture operation expensive. In

our study, serum osmolality and osmoregulatory capacity of *P. vannamei* juveniles did not vary significantly ($p > 0.05$) among the dietary groups probably due to hyperosmotic regulation as serum osmolality is more a function of medium salinity lower than the iso-osmotic values (Castille & Lawrence, 1981; Dall & Smith, 1981). Roy et al. (2007) also reported that dietary supplementation and feeding higher protein level did not affect serum osmolality because it depends only on the salinity of the ambient water. Therefore, feeding low dietary levels could only satisfy the energy need for osmoregulation resulting in poor growth of shrimp because shrimp first needs to ensure its physiological adaptation to the higher or lower salinity than optimum.

In aquatic animals including crustaceans, the process of osmoregulation and ionic regulation generally under the control of branchial Na^+K^+-ATPase activity (Perry & Fryer, 1997) and concentration of K^+ ion triggers the activity of branchial Na^+K^+-ATPase (Mantel & Farmer, 1983). In our study, the activity of branchial Na^+K^+-ATPase of shrimp found similar ($p > 0.05$) among the dietary groups fed with varying level of dietary protein probably due to fortification of water with potassium. This observation was in agreement with the result of Hurtado et al. (2007) who reported that the activity and regulation of branchial Na^+K^+-ATPase was not influenced by protein level of the diet or salinity of the rearing water.

Depending on the physiological status of shrimp, amino acids from haemolymph and haemocyanin amino acid pool could be used for growth, energy production, immune function and osmoregulation during salinity changes (Adachi et al., 2003; Chen & Cheng, 1995). However, 60–97% of the protein in haemolymph is haemocyanin; thus, free amino acid in haemolymph is less, which indicates that shrimp can efficiently store synthesized protein as haemocyanin after salinity acclimatization (Pascual et al., 2003). Haemolymph haemocyanin content and serum total protein concentration increased significantly ($p < 0.05$) with an increase in crude protein level in the experimental diet up to 400 g/kg (TCP_{40}), beyond that it significantly ($p < 0.05$) decreased. Depending on the physiological needs, dietary protein derived amino acids either can be used as osmotic effectors or as a precursor for metabolic satiation energy (Dall & Smith, 1986). Amino acids derived from lower dietary protein probably could be utilized for osmoregulation at the cost of growth, serum total protein level and haemolymph haemocyanin concentration, whereas amino acids from optimum dietary protein could maintain both the osmotic pressure and maximum growth through enhancing synthesis and accretion of body protein (Cuzon et al., 2004; Talukdar et al., 2020b). Probably due to the same reason, 400 g CP/kg (TCP_{40}) diet is attributed to maximum growth with increased haemolymph haemocyanin concentration and serum total protein level of shrimp. However, amino acids derived from preferential catabolism of excess dietary protein can be utilized to meet up the energy satiation to withstand the osmotic stress of shrimp rather than directed towards the formation and accretion of body tissue protein for growth. Several authors (Shahkar et al., 2014; Rosas et al., 2001) also found similar observation in *P. vannamei* juveniles which supports finding our study.

The higher serum glucose indicates stress condition of shrimp with increased energy demand (Shan et al., 2019). Glucose concentration in the serum of shrimp decreased significantly ($p < 0.05$) with increasing dietary CP level might be due to the decreasing level of dietary carbohydrate in the feed. Moreover, shrimp could less efficiently mobilize serum glucose for glycolysis or glycogenesis (Rosas et al., 2000). Accordingly, lower serum glucose concentration in higher protein-fed groups might also be due to the effective metabolic utilization of glucose to satisfy the energy need of shrimp. Our finding accorded the report of Flores et al. (2007) and Wang et al. (2015) in *P. vannamei*. Rosas et al. (2000) reported that white shrimp could have limited capacity to store carbohydrates as glycogenesis could be saturated at 230 g/kg dietary carbohydrate level. In contrast to our findings, Shahkar et al. (2014) concluded that increasing the dietary crude protein level from 250–400 g/kg significantly increases the glucose concentration in the serum of shrimp *P. vannamei* attributed to preferential catabolism of dietary protein than the glucose. Serum cholesterol and triglyceride concentrations are the important biological indicators for growth in relation to dietary formulations (Adhikari et al., 2004; Maheswaran et al., 2008). In our study, serum total cholesterol and triglyceride level showed a significantly increasing ($p < 0.05$) trend in relation to level of crude protein in the diet up to TCP_{50} group. This finding of our study is well corroborated with the study of Xia et al. (2010) who also concluded that feeding higher level of dietary crude protein to *P. vannamei* juveniles could increase the total cholesterol and triglyceride concentration in serum.

P. vannamei possesses the necessary proteolytic enzymes, of which trypsin is considered to be the most important enzyme (Hernández & Murueta, 2009). Further, shrimps can adapt their digestive enzyme activities with their nutrient requirements and dietary substrate availability in the digestive system (Gaxiola et al., 2005). Activities of hepatopancreatic protease and amylase differ significantly ($p < 0.05$) in an increased and decreased fashion, respectively, with an increase in CP level in the diet up to 400 g/kg (TCP_{40}) probably due to the increasing and decreasing availability of dietary protein and carbohydrate in the digestive system of *P. vannamei* juveniles; however, dietary protein beyond 400 g/kg did not show any further significant change in protease and amylase activities. Optimal dietary protein probably could enhance protease of shrimps for hydrolysing the protein and supplying sufficient amino acids for body protein synthesis and metabolic needs leading to maximum growth (Muhlia-Almazan et al., 2003). Our results corroborated the findings of Xia et al. (2010) and Liu et al. (2005), who also reported that activity of hepatopancreatic proteases was maximum by feeding with 400 and 430 g/kg dietary protein, respectively; however in contrast to our finding, beyond that level of dietary protein the activity of hepatopancreatic proteases gradually decreases. Besides, *P. vannamei* probably tries to compensate the stress induced by any deviation from the isosmotic point through enhancing the utilization of energy-producing nutrient with escalating the activities of digestive enzymes (Cuzon et al., 2004; Li et al., 2008).

In the present study, highest activity of the hepatopancreatic amylases was found in the group fed with lowest level of dietary crude protein, which could not exhibit the maximum growth probably due to reduced activity of protease and protein utilization. Thus, the higher activity of amylase does not relate to maximum growth, as seen in the present study (Brito et al., 2001). Similar to our study, several authors could find positive relation between the digestible carbohydrate content of the diet and activity of the hepatopancreatic amylases (Gaxiola et al., 2005; Le Moullac et al., 1997; Rosas et al., 2000; Xia et al., 2010). However, in our present study activity of the hepatopancreatic lipases was found similar ($p > 0.05$) among the treatment groups probably attributed to same level of dietary lipids in all the experimental diets.

5 | CONCLUSION

In conclusion, according to polynomial regression analysis based on WG, SGR and FCR, the optimum dietary crude protein requirement of *P. vannamei* juveniles under the IGSW rearing condition of 15 ppt ambient salinity was found to be within the range of 390.30–397.90 g/kg. Dietary protein beyond the optimal level caused growth retardation of shrimp. The findings of the study will be helpful for developing a nutritionally balanced, environment-friendly and cost-effective diet for *P. vannamei* culture in IGSW of 15 ppt salinity.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Prasanta Jana investigated the study, validated data, implemented software programs and prepared the original draft. Narottam Prasad Sahu conceptualized the study, supervised the study, and reviewed and edited the manuscript. Parimal Sardar designed methodology, involved in visualization and validation, and reviewed and edited the manuscript. Nazeema Shamna involved in visualization and validation, and edited the manuscript. Tincy Varghese and Ashutosh Dharmendra performed data curation and validation. Vungurala Harikrishna supervised the study and edited the manuscript. Mritunjoy Paul performed formal analysis, software implementation and data curation. Hougaina Panmei performed investigation, formal analysis and software implementation. Gyandeep Gupta performed software implementation and validated data. Chinmay Nanda performed formal analysis and data curation. Gopal Krishna reviewed and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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