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Program Based Research Grant (PBRG)

Sub-project Completion Report
on

Exploration of Exogenous Enzymes, Bivalent Efficacy and Omega-3 Fatty Acid of Microbes and Small Invertebrates as Potential Feed Supplement for Enhancing Fish and Shrimp Productivity

Sub-project duration
15 October 2019 to 29 December 2022

Coordinating Organization

Fisheries Division
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

Project Implementation Unit
National Agricultural Technology Program-Phase II Project
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Implementing Organization

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December 2022

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Abbreviation and Acronyms

%	Percentage	EtBr	Ethidium Bromide
<	Less than	eFW	Estimated Fresh Weight
µL	Microliter	EHP	Entero-cytozoon Hepatopenaei
µM	Micro Meter	EMS	Early Mortality Syndrome
A	Area	F	Fishing Mortality
AA	Arachidonic Acid	FA	Fatty Acid
ABW	Average Body Weight	FAME	Fatty Acid Methyl Ester
AD	Antimicrobial Drugs	FAO	Food and Agriculture Organization
AFW	Ash-free Weight	FBM	Final Biomass
AHPND	Acute Hepatopancreatic Necrosis Disease	FCR	Feed Conversion Ratio
ALA	Alpha-linolenic Acid	FE	Feed Efficiency
ANOVA	Analysis of Variance	FER	Feed Efficiency Ratio
AOAC	Association of Official Analytical Chemists	FID	Flame Ionization Detector
ARB	Antibiotic Resistant Bacteria	eFW	estimated fresh weight
BARC	Bangladesh Agricultural Research Council	g	Gram
BFRI	Bangladesh Fisheries Research Institute	GBM	Glioblastoma
BSMRAU	Bangabandhu Sheikh Mujibur Rahman Agricultural University	GC	Gas Chromatograph
cfu	Colony Forming Unit	gDNA	Genomic deoxyribonucleic acid
CGMS	Chromatography Membrane Plasma	GoB	Government of Bangladesh
CH	Chela Height	GR	Growth Rate
Cm	Centimeter	GSI	Gonadosomatic Index
CMC	Carboxy Methyl Cellulose	ha	Hector
Co-PI	Co-Principal Investigator	HP	Hepatopancreas
DGR	Daily Growth Rate	HSD	Tukey's Honest Significant Difference
DHA	Docosahexaenoic acid	IBM	Initial Biomass
DNA	Deoxy Ribonucleic acid	ICW	Internal Carapace Width
rDNA	Recombinant DNA	IDLC	Industrial Development Leasing Company of Bangladesh Limited
DNS	Dinitro Salicylic Acid	IFAD	International Fund for Agricultural Development
DO	Dissolve Oxygen	IPCC	Intergovernmental Panel on Climate Change
DoF	Department of Fisheries	ISO	International Standards Organization
DW	Dry Weight	K	Growth Coefficient
EDTA	Ethylene Diamine Tetra Acetic Acid	Kg	Kilogram

LA	Linolenic Acid	PSU	Practical Salinity Unit
LoA	Letter of Agreement	PUFA	Poly Unsaturated Fatty Acid
LAB	Lysis Activity on the Blood Agar	RGR	Relative Growth Rate
LPW	Lower Paddle Width	RNA	Ribonucleic Acid
LTD	Limited	rpm	Rotation Per Minute
L-W	Length-Weight	rRNA	Ribosomal RNA
M	Natural Mortality	SA	Saturated Fatty Acid
m^2	Square Meter	SAC	SARC Agriculture Center
MALDI	Matrix-Assisted Laser Desorption/Ionization	SBDF	Sea Bream Dry Food
mg	Milligram	SE	Standard Error
mL	Milliliter	SFA	Saturated Fatty Acids
mm	Millimeter	SGR	Specific Growth Rate
MMT	Million Metric Ton	SOD	Superoxide Dismutase
mN	Micronewton	SPSS	Statistical Package for the Social Sciences
MR	Methyl Red	SR	Survival Rate
MUFA	Mono Unsaturated Fatty Acid	SRDI	Soil Resource Development Institute
NARI	National Agricultural Research Institute	SRS	Shrimp Research Station
NATP- II	National Agricultural Technology Program: Phase II	SS	The Sundarbans Site
NB	Nutrient Media	TA	Total Area
NHP	Necrotizing Hepatopancreases	TAE	Tris-acetate-EDTA
NSP	Non-Starch Polysaccharide	TBE	Tris-Borate-EDTA
NSTU	Noakhali Science and Technology University	TCA	Tricarboxylic Acid Cycle
^oC	Degree Celsius	TCBS	Thiosulfate-citrate-bile salts-sucrose agar
OD	Optical Density	TDS	Total Dissolve Solid
Pi	Inorganic Phosphate	THC	Total Haemocyte Count
OMV	*Oncorhynchusmasou* virus	TK	Taka
PBRG	Program Based Research Grant	UK	United Kingdom
PBS	Phosphate Buffer Saline	US$	United State Dollar
PCA	Principal Component Analysis	USA	United State of America
PCR	Project Completion Report	VBGF	Von Bertalenffy Growth Function
PCR	Polymerase Chain Reaction	VP	Voges-Proskauer
P^H	Negative logarithm of Hydrogen Ion Concentration	VPA	Virtual Population Analysis
PI	Principal Investigator	WB	World Bank
PIU	Project Implementation Unit	W/V	Weight By Volume
PL	Propodus Length	WG	Weight Gain
PL	Post Larvae	WSSB	White Spot Syndrome Virus
ppt	Parts per Thousand	Z	Total Mortality
PSM	Phytase Screening Medium		

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Executive Summary

The expansion of global aquaculture production is increasing the demand for aquaculture feeds where more than 70% is dependent upon the supply of external feed inputs. Fishmeal is the main and most critical ingredient in aqua-feed production. But inconsistent supply and rising prices have made prompted the search for a cheaper and available protein sources like plant proteins. However, feed ingredients from plants sources contain some compounds that either the shrimp/fish cannot digest or which hinder its digestive system because they cannot produce the required enzymes to degrade. Different anti-nutritional factors such as protease inhibitors, phytates, glucosinolates, saponins, tannins, lectins, non-starch polysaccharides (NSP) etc. are associated with the most widely used plant materials. Along with the higher demand of feed and its price hike, disease outbreaks are also considered to be a significant constraint to the development of the aquaculture sector, with a global estimate of disease. When faced with disease problems, the common response has been to turn to Antimicrobial Drugs (ADs). Moreover, at this stage, antibiotics are no longer effective in treating bacterial diseases in some cases because the use of ADs in aquaculture has led to the emergence of (ARB). Application of beneficial bacteria (probiotic) may be the alternative of ADs. This treatments may provide a broader spectrum and greater non-specific disease protection through competitive exclusion and immune modulation. Therefore, searching for different target oriented exoenzymes from microbial sources for eliminating the major problems for plant-based aquafeed materials is important. Therefore, the present research attempted to search and identify the suitable indigenous microorganisms for isolating exoenzymes to sustain the commercial aquaculture and identification and culture of locally available potential invertebrates (polychaetes) with higher Omega-3 contents for better feeding management and finally isolation, identification and efficacy assessment of locally available probiotic bacteria to cope with disease outbreak in shrimp aquaculture.

Initially, a total of 520 bacteria were isolated and purified based on characteristics of colony. Among them 109 bacteria were screened as target enzyme (protease, phytase and cellulase) producing bacteria identified by different media-based screening. Among all the pure isolates 44 colonies were screened as cellulase producing (Congo-red media-based method), 58 protease producing (using casein-based media) and 20 phytase producing (Phytate salt-based media). Following screening, different biochemical characterization was done and identified as potent cellulase, protease and phytase enzyme producing colonies. For molecular identification, a total of 80 bacterial DNA was extracted and PCR product was completed and seventy-eight bacteria samples were identified through sequencing analysis and Bruker MALDI Bio type Identification. Among those, *Bacillus, Acinetobacter, Enterobacter* were found and few were identified as *Klebsiella and E. coli* may be due to sampling or other problem. In addition, optimization of enzyme production parameter, crude enzyme production, enzyme activity assay, enzyme purification and pure enzyme activity assay has been completed. For commercial application in aquaculture, plant feed (Soybean) was fermented with the enzyme producing bacteria and proximate composition was analyzed and a significant improvement was observed in term of protein content. Findings of the research revealed that there is a huge opportunity for further research. Different non-conventional plant feed materials can be fermented and species wise feed trial need to be performed.

Apart from bacteria, the aquatic invertebrate “polychaetes”, found in coastal waters are a significant source of polyunsaturated fatty acids (PUFA), particularly Omega-3. Aquafeeds containing omega-3 fatty acids are the highest-quality feed available for aquaculture, particularly for shrimp larvae, matured shrimp, and crab industries worldwide. Under this study total 8 species of polychaetes identified from the Sathkira and Cox's Bazar districts of Bangladesh. *Dendronereis dayi* and *Namalycastis fauveli* are the new records for the marine polychaetes in Bangladesh. In addition, the *Glycera* sp., *Namalycastis* sp., *Neanthes* sp., and *Composetia* sp. are the available important species. The dominating polychaete, *Namalycastis* sp., was chosen for its omega-3 fatty acid content due to its year-round availability. The omega-3 (n-3) fatty acids in the wild and cultivated settings were studied. Wild individuals had fatty acid compositions below detection limits, but cultured specimens fed code:155-FF-02 feed had the highest recoveries. Surprisingly, the fatty acid concentration of cultured individuals fed code:155-FF-03 diet was lower below the detectable limit. Trial on polychaete development and survival in experimental and field environments, considering substrate type and depth, stocking densities, temperatures, and feeds were performed. In the semi-circulatory culture system, 10.0 cm beach sand with 2.0mm grain size showed the most growth and survival of *Namalycastis* sp. out of the six substrate trials. Furthermore, among the three density-dependent studies, this species performed best with a stocking density of 500 indv./m^2. In the semi-circulatory culture system, the maximum growth and survival of the species were observed at 30 ^{0}C. Different treatments of *Namalycastis* sp. cultivated at 26-34 ^{0}C temperature showed a non-significant ($p > 0.05$) relative growth rate but a significant (p 0.05) survival rate. As a result, greater temperatures significantly impact *Namalycastis* sp. survival and growth rate. Among the designed meals, marine fish waste showed the most growth and biomass gain in the system, although tilapia muscles feed had the greatest survival rate in the indoor system. In open water culture systems, commercial feeds demonstrated higher growth of *Namalycastis* sp., although formulated diets showed the maximum survival with the least biomass. Supplementary diets can never replace the natural culture of *Namalycastis* sp. in open water systems with no feeds. For *Namalycastis* sp. culture, the controlled indoor culture provided a higher yield than the natural open water systems.

Various drivers affecting the growth of *Namalycastis* sp. have been optimized through various experiments earlier, however, the growth performance of the commercially important mud crab (*Scylla olivacea*) fed *Namalycastis* sp. was the final step to investigate. Male and female gonads/testis and ovaries were extracted from similarly weighted male and female gonads/testis and ovaries, respectively. For evert crabs, the GSI was calculated. For the growth performances, three natural meals were chosen: live *Namalycastis* sp., Tilapia muscles (*Oreochromis niloticus*), and Little neck clam (*Mercenaria mercenaria*). During the culture period, all diets' growth characteristics improved, and no mortality was observed. Clam feeding had a faster rate of morphometric characteristic than other diets. The GSI was enhanced in all diets but was elevated twice in female crabs fed live *Namalycastis* sp. Further research on breeding behavior and feed formulation of *Namalycastis sp*, could open new sustainable technology for commercial farming of the omega-3-rich *Namalycastis sp*. could be established. This will also ensure the highest quality feed for shrimp and crab but also to prevent significant loss from raw material importation.

Based on the survey information on probiotic application in shrimp farms, efforts were given to identify the local isolates of probiotic under this study. For the determination of monovalent efficacy of *Bacillus spp* as probiotic candidate; In-vitro challenge test were performed to assess the inhibitory effect of *B. subtilis* on the growth of *V. parahaemolyticus*. Study revealed the highest production from T1, where shrimp was supplied with *B. subtilis* treated diet @2* 10^8 cfu/g feed, whereas the control diet produced the lowest production. Highest survival was found also in T1and lowest survival was found control pond. Under the screening process of the probiotic bacteria, different new strains of probiotic bacteria were identified by metagenomic study. Among the isolates, 5 non-conventional probiotic candidates under the genus of *Lactobacillus*, *Virgibacillus*, *Bacillus, Streptobacillus & Shewanella* were found most effective based on wide range of organic carbon degradation range along with higher tolerance to temperature fluctuation.

The efficacy of the locally isolated *Bacillus* sp. was also determined both *in vitro* and *in vivo*. Almost three times inhibitory effects of *Bacillus* sp. on pathogenic *Vibrio* sp. recorded. In total colony forming unit (cfu) count of experimental and control pond water in TCBS selective agar media, total *Vibrio* were observed almost ten times higher in control pond than the treatments. This showed much satisfactory results about the inhibitory effects of combined *Bacillus* sp. on pathogenic *Vibrio* sp. *in vivo*. To determine the effects of probiotics on growth and survival of *Penaeus monodon* and assessment of the production feasibility, monitoring of growth and survival of the stocked PLs and comparative bacterial colony count were done fortnightly. *Vibrio* load was always found higher in the control ponds and lower in treatment ponds. During the 60 days of culture, growth performance found significantly better in the treatment ponds where locally isolated probiotics were applied. The average body weight in control pond found was 12.87±1.79 g whereas the body weight from the treatment pond was 22.31±1.54 g which clearly indicate the positive effect of probiotics. The immune parameters such as Superoxide Dismutase (SOD) activity, Total Haemocyte Count (THC) indicated better immune responses and healthy condition of shrimp in treatment ponds.

Keywords: Omega-3, Exoenzymes, Polychaete, Mud crab, Local isolates, Probiotics, Shrimp (*P. monodon*), Disease, Superoxide Dismutase, Immune responses

PBRG Sub-project Completion Report (PCR)

A. Sub-project description

1. **Title of the PBRG sub-project**

   Exploration of Exogenous Enzymes, Bivalent Efficacy and Omega-3 Fatty Acid of Microbes and Small Invertebrates as Potential Feed Supplement for Enhancing Fish and Shrimp Productivity**.**

2. **Implementing organization (s)**

   Department of Fisheries Biology and Aquatic Environment, BSMRAU, Gazipur-1706; Department of Fisheries and Marine Science, Noakhali Science and Technology University, Noakhali-3814 and Bangladesh Fisheries Research Institute, Shrimp Research Station, Bagerhat-9300.

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4. Sub-project budget (Tk)

4.1	Total:(in Tk as approved)	: Tk. 28040000.00
4.2	Latest Revised (if any)	: Tk. 32525400.00
i.	Coordination component	: Tk. 1830000.00
ii.	Component 1 (BSMRAU)	: Tk. 10717400.00
iii.	Conponent 2 (NSTU)	: Tk 13868000.00
iv.	Component 3 (BFRI)	: Tk. 6110000.00

5. Duration of the sub-project:

5.1 Start date (based on LoA signed) : 15 October 2019

5.2 End date : 29 December 2022

6. Background of the sub-project

Bangladesh is one of the best countries for aquaculture, and the country's present aquaculture production is highly adequate and improving with time (Ahmed et al. 2012). Bangladesh has risen to fifth place in the world in terms of overall aquaculture production (El-Sayed et al., 2015) due to increased aquaculture production. However, due to increased prices and poor quality of fish feed, the profitability of this crucial industry has been steadily declining (El-Sayed et al., 2015; Yuan et al., 2017; Yang et al., 2019; Nguyen et al., 2020). In most aquaculture systems, feed accounts for 50–60 percent of overall operational costs (Mzengereza et al., 2014; Daniel 2018). Furthermore, feed quality, acceptability, and utilization have major effects on water quality, growth, survival, and, ultimately, profitability and sustainability in this industry (Singha et al., 2020; Guo et al., 2020; Kong et al., 2020). As a result, high-quality feed is essential for the aquaculture industry's successful, long-term, and lucrative fish production.

Enzymes are the bio-catalysts playing an important role in all stages of metabolism and biochemical reactions. Microbial enzymes are known to be superior enzymes obtained from different microorganisms, particularly for applications in industries on commercial scales. Though the enzymes were discovered from microorganisms in the 20th century, studies on their isolation, characterization of properties, production on bench-scale to pilot-scale and their application in bio-industry have continuously progressed, and the knowledge has regularly been updated. Many enzymes from microbial sources are already being used in various commercial processes. Selected microorganisms including bacteria, fungi and yeasts have been globally studied for the bio-synthesis of economically viable preparations of various enzymes for commercial applications. Applying enzymes in feeds to enhance feed utilization is an idea that has been explored well in terrestrial animal feeding especially for the poultry and swine. However, utilization of exogenous enzymes in aquaculture is still in infancy. With pressure to find more sustainable sources of fish feed for aquaculture, researchers have been trying to improve the nutritional value of fish feed by enzyme supplementation.

In Bangladesh, the increasing cost of fish meal has encouraged feed manufacturers search for cheaper alternative protein sources such as plant proteins. However, feed ingredients from plants sources contain some compounds that either the shrimp/fish cannot digest or which hinder its digestive system because they cannot produce the require enzymes to degrade. Anti-nutritional factors in plant materials have an adverse impact on the digestion of feed and its efficiency. There are many kinds of anti-nutritional factors and they are associated with the most widely used plant materials like trypsin inhibitor proteins, glucosinolates, non-starch polysaccharides (NSP) and phytate. Thus, scientists are now searching different target oriented exoenzymes from microbial sources which can eliminate the major problems for plant-based aquafeed materials. Supplementation with enzymes can help to eliminate the effects of antinutritional factors and improve the utilization of dietary energy and amino acids, resulting in improved performance of fish/shrimps. Feeding enzymes to shrimps and fishes is one of the major nutritional advances in the aquaculture sector since last few years. But efficacy of those exoenzyme depends on the sources of microbes, type of microbes, other properties of enzymes such as temperature stability, pH stability etc. So, researches on their isolation, characterization of properties, production on bench-scale to pilot-scale and their application in aquaculture industry is of prime importance. Therefore, this proposed part of research is designed with a view to search and identify the suitable indigenous microorganisms for isolating exoenzymes to sustain the commercial aquaculture.

On the other hand, feed is considered as the major contributor in the aquaculture and shares about 50-80% of the total production costs (SARC agriculture center, SAC 2017). In Bangladesh, the market size of total commercial feed (poultry, cattle and fish) stands to be at 5.03 MMT with an estimated market turnover of US$ 2.5 billion while fish feed shares 23% (IDLC, 2020; Rashid et al., (2013) reported that Bangladesh produces 50–55% of fish feed ingredients, with the remainder imported yearly. Therefore, Bangladesh spent a significant amount (0.23-0.25 billion US$) of money behind importing fish feed’s raw materials (fish meal, meat, and bone meals) every year. Alternative way through utilizing our natural resources for the best quality animal protein source to supplement high quality fish feed could be a new horizon to save such significant economic loss as well as to ensure food security in Bangladesh.

Shellfish aquaculture in Bangladesh is widespread in Satkhira, Khulna, Cox's Bazar and Bagerhat District in Bangladesh. Tiger Shrimp *Panaeous monodon* and giant prawn *Macrobrachium rosenbergii* are the species of shellfish cultured in those areas. M. rosenbergii is largely cultured in southwest region of the country (DoF, 2020).

It is widely reported that coastal water polychaetes, as a potential nutrient rich fish feed ingredient with Omega-3 fatty acid, ensure fecundity increment, early maturation and immunity of fishes and shell fishes by supplying significant amount of polyunsaturated fatty acids and omega-3 fatty acids. Therefore, polychaetes as alternative fish feed supplements might be good search for quality shellfish and/fish feed in Bangladesh.

Polychaetes are recently been recognized as potential feed ingredients due to its excellent nutritional content, particularly their high unsaturated fatty acid and lipid profile (Pauls 2009). These worms are widely feed to the brood-stock shrimp because they improve the gravid shrimps' gamete cells quality (Yuwono et al., 2002). In marine environment, size-based assessments may easily reveal any community structure and function, and this has been the subject of many studies (Robinson et al. 2010; Hua et al. 2013; Górska and Włodarska-Kowalczuk 2017). The length-weight (L-W) relationship of polychaetes worms can be used to estimate biomass, population density, growth, evolution (Costa-Paiva and Paiva 2007). They can also be used to examine food webs (Woodward et al., 2005; Nakagawa and Takemon 2015). Moreover, this fundamental model determines secondary productivity and trophic potentiality (Mistri et al., 2001). The measurement of fresh weight has further aquaculture benefits, such as effects on growth and survival of polychaetes in variable diets, temperature, salinity, etc. can be evaluated under controlled culture conditions.

Marine fish and shrimp brood-stock largely rely on the adequate supply of indispensables fatty acids which they cannot synthesize themselves are typically marine origin and harvested as trash fish being processed to fish meal and fish oil (Tacon and Metian, 2015; Cashion et al., 2017). Most natural fish stocks have been overexploited (Pauly and Zeller, 2016); therefore, searching for novel feed sources is necessary to sustain the aquaculture industry (Pauls 2009) which is also true for Bangladesh. Plant source feed ingredients like soybean, maize, and oilseed cakes are extensively applied as cheaper fishmeal and fish oil (Alam et al., 2018). Nevertheless, these derivatives lack essential amino acids and fatty acids and carry antinutritional factors affecting fish growth (Francis et al., 2001). Several studies have also focused on intensifying aquaculture production in suitable artificial conditions, such as considering the salinities, substrates, feeding ratios, stocking densities, feeding with high protein feeds and macroalgae, etc. Polychaetes such as, *Hediste diversilocor* (Fidalgo e Costa et al., 2000; Nesto et al., 2012; Santos et al., 2016; Wang et al., 2019; Jerónimo et al., 2021), *Marphysa sanguinea* (Parandavar et al., 2015; Parandavar et al., 2018; Thu et al., 2019) have been investigated under different salinity treatments and fatty acid profile has been studied. Very few studies investigated the growth and fatty acid composition of the *Namalycastis* sp. as potential use feed for mud crabs (Lim et al., 2021).

Therefore, polychaetes cultivation can provide natural quality food and reduce the dependence on nature by artificial seed propagation and biomass intensification. Furthermore, they can potentially substitute fishmeal as raw material for shrimp feed (Yuwono et al., 2002). Cox’s Bazar and Sundarbans mangrove are home to Bangladesh’s greatest coastline areas. These intertidal habitat shelters several macrobenthos including Polychaetes. Some species of polychaetes have been identified from these coasts (Pramanik et al., 2009). The proposed research will investigate the number of polychaetes species found in the coastal and marine areas of Bangladesh. The study will also deal with population dynamics of polychaete, *Namalycastis* sp. optimalizations of environmental (substrates, density, temperature, salinity) and dietary (different feeds) drivers for intensifying the biomass production of *Namalycastis* sp. and its fatty acid profile. Finally, we explore the growth performance and gonadosomatic index of shrimp/mud crab (*Scylla olivacea*) supplying polychaetes and other natural diets. In the fisheries sector of Bangladesh, bacterial diseases are considered to be a threat in the intensive larval culture and production of aquaculture species owing to the large-scale mortality they cause in hatcheries and culture systems (Cheng et al., 2004). For prevention and control of the diseases, antibiotic, pesticides, and other toxic chemicals are used possibly creating antibiotic resistance bacteria, persistence of pesticides and other toxic chemicals in aquatic environment and creating human health hazards (Devi *et al.,* 2015).

The major cause of fish/shrimp loss is disease infection. Among the known disease, *P. monodon* facing devastating crop loss due to White Spot Disease outbreak in Bangladesh. In 2010-2013, intensive research on the risk factors associating white spot syndrome virus (WSSV) infection into shrimp was carried out and the findings were disseminated to the farmers to reinforce their hold to cope with WSSV infection (Islam *et al*., 2014). But interestingly, since last two years, mortality pattern due to WSSV infection found following a different pattern and showing differences in virulence as well. Apart from WSSV, several symptoms of shrimp mortality indicating the probability of presence of some non-reported disease to Bangladesh, *viz*., Necrotizing Hepatopancreatitis (NHP), Bacterial White Tail Disease, Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) and Enterocytozoon hepatopenaei (EHP). Among these diseases, EMS found positive to a number of shrimp farms (Personal Communication with the PL nurturers and farmers).
Rengpipat et al. (2000) mentioned that the use of *Bacillus* sp. (strain S11) provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp (*P. monodon*). Balcazar (2003) demonstrated that the administration of a mixture of bacterial strains (*Bacillus and Vibrios* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity. Chythanya et al. (2002) showed that *Pseudomonas* I2, was isolated from estuarine environmental samples that produced inhibitory compounds against shrimp pathogenic vibrios. Isolated two strains of *Vibrio* spp. NICA 1030 and NICA 1031 from a black tiger shrimp hatchery displayed antiviral activities against IHNV and *Oncorhynchusmasou* virus (OMV), with percentages of plaque reduction between 62 and 99%, respectively (Direkbusarakom*et al*., 1998).

Microbes play very important role in aquaculture systems, both at hatchery and grow-out levels as water quality and disease incidences are directly affected by microbial activity (Kumar *et al.,* 2006). Probiotics are known to control pathogens through a variety of mechanisms; hence, they are exploited as an alternative to antibiotic treatment. Fuller (1992) defined probiotics as live microbial feed supplements which are beneficially affect the host by improving its intestinal microbial balance. There is a national and international concern for preventing and controlling the diseases through new scientific approaches in order to make health-safe aquaculture product. Probiotics can be one of the alternatives to improve culture friendly water and soil quality, prevention of disease producing pathogen, increasing digestibility and immune competence of prawn. Thus, it is also important to isolate potential probiotics bacteria from the culture environment and go for their mass production in laboratory and pond.

7. Sub-project general objective (s)

Development of technology for reducing fish/shrimp mortality by improves feed digestibility and disease resistance.

8. Sub-project specific objectives (component wise)

Coordination component (BARC)

1) To ensure smooth and efficient implementation of sub-project activities to achieve desired sub-project outputs within the stipulated timeframe under strengthened capable research management system;
2) To coordinate sub-project implementation efforts and integration of activities to generate desired information /technology as per methodology of the sub-projects;
3) Identify operational deviations and addressing constraints/problems (if any) under a process of strong and regular monitoring of the sub-project activities;
4) To upgrading the level of output of the sub-project through reviewing of yearly technical progress;
5) Collect and collate sub-project data, finding and observation and production of compiled Project Completion Report (PCR).

Component 1 (BSMRAU)

1) To search and identify the suitable indigenous microorganisms for isolating bioactivity targeted exoenzymes;
2) To purify and characterize the isolated exoenzymes for commercial application in aquaculture;

Component 2 (NSTU)

1) Identification and distribution of polychaete worms in the coastal and/marine waters;
2) To analyze quality and quantity of Omega-3 fatty acid content of those identified species;
3) To develop mass culture method of Omega-3 fatty acid containing polychaetes for commercial applications in the feed industries;
4) To explore growth performance of shrimp/mud crab using formulated supplementary feed from polychaetes;

Component 3 (BFRI)

1) to determine the monovalent efficacy of locally isolated *Bacillus* sp.;
2) to determine the combined effect of *Bacillus* sp. on growth and survival of *Penaeus monodon;*
3) To assess the production feasibility of *P. monodon* using combined *Bacillus* sp.;

9. Implementing location (s)

i) Department of Fisheries Biology and Aquatic Environment, BSMRAU, Gazipur-1706;
ii) Sundarbans and Cox's Bazar coastal areas and Dept of Fisheries & Marine Science, NSTU; and
iii) Shrimp research Station, BFRI, Bagerhat;

10. Methodology in brief

10.1. Activity implementation approach for the Coordination component

The Coordination component, as the responsible unit of the sub project to initiate all potential efforts in the process of implementation of each component under the sub project so that the general objectives and goal of the sub project can be achieved through smooth and successful completion of each of the specific objectives as per activity time plan of the sub-project document. To ensure that, the Coordination component, taken into consideration its own activity and objectives and duration of the sub-project, thus accordingly designed its own plan of activity (approach) for the proposed period. Following are the major activities carried out by the Coordination component under the plan:

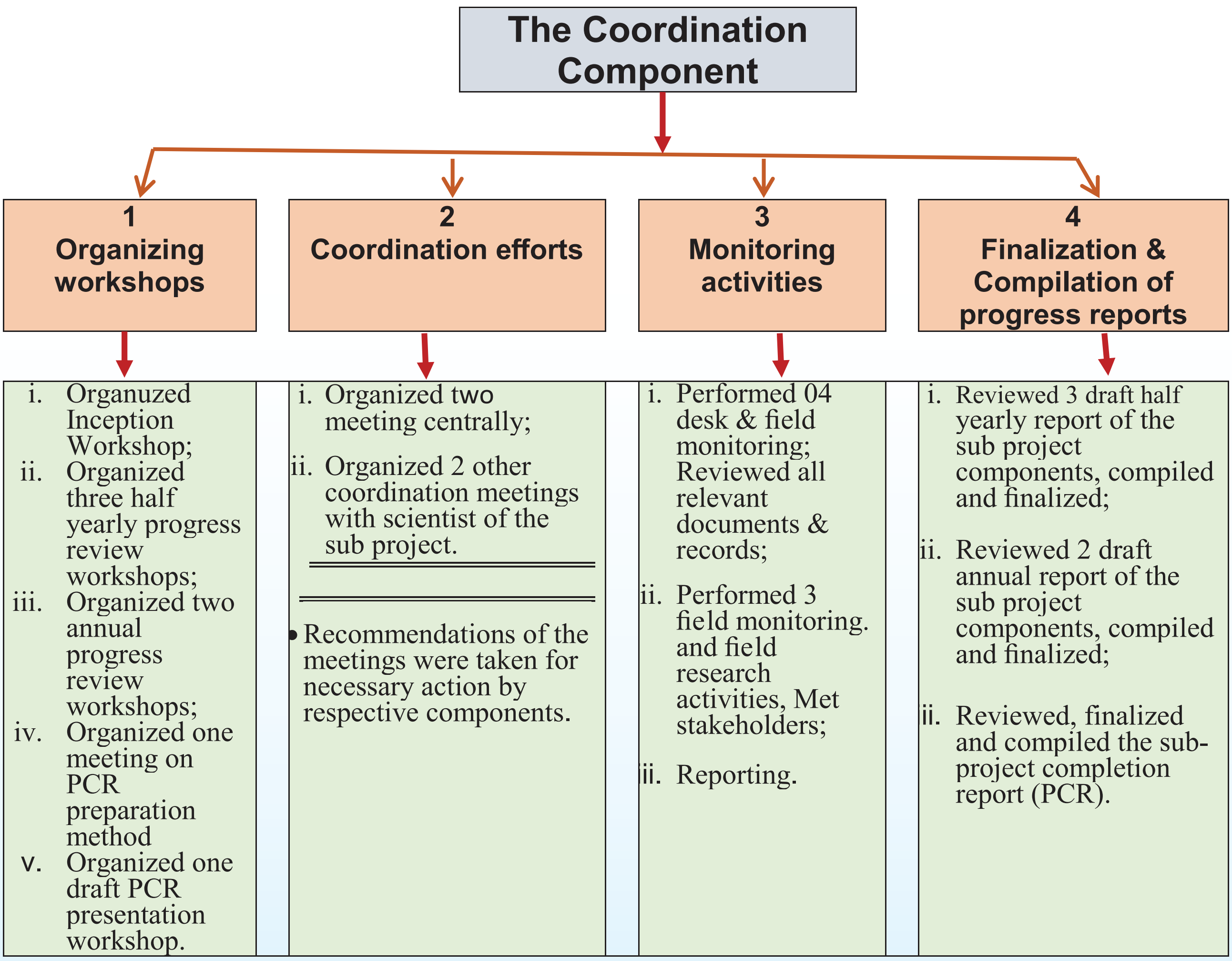
a. Organize seminars/workshops/coordination meetings;

b. Monitor of the sub-project activities;

c. Coordination of activities within the components of the sub-projects.

d. Review and compilation of half yearly and annual research progress reports and PCR.

The implementation approach and activities there under for the Coordination component of the sub-project are shown in the following diagram:



Recommendations of the inception workshop, half yearly and annual research progress review workshops and different coordination meetings are furnished hereunder in Annexures: BARC (A – E).

Following table presenting the summary statement of achievements performed by the Coordination component of the sub project

Summary statement of achievements		
Name of activities	**Performance against each component of the sub-project**	**Remark**
Inception workshop	Organized centrally at BARC in November' 2018	Attended all PI, Co-PI & expert members.
Revision of PP	Done as per recommendations of inception workshop	Mpleted as per plan
Half yearly progress review Workshop	Organized centrally at BARC in March 2020, January 2021 & June 2022.	Attended all PI, Co-PI & expert members
Workshop on Draft PCR	01 (24/10/22 & 25/10/22)	Review of the respective draft PCR done
Ann. Progress review Workshop	Organized centrally at BARC in December 2020 & in April 2022	Attended all PI, Co-PI & expert members.
Coordination meetings	04 *(two meeting held virtually)*	All coordination meetings held centrally.
Monitoring of field and Lab activities	04 *(BSMRAU, NSTU & BFRI)*	Covered all components under the sub-project.
Training/orientation	01 (11.05.22)	Orientation workshop of 09 sub-project PIs on PCR development Prtocess
Financial achievement	Approx. 87% of total approved budget & 100% of released money	Delay in procurement plan approval and Covid-19 pandemic hampered desired progress
Reporting performance	Provided sub-project inception report, SoE, Half yearly and Annual compiled progress reports of all sub project components as per planned time frame.	Major reports/proceedings: • Inception report (1 no); • Compiled half yearly progress report (3 no); • Compiled annual progress report (2 no); • Monitoring reports (4 no); • Workshop proceeding (6); • Project Completion Report (1);

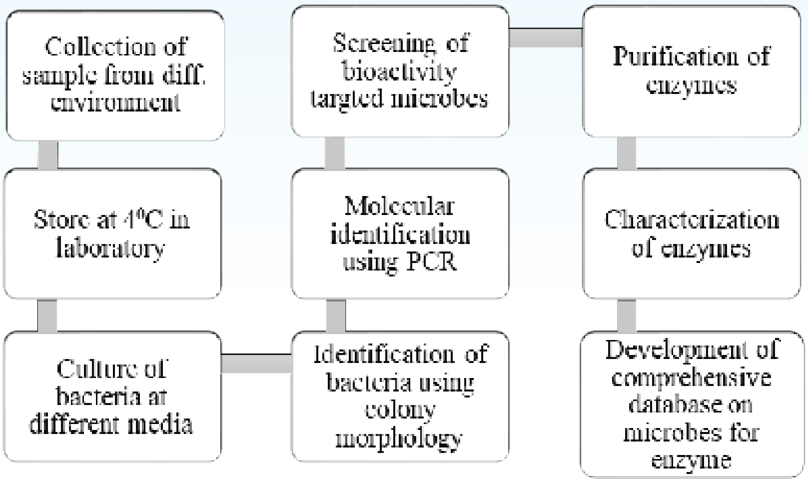
Pictorial views of
different workshops, coordination meetings and field monitoring activities

10.2. Component 1 (BSMRAU)

10.2.1. Approach

Approach followed for collection of microbial samples from different geographical locations and environment of the country, culture and screening of bioactive target microbes, molecular identification and identification through colony morphology, purification and characterization of enzymes and database preparation activities are presented stepwise in the following flow chart as follows:

Research implementation approach of the component 1(BSMRAU)



10.2.2. Collection of samples

Different type of sample like soil, water, yogurt, spring water, sand, fish gut and Nga-pi (fermented fish) and fermented bamboo were collected from different selected areas like Cox's bazar, Rangamati, Naogaon, Mymensingh, Bogura, Moulavibazar, Tangail, Potuakhali for isolating the bioactivity targeted enzymes. Samples were transported to the laboratory and stored in refrigerator (4^0C) for further analysis.

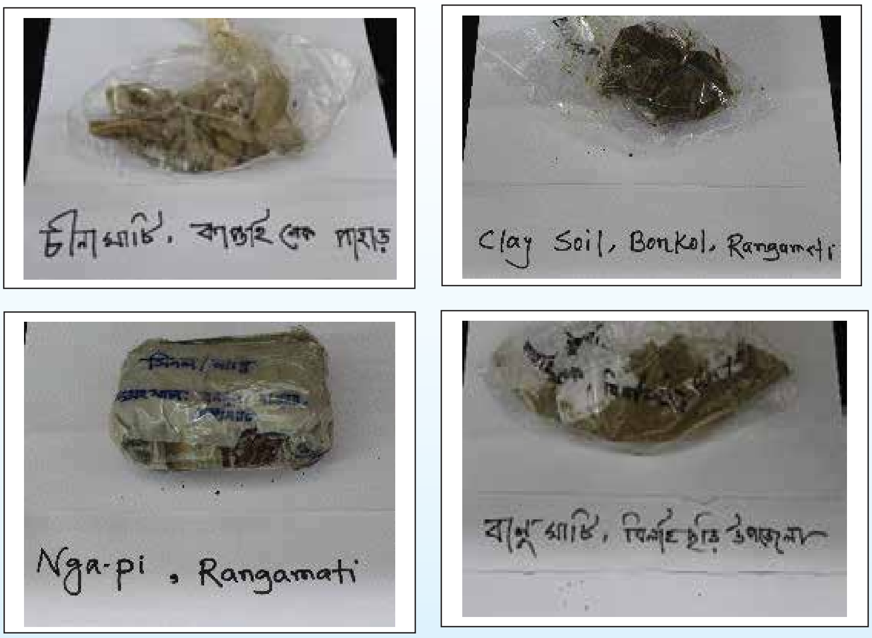


Plate 1. Samples collected from different locations/environment

10.2.3 Isolation of microorganisms

Briefly, 1 g of each sample was suspended in 10 ml of sterile potassium phosphate buffer solution (PBS) or Tween 80 by vortexing for 2 min on maximum speed. Following, aliquot (0.1 ml) of this suspension was streaked on different nutrient agar media and incubated at different range of temperature 25°C-37^{0}C for 24 hr. Various colonies were selected and cultured separately to ensure purity.

10.2.4. Pure culture of isolated microorganisms

Initially the microorganisms were isolated on new cultured plates by their visual colony characteristics (color, opacity, surface, border structures and diameter size). These plates were kept in incubation at previously described temperature. After pure colony streaking, each isolate was labeled properly as different number to avoid any kind of misleading activities. Continuous observation was done and incubation time required for proper growth is noted properly. These plates are kept in 4^0 C temperatures for short time until the final stocking is done.

***10.2.5.* Screening of microbes**

10.2.5.1. Cellulase activity

A volume of 5µl of each broth culture was dropped onto a plastic Petri dish containing carboxymethyl cellulose (CMC) agar with Congo-Red and then incubated for 48 h at 28 °C. After the allotted growth time, the CMC agar plates with the isolates will be stained with Grams iodine solution (2.0g Kl and 1.0g l, per 300ml ddH_2O) to visualize the cellulase activity. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria (Lu et al. 2004). Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies (Plate 2a)

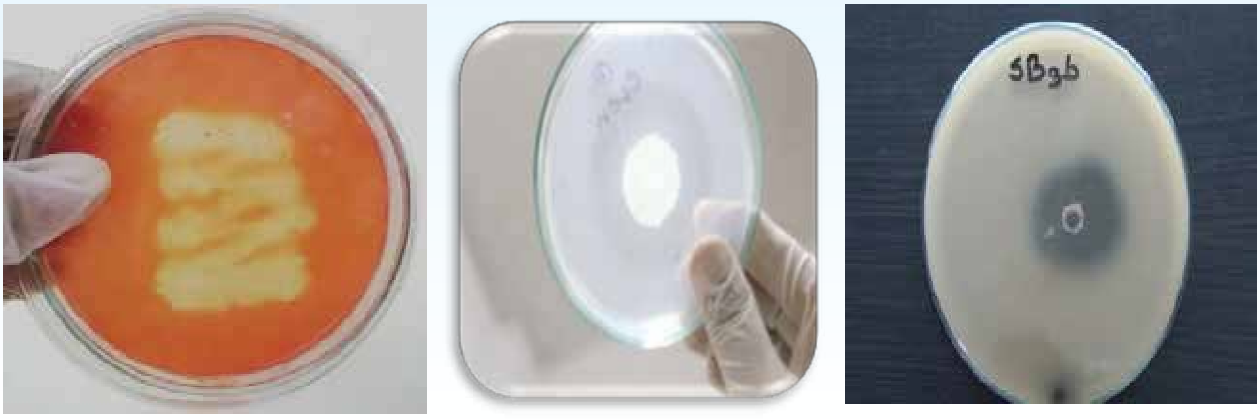
10.2.5.2. Proteolytic activity

Proteolytic production of the bacterial strains was screened on agar plates supplemented with 1% NaCl, 5% casein and Nutrient media (NB). A volume of 5µl of each broth culture was dropped onto the agar plates and incubated for 24 hours at 37 °C. After incubation, clear hydrolytic zone forming bacterial strains were taken as protease producing bacterial colonies (Plate 2b).

10.2.5.3. Phytase activity

Bacteria was dropped on both paper disc and hole in phytase screening medium (PSM) plate containing (gl^{-1}) dextrose 20, tryptone 10, NaCl 5, KCl 0.1, Sodium Phytate 5, agar 20. The pH was adjusted to 8.0.

The incubation was carried out for 24 h at 37 ± 1 C. The zones of clearance flooding of the plate with 2% cobalt chloride for 5 min, after which the solution was replaced and incubated for 5 min at room temperature with freshly prepared colouring reagent containing equal volumes of 6.25% (w/v) aqueous molybdate and 0.42% (w/v) ammonium vanadate solution (Plate 2c).



(a) (b) (C)

Plates 2. a) Cellulase screening, b) Protease screening and c) Phytase screening

10.2.6. Biochemical characterization of the isolates

The isolated microorganisms were characterized by biochemical tests such as Gram test (Aditi et al. 2017), catalase test (Reiner K, 2010), oxidase test (Shield *et al.,* 2010), triple sugar iron test (Capuccino and Sherman 2005), citrate test (Capuccino and Sherman 2005), gelatin test (Cruz and Torres 2012), hemolysis test (Aryal 2015) and starch hydrolysis test (www.vumicro.com) were performed.

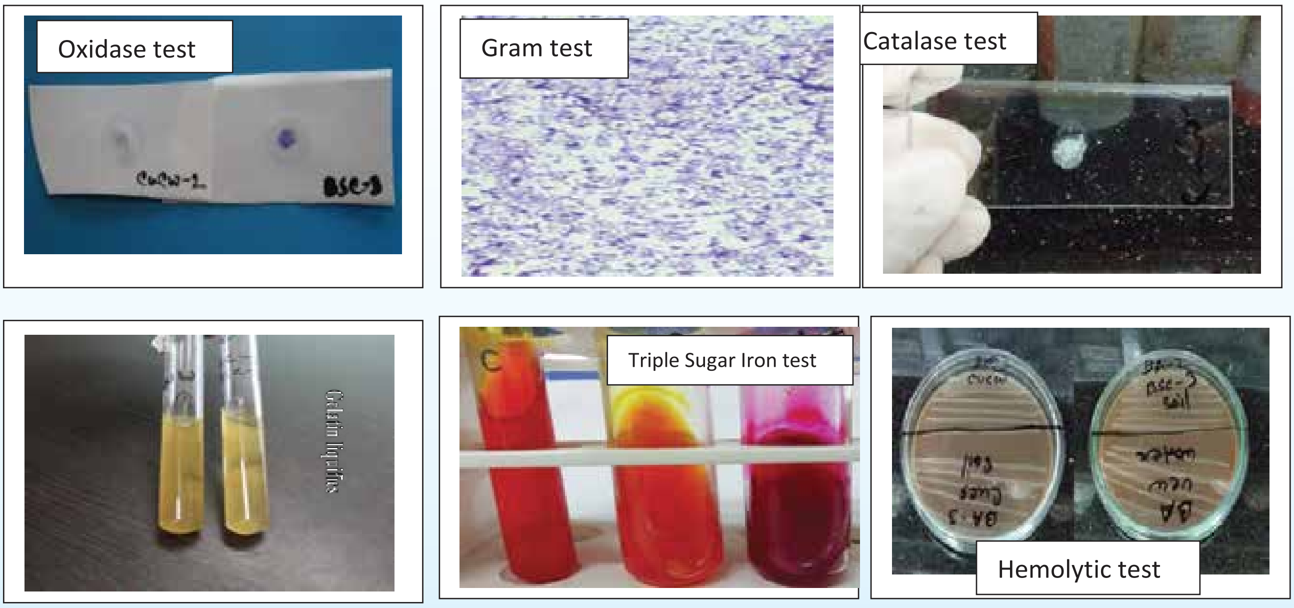


Plate 3. Biochemical characterization of pure isolates

10.2.7. Molecular characterization

10.2.7.1. Extraction of DNA from bacteria

Fresh bacterial culture was done before using the bacteria. Extraction of DNA from bacterial cell was carried out using Monarch® Genomic DNA Purification Kit following the procedure supplied from kit manufacturer. Following procedure was used for DNA extraction:

a. Sample lysis

Prepare pellet of bacterial cells by centrifugation at 1,000 x g for 1 minute and removed the supernatant and resuspended in 100 µl cold PBS by carefully pipetting up and down 5-10 times until the pellet is resuspended completely.

Then 1 µl Proteinase K was added and 3 µl RNase A to the resuspended pellet and mixed by vortexing briefly to ensure the enzymes are efficiently dispersed.

Then 100 µl cell lysis buffer was added and vortex immediately and thoroughly. The solution was rapidly become viscous.

Then incubated it for 5 minutes at 56°C in a thermal mixer with agitation at full speed (~1400 rpm).

After that we proceeded to the next step: Genomic DNA Binding and Elution

b. Genomic DNA binding and elution

Firstly 400 µl gDNA Binding Buffer was added to the sample and mix thoroughly by pulse-vortexing for 5-10 seconds.

Then transferred the lysate/binding buffer mix (~600 µl) to a gDNA Purification Column pre-inserted into a collection tube, without touching the upper column area. Proceeded immediately to Step 3.

Closing the cap and centrifuge: first for 3 minutes at 1,000 x g to bind gDNA (no need to empty the collection tubes or remove from centrifuge) and then for 1 minute at maximum speed (> 12,000 x g) to clear the membrane. Then we discarded the flow-through and the collection tube.

Then transferred column to a new collection tube and 500 µl gDNA Wash Buffer was added and closed the cap and inverted a few times so that the wash buffer reaches the cap. Centrifuge immediately for 1 minute was done at maximum speed and discarded the flow through. Inverting the spin column with wash buffer prevents salt contamination in the eluate.

Reinsertation the column into the collection tube was done. 500 µl gDNA Wash Buffer was added and closed the cap. Centrifugation immediately for 1 minute at maximum speed was carried out and discarded the collection tube and flow through.

gDNA Purification Column was placed in a DNase-free 1.5 ml microfuge tube (not included).

35-100 µl preheated (60°C) gDNA Elution Buffer was added, closed the cap and incubated at room temperature for 1 minute. Then Centrifugation for 1 minute at maximum speed (> 12,000 x g) was carried out to elute the gDNA.

c. Confirmation of extracted DNA via agarose gel electrophoresis

Extracted DNA often contains a large amount of RNA and pigments, which cause over valuation of DNA concentration during Nanodrop reading. DNA samples were evaluated qualitatively using 1% agarose gel electrophoresis.

d. Preparation of 1% agarose gel (100 ml)

i. A clean 250 ml conical flask is filled with 100 ml 1X TAE (electrophoresis buffer), then 1.0g agarose was added in to it.

ii. After that flask-head was covered with aluminum foil and the mixture was heated in micro-oven for 2 minutes to get uniform suspension until the solution become transparent;

iii. The gel was turned cold at 60^0 C, then poured onto gel casting tray carefully;

iv. In the meantime, comb was inserted with the bottom of the teeth;

v. Wait, until the gel became solidified, then carefully removed the comb from the gel;

vi.Later, the gel was sunken into TAE buffer, and ensures that, the final level of the buffer must be 1cm above from the gel. The gel was then ready for loading the DNA samples;

E. Preparation of DNA for electrophoresis

i. Loading-dye and DNA marker were required for this step;

ii. At first, 1µl loading dye was dropped on a parafilm for each sample by using micropipette;

iii. 4µl extracted DNA sample was added to that drops and mixed well, then the mixture was loaded in the lane of the gel;

iv. The known DNA marker was loaded in the first lane of the gel;

v. The gel tank was covered and the electrophoresis apparatus were connected to the power supply unit;

vi. After switching on, DNA was migrated from negative to positive electrode;

vii. The migration process was monitored by the movement of the dye in the loaded gel;

viii. Electrophoresis was carried out at 100 volts for 20 minutes;

ix. When the bromo-phenol blue dye had reached at 4/5 of the gel length, the electrophoresis was stopped and the gel with bed was picked up from the buffer tank;

x. Subsequently, the gel was immersed in 1.0 µg/ml ethidium bromide (Lee et al.,2012) solution, and stained for 30 minutes;

f. *Documentation of DNA samples*

After electrophoresis and ethidium bromide staining, the gel was placed on the UV trans-illuminator in the dark chamber of the gel documentation system to observe DNA band. The UV light of the system was switched on; the image was viewed on the monitor and data were recorded.

g. Amplification of housekeeping gene by PCR (polymerase chain reaction)

To amplify the 16S rRNA gene, a single oligo-nucleotide of an arbitrary DNA sequence is mixed with genomic DNA in the presence of a GoTaq® G2 Green Master Mix with a suitable buffer. The products of the reaction depend on the sequence and length of oligonucleotide, as well as reaction conditions. At an appropriate annealing temperature during the thermal cycle, the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other and a discrete DNA segment is produced. The presence or absence of this specific gene, although amplified with an arbitrary primer will be diagnostic for the oligonucleotide-binding sites on the genomic DNA.

h. Preparation of PCR reaction mixture

The following components were used to prepare a PCR reaction mixture. The total volume of reaction mixture was 50µl per sample. The amplification conditions originally recommended by Williams et al. (1990) were applied with slight modifications. Master mixture (except template DNA) was prepared using all components for total samples and then distributed to individual PCR tubes; then the template DNA was added in the PCR tubes.

Table 1. PCR reaction mixture**:** For a 50 µl reaction volume

Component	Volume
PCR Master Mix, 2X	25.0 µl
upstream primer, 10µM	2.5µl
downstream primer, 10µM	2.5µl
DNA template, <250ng	10 µl
Nuclease-Free Water to	50 µl

i.Thermal profile for the amplification of 16S rRNA gene

DNA amplification was performed in a thermal cycler. The PCR tubes were set on the wells of the thermo-cycler. Then the machine was run according to the following sequential setups:

i. Initial denaturation at 94^0 C for 5 min

ii.Denaturation at 94^0 C for 1 min

iii.Annealing at 55^0 C for 45 Sec

iv. Elongation at 72^0 C for 2 min

v. Final elongation at 72^0 C for 7 min

vi. Completion of cycling program, reactions were held at 4^0C.

Plate 4. Samples running at PCR

j. Documentation of the amplified 16S rRNA gene through electrophoresis

The amplified products were separated by electrophoresis on 1.0% agarose gel in 1X TAE buffer at 100 volts for 20 minutes. Molecular weight marker 1Kbp ladder was electrophoresed alongside the PCR products. After completion of electrophoresis, the gel was soaked in ethidium bromide solution for 25-30 minutes. After staining, the gel was taken out carefully from the staining tray and placed on the UV trans-illuminator in the dark chamber of the gel documentation system to observe amplified bands and saved the records.

k. PCR product purification for gene sequencing

To protect the PCR product from damage, it must be preserved in purified condition for sequencing purpose wher the procedureal steps were as follows:

After confirming the amplified gene via electrophoresis, PCR products were kept in an eppendorf (500 µl) tube and then 10 times ethanol (100%) was mixed with the PCR product. After proper mixing with 100% ethanol, equel amount of ammonium acetate was added and mixed by inverting and the centrifuged at 13000 rpm for 20 minutes at 4 ^{0}C; supernents were discarde and again mixed 70% ethanol with remaining PCR products and centrifuged at 13000 rpm for 20 minutes at 4^0C and discarde supernatents and dried the pellet. Finally, equel amount of nuclease free water was added to PCR products which was used for sequencing.

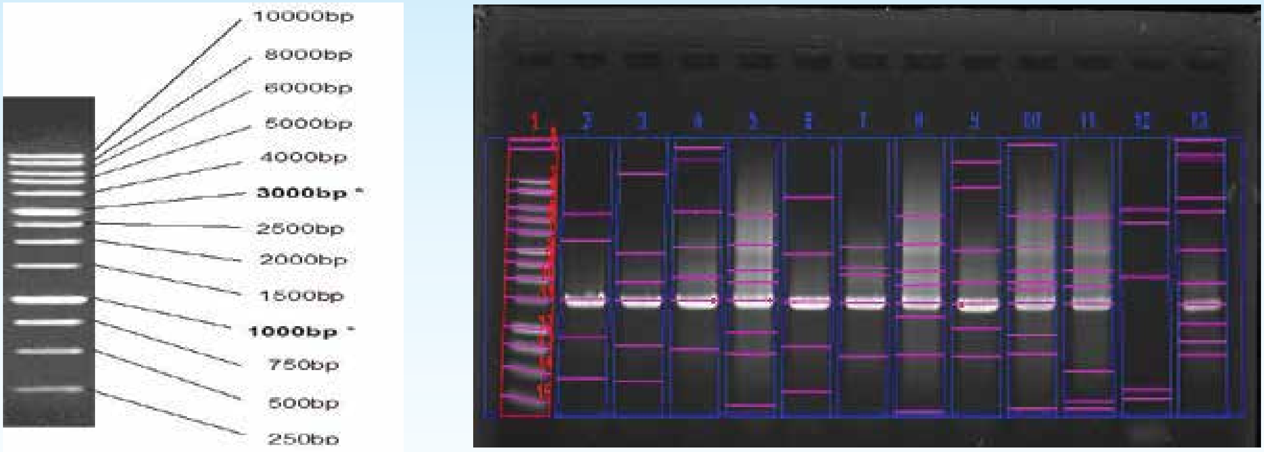
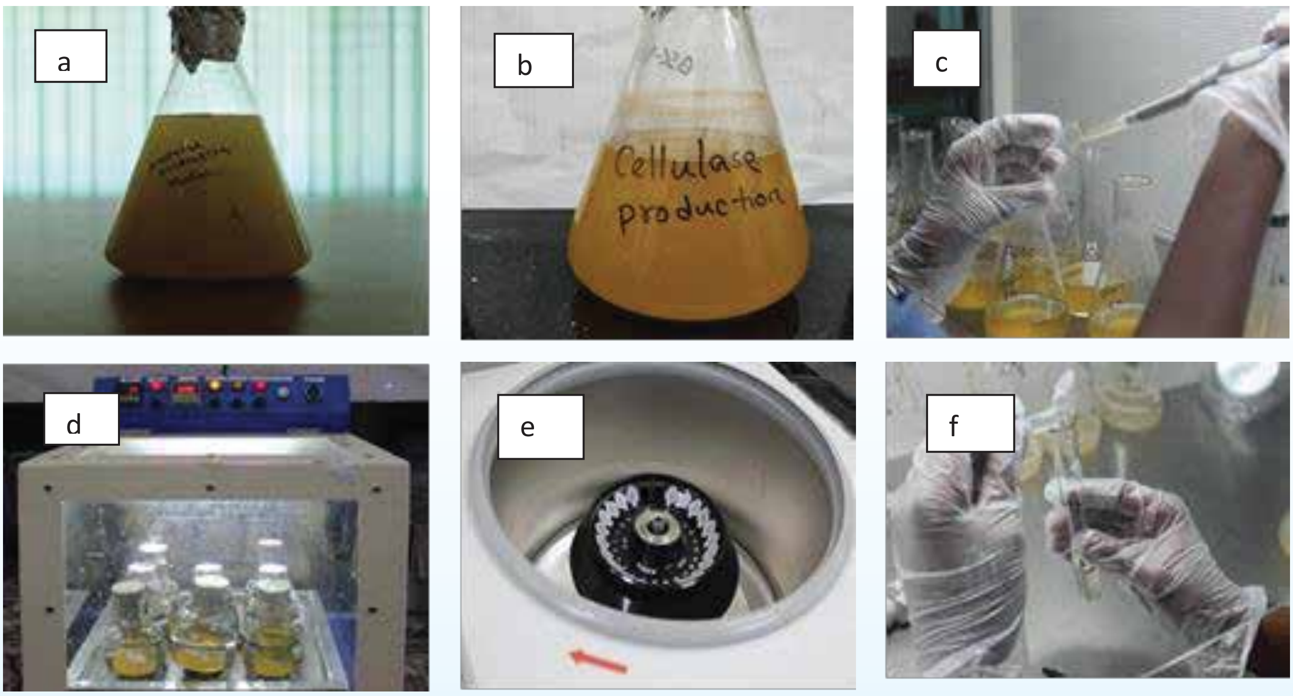


Fig 1. Purified PCR product of target sample

10.2.8. Production of crude enzymes

The screened isolates of protease, cellulase and phytase were then cultured in broth medium and incubated at 37 °C for overnight. Then the cultures were inoculated in the production media of cellulase, protease and phytase enzyme. The production of cellulase, protease and phytase enzymes were done according to following protocol.



Plates 5. a) Protease production media, b) Cellulase production Media, c) Inoculation of bacteria. d) Enzyme production, e) Centrifugation and f) Crude enzyme collection

a.Procedures of cellulase enzyme production

- Production medium contained (g/L) glucose 0.5 gm, peptone 0.75 gm, $FeSO_4$ 0.01 gm, KH_2PO_4 0.5 gm, and $MgSO_4$ 0.5 gm
- Ten millilitres of medium were taken in a 100 mL conical flask. The flasks were sterilized in autoclave at 121°C for 15 min, and after cooling, the flask was inoculated with overnight grown bacterial culture
- The inoculated medium was incubated at 37°C in shaker incubator for 24 h
- At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme
- The supernatant was collected and used for further experiments.

b.Procedures of protease enzyme production

- For enzyme production media consisting of Casein 2.0%, Dextroses 1.0%, Peptone 1.0%, KH_2PO_4 2.0%, $NaCl_2$ 0.2%, $CaCl_2$ –0.002% at PH 7.0 was used
- Inoculums (OD 660 nm) were developed by growing the isolate in nutrient broth for 24 h.
- For production of enzyme, 1.0% inoculums was added to 50 ml production medium in 250 ml conical flasks and then incubated at 37°C for 3 – 4 days
- Sample withdrawn at specific time intervals were centrifuged at 10,000 rpm for 10 min
- The supernatant was collected and used for further experiments.

c. Procedures of phytase enzyme production

- Media was prepared with 500 ml Heart infusion broth supplemented with 10 mM $CaCl_2$, 1% glucose, 1% D Mannose, 0.5% Yeast extract
- Phytase activity is routinely measured by incubating 150 µL enzyme solution with 600 µL of 2 mM Na-phytate in 0.1M tris-HCl buffer supplemented with 2mM $CaCl_2$
- After 15-30 min of incubation reaction was stopped by adding 750µl 5% TCA
- 1.5 ml color reagent (1.5% ammonium molybdate in 5.5% Sulfuric acid and 2.5% Ferrous sulfate in 4:1 ratio) was added
- Liberated Pi was measured at 700 nm

10.2.9. Enzyme activity assay

10.2.9.1. Cellulase assay

Cellulase activity was measured according to the method of Denison and Kohen (1977) using 1% CMC in citrate bu¡er (0.1 M, pH 5.0) as substrate. The production of reducing sugar (glucose) from CMC substrate because of cellulolytic activity was measured at 540 nm by dinitrosalicylic acid method using glucose as the standard. One cellulase unit is defined as the amount of enzyme per milliliter culture filtrate that released1micro-gram glucose per minute.

a. Steps for cellulase enzyme assay

Enzyme production

Production media was prepared (0.01% $MgSO_4$, 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.05% Sodium citrate,0.1% carboxymethyl cellulose (CMC)). After inoculation of bacteria were incubated at 37 °C in 200 rpm shaker incubator for 72 hrs.

b. Crude enzyme collection

After 72 hrs of culture, culture media was centrifuged at 10,500 rpm for 10 min. After centrifugation supernatant was collected as crude cellulase enzyme.

Reagent preparation for assay:

DNS reagent

30g potassium sodium tartarate in 50 ml DW + 1g DNS powder + 20 ml 2N NaOH, made the volume 100 ml

Substrate solution

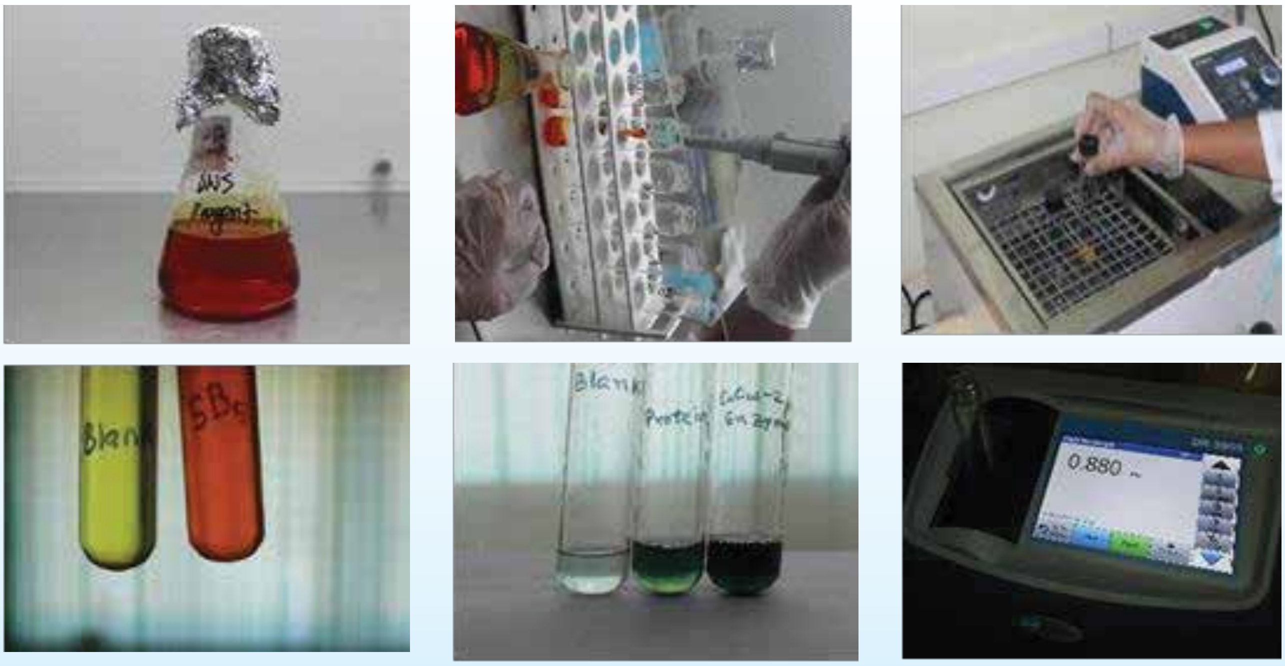
To make phosphate buffer we added 0.315g KH_2PO_4 and 0.467g K_2HPO_4 and made the volume 100 ml. Then we added 1% CMC in that buffer that will be used as substrate solution

Substrate Blank

1% CMC in citrate buffer (2.1 g citric acid +0.55g NaOH and made volume 100 ml)

c. Assay procedure

- Preparation of substrate solution (1% CMC in 0.05M phosphate bufferate buffer)
- 10 ml of culture centrifuged at 5000 rpm for 15 min
- 0.5 ml Culture supernatant + 0.5 ml substrate solution
- Incubate at 50 degrees for 30 min
- Add 3 ml DNS reagent, reaction mixture boiled for 5 min
- 20 ml distilled water added and OD measured at 540 nm



Plates 6. Protease, cellulase and phytase enzyme assay

10.2.9.2. Protease assay

a. Preparation of reagents

A 50 mM Potassium Phosphate Buffer, pH 7.5

1.14 % of potassium phospate dibasic, trihydrate in purified water and adjusting pH with 1 M HCl. This solution is placed at 37 °C before use.

A 0.65% weight/volume casein solution

0.65 % casein in the 50 mM potassium phosphate buffer. The solution temperature is gradually increased with gentle stirring to 80-85 °C for about 10 minutes until a homogenous dispersion is achieved. Do not to boil the solution. The pH is then adjusted, if necessary, with NaOH and HCl.

A 110 mM Trichloroacetic acid

Prepared by diluting a 6.1 N stock 1:55 with purified water

0.5 M Folin & Ciolcaltea's, or Folin's Phenol Reagent

A 500 mM Sodium Carbonate solution

5.3 % of anyhydrous sodium carbonate in purified water.

An enzyme diluent solution

Which consists of 10 mM Sodium Acetate Buffer with 5mM Calcium acetate buffer, pH 7.5, at 37 °C.

1.1 mM L-tyrosine Standard stock solution

0.02 % L-tyrosine in purified water and heated gently until the tyrosine dissolves. Allow the L-tyrosine standard to cool to room temperature. This solution will be diluted further to make the standard curve.

b. Assay Procedure

- 5ml alkaline solution + 1ml test solution were taken and mixed properly
- After thoroughly mixing stand at room temp for 10 min or longer
- 0.5ml FCR was added and mixed immediately
- After 30 minutes the OD was checked at 750 nm

10.2.9.3. Phytase assay

***a. Reagents*:**

- 0.1mM tris-HCl buffer supplemented with 2mM $CaCl_2$
- mM Na-phytate
- Color reagent (1.5% ammonium molybdate in 5.5% Sulfuric acid and 2.5% Ferrous sulfate in 4:1 ratio)

b. Assay Procedure

- Phytase activity is routinely measured by incubating 150 µL enzyme solution with 600 µL of 2 mM Na-phytate in 0.1M tris-HCl buffer supplemented with 2mM $CaCl_2$
- After 15-30 min of incubation reaction was stopped by adding 750µl 5% TCA
- 1.5 ml color reagent was added
- Liberated Pi was measured at 700 nm

10.2.10. Enzyme purification

a. Procedure

- Pipet 4 ml of the enzyme solution into a test tube.
- While stirring, add the saturated ammonium sulfate solution drop-wise from a burette to the protein solution until precipitates start to form.
- Record the volume of the saturated ammonium sulfate solution needed to cause precipitation and collect the protein precipitates. Also note that protein precipitation is not instantaneous; it may require 15--20 minutes to equilibrate.
- Centrifuge the mixture at 10,000 g for 15 minutes. Collect the precipitate by carefully discarding as much supernatant as possible.
- The precipitates may be collected by following a similar washing procedure as first adding approximately 2 ml of water to the centrifuge tube, shaking the tube to redissolve the precipitate, and transferring as much as possible the protease solution from the centrifuge tube into a test tube with a pipet while noting the volume.
- Try not to dilute the enzyme solution too much

b. Calculation of enzyme activity

$$\text{Units/mL Enzyme} = \frac{(\mu\text{mol tyrosine/glucose/phosphate equivalents released}) \times (\text{total volume of assay})}{(\text{Volume of enzyme used}) \times (\text{time of assay}) \times (\text{volume used in colorimetric Determination})}$$

10.3. Component 2 (NSTU)

10.3.1. Study area

Based on primay information on availability of polychaetes, Malancha River of Satkhira range (Sundarbans) and Bakkhali River, Moheskhali Channel, Cox’s Bazar were selected for polychaetes sample collection (Fig 2 & 3). Therefore, field sampling was repeatedly performed in these areas from March 2020 onward to April 2022.

Based on locat information and secondary information fom literatures, four sites were selected from the Sundarbans mangrove areas and five siters were selected from Cox’s Bazar areas. Regionwise sampling sites are listed as follows:

<u>Sunderbns area</u>

SS-1 (Chunkuni River), sampling depth, 15 – 25 cm

SS-2 (Malancha River), sampling depth, 20 – 25 cm

SS-3 (Malancha River), sampling depth, 15 – 23 cm and

SS-4 (Tader Varani River), sampling depth, 12 – 20 cm.

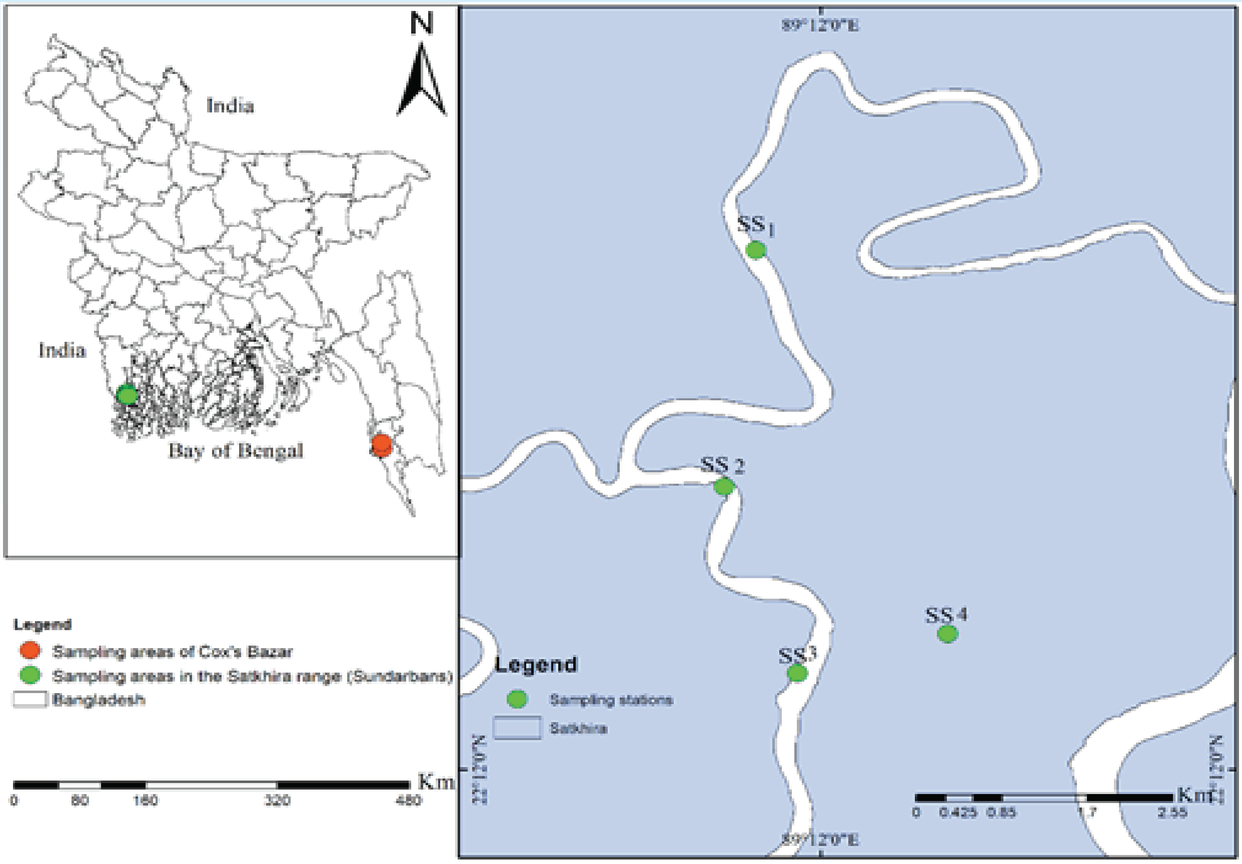


Fig 2. Sampling areas in the Satkhira range (green circled) and Bakh Khali River, Cox's Bazar (red circled).

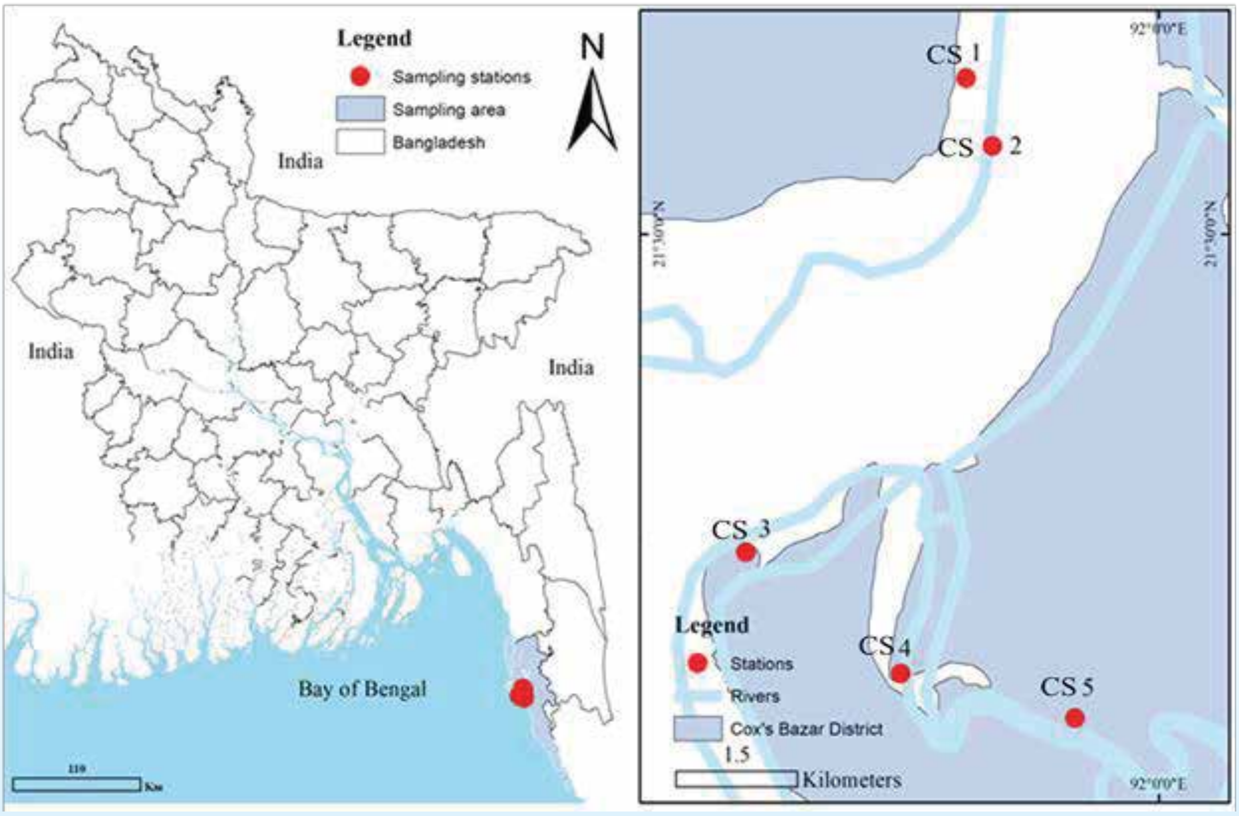


Fig 3. The map shows the cox's Bazar district and the sampling stations (red circled)

Cox'sBazar area

CS-1 (Bakkhali River), sampling depth, 12 – 30 cm
CS-2 (Bakkhali River), sampling depth, 15 – 30 cm
CS-3 (Charpara), sampling depth, 12 – 25 cm
CS-4 Moheshkahli channel, sampling depth, 15 – 35 cm
CS-5 Moheshkahli channel, sampling depth, 15 – 35 cm

10.3.2. Polychaetaes collection and identification

Monthly sampling was performed during low tide from March 2020 to April 2022 using a square-shaped mud corer with a 0.093 m^2 (or 1.0 ft^2) opening. Each station had three sediment samples taken at 1.0 m interval. Specimens were collected from 12.0-35.0 cm depth, depending on where the intertidal zone was located (typically middle to lower-middle). Monthly water temperature and salinity data were collected using a multiparameter sonde (HANNA, model: HI9829, Romania). The sediments were sieved (0.5 mm mesh size), and polychaetes specimens were deposited into a previously prepared bucket (3.0 cm sand bed with natural seawater). The samples were brought to the research station for further study. The specimens were counted, and only complete samples were considered for culture and length-weight (L-W) data. During the study period, approximately 8,094 polychaetes specimens were collected, with 1300 specimens (n) being measured for L-W, while rest of the specimens were introduced for culture and preserved in refrigerator (- 22.0 ˚C) for further study. A simple centimeter (cm) scale and a digital weighing scale (Model: PS. P3.310, P-Scale, Taiwan) were used to take L-W data of individual samples. The specimens were identified as *Dendronereis dayi*, *Glycera* cf. *sheikhmujibi*, *Glycera* sp., *Lycastonereis indica*, *Namalycastis fauveli*, *Namalycastis* sp., *Neanthes* sp., and *Composetia* sp. based on the morphological features and taxonomic keys provided Glasby (1999: 100 – 103); Pramanik et al., (2009: 62 – 63); Muir and Hossain (2014) Conde-vela (2017), Hsueh 2019, Hossain and Hutchings (2020) and other relevant literature.

Only *Namalycastis* sp. was available throughout the year, that's why this species was chosen for population dynamics and culture research.

Plate 7. Polychaete collection from the field

10.3.3. Population parameter study

Length-weight (L-W) determination

For determining L-W relationship, the following equation is applied for polychaetes (Paavo et al. 2008; Rosati et al. 2012).

$$W=aTL^{b}, \quad (1)$$

where, W= BW-body weight in g of *Namalycastis* sp., a = intercept, b= slope, and TL = total length in cm. Usually, this equation is solved by adding $\log_{10}$ base as follows:

$$\mathbf{Log_{10}(BW) = Log_{10}(a) + b \times Log_{10}(TL)}$$

If b > 3, it indicates positive allometric growth, b = 3 denotes isometric growth, which means a perfect cubic relationship between length and mass. Moreover, b < 3 indicates negative allometric growth of *Namalycastis* sp.

10.3.4. Analysis of population dynamics of *Namalycastis* sp.

The recorded data were analyzed using the FiSAT-II (FAO-ICLARM Stock Assessment Tools) following the revised version explained by Gayanilo et al., (2005). The asymptotic total length (TL_∞) and growth co-efficient (K) of the von Bertalanffy equation for growth considering the TL of a *Namalycastis* individual was calculated by ELEFAN-I (Electronic Length Frequency Analysis) incorporated in the FiSAT-II software. The TL_∞ and K were measured by using the formula 2 as follows:

$$TL_t = TL_\infty (1-e^{-K(t-t_0)}), \quad (2)$$

where, t denotes the age of the *Namalycastis* sp. (year), TL is the mean total length at age t (cm), t_0 is the hypothetical age when TL was zero, and K represents a growth coefficient ($year^{-1}$).

The growth performance index (ϕ) of *Namalycastis* sp. Was estimated using the K and TL_∞ values using the Pauly and Munro's (1984) equation (3) below:

$$\phi' = \log K + 2 \log TL_\infty, \quad (3)$$

Beverton (1956) length converted catch curve method was applied to calculate the total mortality (Z) incorporating the average annual habitat temperature 28.58 ˚C measured during the sampling period.

Natural mortality (M) was calculated by applying the empirical relationship of Pauly (1980). Once Z and M were estimated, fishing mortality (F) was obtained using the equation no. 4 as follows:

$$F = Z - M, \quad (4)$$

The exploitation level € was determined by the equation no. 5 developed by Gulland (1971) as follows:

$$E = F/Z, \quad (5)$$

Recruitment pattern was estimated by the backward projection method on the TL axis of a set TL-frequency data as explained in the FiSAT-II management software. The 'probability of capture' routine was applied to calculate the possibility of *Namalycastis* sp. Capture, and the FiSAT-II analyzer was applied to estimate the approximate TL structured virtual population.

10.3.5. System setup and stocking density

A total of 9 identical sizes aquariums (46 cm length × 35.5 cm breadth × 30.5 cm height) were used in different treatments (e.g., T1, T2, T3) for the growth and survival of *Namalycastis* sp.(Fig 4)

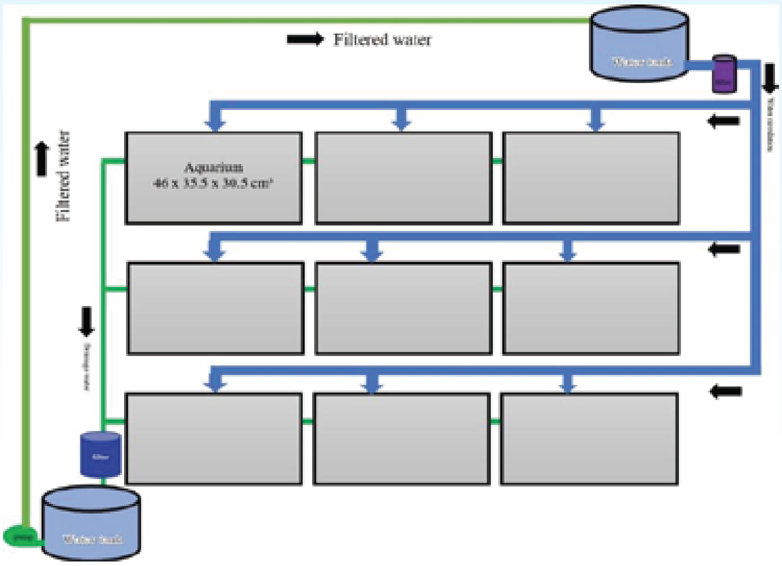


Fig 4. An indoor semi-circulatory culture system is showing its components (e.g., aquariums, filtrations, and pumps).

Natural sand bed (10.0 cm depth, ≤ 500 µm grain size) was dried in sunlight and heated at 90 °C for overnight in a hot air oven to kill any microorganisms (Fidalgo e Costa et al., 2000). An inlet and outlet water pass (2.3 L min^{-1}) systems were kept avoiding any gaseous and excretory metabolites formation in the aquarium. As there are no commercial polychaetes supplier in Bangladesh, we collected from the wild. Continuous oxygen supply was provided by two aerators (Model no. ACO-003, 35 W, Resun, China). The collected *Namalycastis sp.* specimens were sorted out and acclimatized for 24 hrs and were stocked as 200 (1225 specimens m^{-2}) specimens in each aquarium. Water quality parameters such as temperature (26 ± 2.0 °C), DO (4.80 ± 0.70 mg/L), pH (7.30 ± 0.30) salinity (17.0 ± 2.0 PSU) and TDS (20.0 ± 2.0 ppt) were maintained and measured daily. A 16L:8D photoperiod was maintained with an average 130 Lux light intensity. The survival rate and fresh weight were taken fortnightly.

Plate 8. Indoor semi-circulatory culture system with required facilities (Thermostat water heater, aquariums, filtrations, and pumps).

10.3.5.1. Feeds and Feeding

Three commercial diets characterized by different protein and fat content were labeled by the feed company. The specimens were fed treatment-wise (T1, T2 & T3) with one of the 3 types of feed namely T-1(code:155-FF-01,Belgium), T-2(Code:155-FF-02,Vietnam) and T-3(Code:155-FF-03,Thailand). These feeds are locally available and widely applied as commercial shrimp feeds [Code:155-FF-01 (protein: 42.00%, fat: 7.00%), code:155-FF-02 (protein: 42.00%, fat: 4.00%) and Code:155-FF-03 (protein: 38.00%, fat: 5.00%)] The polychaetes were cultured for 90 days from October 2020 to January 2021. Feeding was provided once a day (afternoon) at 3% body weight of polychaetes.

Plate 9. *Namalycastis sp*

10.3.5.2. Final Weighing and calculations

At the end of 90 days experimental period, all individuals were counted and weighed (wet weight). Then the samples were stored at -80°C freezer for fatty acid analysis. The following calculations were made from the cultured data:

- **Specific Growth Rate (SGR):** According to Nielsen et al., 1995; Fidalgo e Costa et al., 2000; Batista et al., 2003; Parandavar et al., 2018

$$SGR\ (\%\ d^{-1}) = \frac{100[\ln(final\ wet\ weight) - \ln(initial\ wet\ weight)]}{duration\ (90)} \quad \text{...............(1)}$$

- **Daily Growth Rate (DGR): (**Fidalgo e Costa et al., 2000)

$$DGR\ (mgd^{-1}) = \frac{(final\ wet\ weight - initial\ wet\ weight)}{duration} \quad \text{.........................(2)}$$

- **Feed Conversion Ratio (FCR): (**Parandavar et al., 2018)

$$FCR = \frac{Feed\ fed}{(final\ wet\ weight - initial\ wet\ weight)} \quad \text{...................................(3)}$$

- **Feed Efficiency Ratio (FER): (** Batista et al., 2003)

$$FER = \frac{(final\ wet\ weight - initial\ wet\ weight)}{feed\ dry\ weight} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

- **Weight Gain (%): (**Parandavar et al., 2018; Pajand et al., 2020)

$$WG\ (\%) = \frac{Final\ biomass\ - Initial\ biomass}{Initial\ biomass} * 100 \ldots\ldots\ldots\ldots\ldots(5)$$

- **Survival Rate (SR): (**Parandavar et al., 2018; Thi Thu et al., 2019; Pajand et al., 2020)

$$SR\ (\%) = \frac{Final\ no\ of\ specimen\ survived}{Initial\ no\ of\ specimen} * 100 \ldots\ldots\ldots\ldots\ldots\ldots(6)$$

10.3.6. Fatty acids analysis

The stored polychaetes were thawed and heated in hot air oven at 75°C for overnight for extraction. Then the extracted samples (12.0 g of each) were weighted and placed in paper thimble. The crude fat was extracted in Soxhlet apparatus for 3.5 h heating at 70 °C following the AOAC (2000) certified standard methods.

Fatty acid methyl esters (FAMEs) were prepared from the extracted crude fat (50 gm from each sample) and mixed with 2 ml of solvent (methanol/hexane, 4:1), and 200 µl of acetyl chloride was steadily added. The aliquot was heated at 100°C in a heating box for one hour. Following this, 5 ml of 6% K_2CO_3 and 2 ml of hexane were gradually added. After that the solution was centrifuged at 3000 rpm for 15 min. Finally, the hexane layer was collected and dried with sodium sulphate (Na_2SO_4).

Fatty acid composition was determined by preparing FAMEs and was analysed them by gas chromatography (AOCS, 1992). The gas chromatography (Model no. 14B, SHIMADZU, Japan) with flame Ionization detector (GC-FID) was loaded with software class GC-10 (Version-2.0). The GC was prepared with flame ionization detector (FID) and capillary column, with dimension 15 m x 0.25 mm ID 0.2 µm. The functional condition was automated at oven temperature 150°C (hold time 5 min), 8°C /min-190°C (hold time 0 min), 2°C/min-200°C (hold time 10 min), injection port temperature 250°C and detector temperature 250°C. Fatty acid peaks were identified from standard fatty acid mixtures. Nitrogen was used as carrier gas, flow rate 20 mL/min and aliquots of 1 µL FAME (formed by esterification of polychaetes oil samples) was injected and the peaks of fatty acids were documented for their holding time and areas by the data processor unit of GC.

Statistical analysis

Data were checked for normality and homoscedasticity. All data were subjected to one-way analysis of variance (ANOVA) to assess differences between diet treatments concerning growth and survival rates. When the conditions were not fulfilled, Kruskal-Wallis non-parametric test was performed. When differences were found, Tukey HSD post-hoc tests were employed. Where applicable, results are presented as mean ± SD. Microsoft Excel, SPSS (version: 24.0), PAST (version 4.03) used for single-factor ANOVA, Tukey-Kramer test, and other statistical analysis.

10.3.7. An alternative freeze-drying method was used for total fat and fatty acid analysis

Both the wild and cultured worms (for salinity effect in 15, 20, 25, 30, 35 and 40 PSU) were rinsed with deionized water five times each, blot-dried with paper towels and then weighed in 50 mL Falcon centrifuge tubes. The samples were frozen at −80°C for at least 24 h, freeze-dried for 72 h using a freeze dryer (VaCo 2 series, Zirbus Technology, Germany). The samples were manually crushed into finer bits and consolidated into seven samples namely, WN (wild), 15, 20, 25, 30, 35 and, each with dry weight of 10 g. The samples were then sent to a commercial service (Setsco Services Pte Ltd, Singapore) for total fat and fatty acid analysis. The analysis methods used were the AOAC Official Method No. 991.36 (2006) for total fat and the AOAC Official Method No. 996.06 (2008) for fatty acids by GC-FID Agilent technologies 7820A, China at 0.01% detection limit.

10.3.8. Develop of mass culture method of Omega-3 fatty acid containing Polychaetes

Under this section methodology for culture system and stocking density studies were followed as per objective II with necessary modification. The culture parameters (such asspecific growth rate, weight gain, etc.) were also calculated following the procedure as of objective II.

Methodlogy followed for relevant other studies such as habitat preferences and density-dependent growth, growth optimizing and survival, effect of different formulated feed on growth and survival and unraveling the growth and survival of the *culture animal* on four variable diets in an open water culture system were carried out as per following experiments**.**

10.3.8.1. Exp-1: Habitat preferences and density-dependent growth of Namalycastis sp. (Polychaetes, Nereididae) in semi-circulatory culture systems

For density dependent growth, *Namalycastis* sp. specimens were stocked at 500, 1000 and 2000 indv. /m^2 in DT-1, DT-2 and DT-3 treatment (triplicating of each), respectively in 9 identical aquariums (46 cm length × 35.5 cm breadth × 30.5 cm height). Six types of substrates namely: sand beds at 3.0, 7.0, 10.0 cm depth (≤ 500 μm grain size), mixer of silty sand and ≤ 7.0 mm diam. gravel (4.0 cm + 2 cm), silty sand (6.0 cm) and unmodified mangrove mud (8.0 cm) were prepared for T-1, T-2, T-3, T-4, T-5 and T-6, respectively in 18 identical aquariums. Treatments were stocked at 500 indv. /m^2 and 3 % body weight/day. Fish nursery powdered feeds code: 155-FF-08, Protein 40 %, Carbohydrate 22 %, Moisture 12 %, Lipid 5%, Ash 16 %, and Fiber 5 %) was provided. Water quality parameters such as temperature (26 ± 2.0 °C), DO (4.80 ± 0.70 mg/L), pH (7.30 ± 0.30) salinity (17.0 ± 2.0 PSU) and TDS (7.0 ± 5.0 ppt) were maintained and measured daily. A 16L:8D photoperiod was maintained with an average 130 Lux light intensity. The survival rate and fresh weight were taken fortnightly. Both the treatments were cultured for 90 days.

10.3.8.2. Exp-2: Optimizing the growth and survival of Namalycastis sp. (Polychaetes, Nereididae) in variable temperature

Namalycastis sp. was stocked at density 500 indv. /m^2 at five treatments namely: T26, T28, T-30, T-32, and T-34 at 26, 28, 30, 32 and 34 ˚C, respectively in 15 aquariums (22 cm length × 10 cm breadth × 30.5 cm height). Commercial shrimp nursery feeds (code:155-FF-09, 40 % protein, 10 % moisture, 9% lipid, 14 ash % and 5 % fiber) were provided at 4 % body weight/day. The stocked was cultured for 40 days and sampled at five days interval.

10.3.8.3. Exp-3: Effect of growth and survival of Namalycastis sp. (Polychaetes, Nereididae) in different formulated diets in a semi-circulatory culture system.

A total of four feeds were prepared as T1 (code:155-FF-04), T2 (code:155-FF-05), T3 (code:155-FF-06) and T4 (code:155-FF-07), respectively in 12 identical aquariums (46 cm length × 35.5 cm breadth × 30.5 cm height). *Namlycastis* sp. was stocked at density 500 ind./m^2 with 3% feeds body weight once in a day. Specimens were sampled fortnightly and culture duration was 75 days.

Plate 10. Mass culture method of *Namalycastis sp.* Indoor culture system

10.3.8.4. Exp-4: Unraveling the growth and survival of Namalycastis sp. (Polychaetes, Nereididae) on four variable diets in an open water culture system

A total of 15 circular sized (inner diameter: 81.28 cm and height: 30.48 cm) concrete ring was used to stock *Namalycastis* sp. *Namalycastis* sp. was stocked at 500 ind./m^2 by calculating the inner walls the area (πr^2). The rings were divided into five treatments (T1 to T5) triplicating of each supplied with commercial feeds (T1 and T2), formulated feeds (T3 and T4), and no feed (T5) were selected. Commercial feeds T1 (code:55-FF-03, protein 38 %, fat 5%, fiber 4 %, moisture 12%), T2 (code:155-FF-01, protein 42 %, fat 7%, fiber 3 %, moisture 9%), formulated feeds T3 (code:155-FF-04), T4 (tilapia fins: intestine: potato peels at 1:1:1) and T5 (no feed) were selected, respectively. *Namalycastis* sp. was sampled every 10 days interval and cultured from March to April 2022 (60 days).

Culture and growth parameters such as Specific Growth Rate (SGR), Daily Growth Rate (DGR), Feed Conversion Ratio (FCR), Feed Efficiency Ratio (FER), Weight Gain (WG) and Survival Rate (SR)were calculated as per formulae described under the previous section 10.3.5.2.

Plate 11. Outdoor culture experiment of *Namalycastis* sp.

10.3.9. Explore growth performance of shrimp/mud crab using formulated supplementary feed from polychaetes

Under this section methodology for culture system and stocking density studies were followed as per objective II with necessary modification. The culture parameters (such asspecific growth rate, weight gain, etc.) were also calculated following the procedure as of objective II.

A total of 125 male and female *S. olivacea* were used for this experiment. Moropometric traits namely: internal carapace width (ICW), propodus length (PL), chela height (CH), lower paddle width (LPW) was taken by measuring taps before and after the experiment. Similarly weighted male and female were sorted out and dissected to extract gonads/testis and ovaries, respectively. The gonadosomatic index (GSI) = (wet weight of gonad/wet weight of the crab) × 100 (Barber & Blake, 2006) was calculated for evert crabs. Three natural diets namely: Polychaetes *(Namalycastis* sp.), Tilapia muscles (*Oreochromis niloticus*) and little neck clam (*Mercenaria mercenaria*), respectively were selected for the growth performances. For each diet, five replicates were prepared and total of thirty crabs were cultured dividing into three diets performances. Feeding was performed once a day at 7 % body weight and daily 100 % water was exchanged. The crabs were cultured for 25 days.

Plate 12. Culture experiment with various diets for mud crab growth performance.

In addition to the above, the male and female *Scylla olivacea* were identified by observing the narrower abdominal flap like a “V” shape for male craband wider abdomen like “U”-shape for frmale crab

Plate 13: *Scylla olivacea*

Plate 14. Identification of male and female *Scylla olivacea*

Plate 15. Measuring the morphological traits of *Scylla olivacea*.

10.3.9.1. Measuring the morphometric traits

Before and after introducing into the indoor culture, the morphological traits of male and female specimens were measured. For example, the internal carapace width (ICW), propodus length (PL), chela height (CH) and lower paddle width (LPW) were measured.

Isolating the male and female gonads and calculating the gonadosomatic index (GSI):

Gonad/Testis(G/T) **Ovary (OV)**

Plate 16. Isolating the male gonad/testis and female ovaries from Scylla *olivacea*.

10.3.9.2. Gonadosomatic index (GSI)

GSI was calculated based on the equitation applied by Barber & Blake (2006):

GSI = (*wet weight of gonad/wet weight of the crab*) × 100

Plate 17. Weighing (g) of mud crab

10.4. Component-3 (BFRI)

10.4.1. Exp I: Determination of monovalent efficacy of B. subtilis as probiotic candidate

10.4.1.1. In-vitro challenge test for antagonistic effect of Bacillus subtilis against V. parahaemolyticus

For in-vitro challenge test, the method provided by International Standards Organization (ISO) is being followed with some modifications. *Bacillus subtilis* was isolated from shrimp intestine and pure culture was done. Characterized *Vibrio parahaemolyticus* and *B. subtilis* was cultured separately in nutrient broth media and centrifuged at 3500g for 5 min. The supernatants were discarded, and *V. parahaemolyticus* as well as *B. subtilis* were separately re-suspended in peptone water. The *V. parahaemolyticus* suspension was diluted to approximately 10^4 colony forming units (cfu)/mL and the *B. subtilis* suspension was diluted to 10^6 cfu/mL, called test solutions. After that, 0.5 ml of each test solutions was mixed together. Then the mixer solution was inoculated in TCBS Agar media at subsequent intervals of 4 h up to 12 h. Test solution of *V. parahaemolyticus* without *B. subtilis* was also inoculated in TCBS Agar media at 0, 4, 8 and 12 h subsequently. All the inoculated Agar plates were incubated at 37 ºC for 12-24 h. Standard plate count was done after incubation.

10.4.2. Expt II: Determination of efficacy of *B. subtilis* on growth and survival of *P. monodon*

For this experiment, four ponds of SRS pond complex were partitioned into two parts and the ponds were prepared following drying, liming, and fertilization. Then the ponds were stocked according to the experimental design given in Table 2.

Table 2. The experimental design

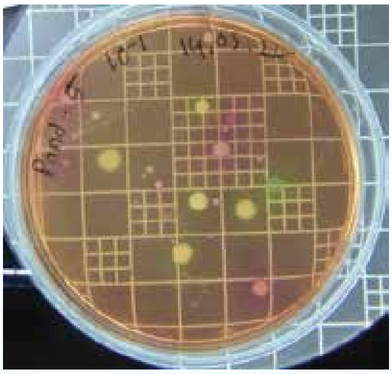
Treatment	Dose (cfu/g feed) of *Bacillus Subtilis*	Mode of application	Days of culture	Replication
T1	$2*10^{8}$	Dietary supplementation	80	2
T2	$2*10^{9}$			
T3	$2*10^{10}$			
T4 (Control)	-			

10.4.3. Exp III: Determination of the combined effect of *Bacillus sp.* on growth and survival of *Penaeus monodon*

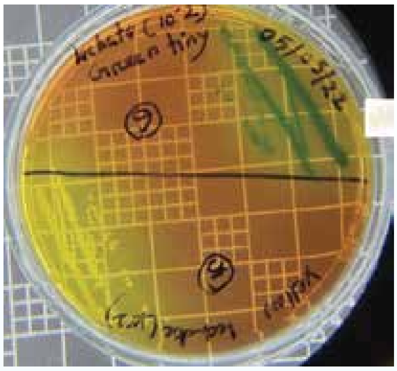
10.4.3.1.Screening and Isolation of probiotic bacteria (Bacillus sp.) from local sources using selective agar media

Water and soil samples were collected from local gher and river and fish samples (Tilapia) were collected from local fish market. Then these samples were transported to Shrimp Health Management laboratory, SRS. Then, bacteria were isolated (initially based on colony morphology, *viz*., shape, size and color) from the samples using Hi-chrome Bacillus Agar media. After that, streak plate technique was used for the purification of those bacteria.

Metagenomics were done to get an idea about the probiotics available in the soil and water and tilapia intestine



Isolation of *Bacillus* sp. by spread plate technique



Pure culture of *Bacillus* sp. by streak plate technique

Plates 18. Isolation and purification of *Bacillus* sp. from soil and water sample of local gher and river.

10.4.3.2. Assessment of invitro growth inhibition of pathogenic bacteria

The selected probiotic bacteria were subjected to mass culture initially in lab condition to test invitro efficacy. Individual probiotic bacteria were assessed against the pathogenic bacteria. For invitro growth inhibition, individual probiotic bacteria were co-culture overnight with the pathogenic bacteria at a same concentration. Another pure culture of the pathogenic bacteria was grown overnight simultaneously. Both cultures were plated on TCBS agar plate and inhibition was assessed by comparing the total plate count of both co-cultured and pure cultured pathogenic bacteria.

10.4.3.3. Mass culture of Probiotic bacteria and dose optimization for application in shrimp culture pond

Mass culture of combined *Bacillus* sp. was done in growth media and dose was optimized for pond application by measuring total bacterial count in Hi-chrome Bacillus Agar media.

a.Pond preparation, prebiotic (Yeast Molasses leachates), probiotic application and water quality checking

Control and treatment ponds were prepared, prebiotic (Yeast Molasses leachates) application was done and mass culture of probiotic bacteria was also applied at optimum dose in the treatment ponds. Different Physicochemical characteristics of the ponds were recorded fortnightly.

b.Collection and stocking of PL: The post larvae (PL) were acclimatized and stocked in the ponds on April 05, 2022 with a stocking density of 4 ind./m^2.

Plate 19. Stocking of PL to the experiment ponds

***c.Feed and feeding management*:**

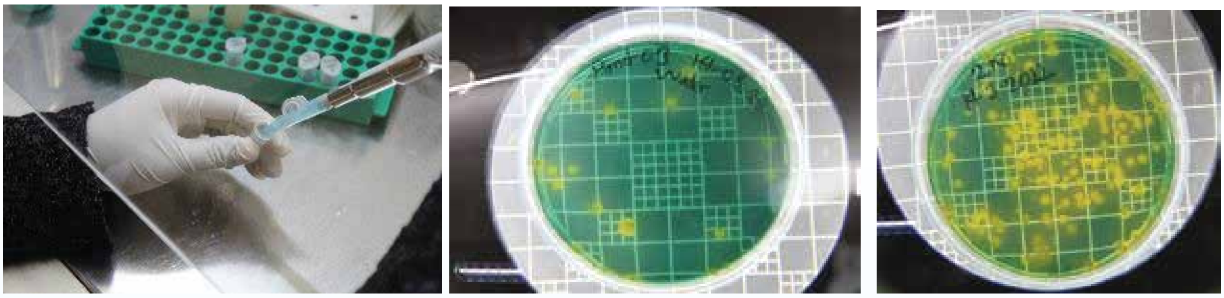
The shrimps have been weighed initially and weekly and the feeding rate adjusted according to their body weight (Table 3).

Table 3. Feeding rate at different days after stocking.

Days After Stocking	Daily Ratio (% of BW)
7	200
14	100
21	40
21-Until harvesting	5-10

***d.Monitoring of physico-chemical water parameters*:** Water quality parameters (temperature, salinity, ammonia, and pH) are being checked daily after stocking. Temperature has been monitored using digital thermometer. A digital DO meter (HACH, USA) has used to determine the dissolved oxygen content of water. pH has been measured using a digital pH meter (HACH, USA). Salinity is determined using a refractometer. Ammonia (mg/l) is determined by test kit.

***e. Vibrio load count*:** *Vibrio* bacterial load of the collected samples were analyzed in the laboratory of Shrimp Research Station, Bagerhat after probiotic application in treatment ponds. Specific media was used to identify *vibrio* specific bacteria. Water from pond, Hepatopancreas (HP) and gut content of shrimp from the project pond was collected and the sample was streaked in *Vibrio* specific agar by spread plate method. Then incubated at 37°C for overnight. Then colony is counted using digital colony counter.



Plates 20. *Vibrio* load count

***f. Monitoring of growth and estimation of production*:** Health condition and growth of shrimp was monitored fortnightly during the full and new period. Around 5% of shrimp was collected by a cast net in early morning. Length was measured by using a centimeter scale and the weight by a portable weighing balance. After experimental period, all shrimp were harvested with seine net and the parameters were calculated.

10.4.3.4. Analysis of immune parameters

a. ***Superoxide Dismutase Activity:*** SOD activity was determined according to the protocol of Creative Enzymes (EC 1.15.1.1).

***j. Haemocyte Collection and Total haemocyte count (THC)*:** From each shrimp 0.1 ml haemolymph were collected into a 26-gauge 1 ml sterile syrange containing 0.2 ml of anticoagulant (Trisodium Citrate 30mM, NaCl 338mM, Glucose 115mM, EDTA 10mM).

THC= average of 4 blocks × dilution correction factor ×10^4

Dilution correction factor =

$$\frac{volume\ of\ hemolymph\ extracted + volume\ of\ anticoagulant\ used}{volume\ of\ hemolymph\ extracted}$$

11.Result and discussion

11.1. Component 1 (BSMRAU)

11.1.1. Sample collection

A total of 350 samples of soil, water, yogurt, live fish, fermented bamboo, Nga-pi, spring water, deep soil sand etc. were collected from Bogura, Sylhet, Cox's bazar, Rangamati, Khagrachori, Bandarban, Tangail, Barisal and many other places in Bangladesh. (Fig 5)

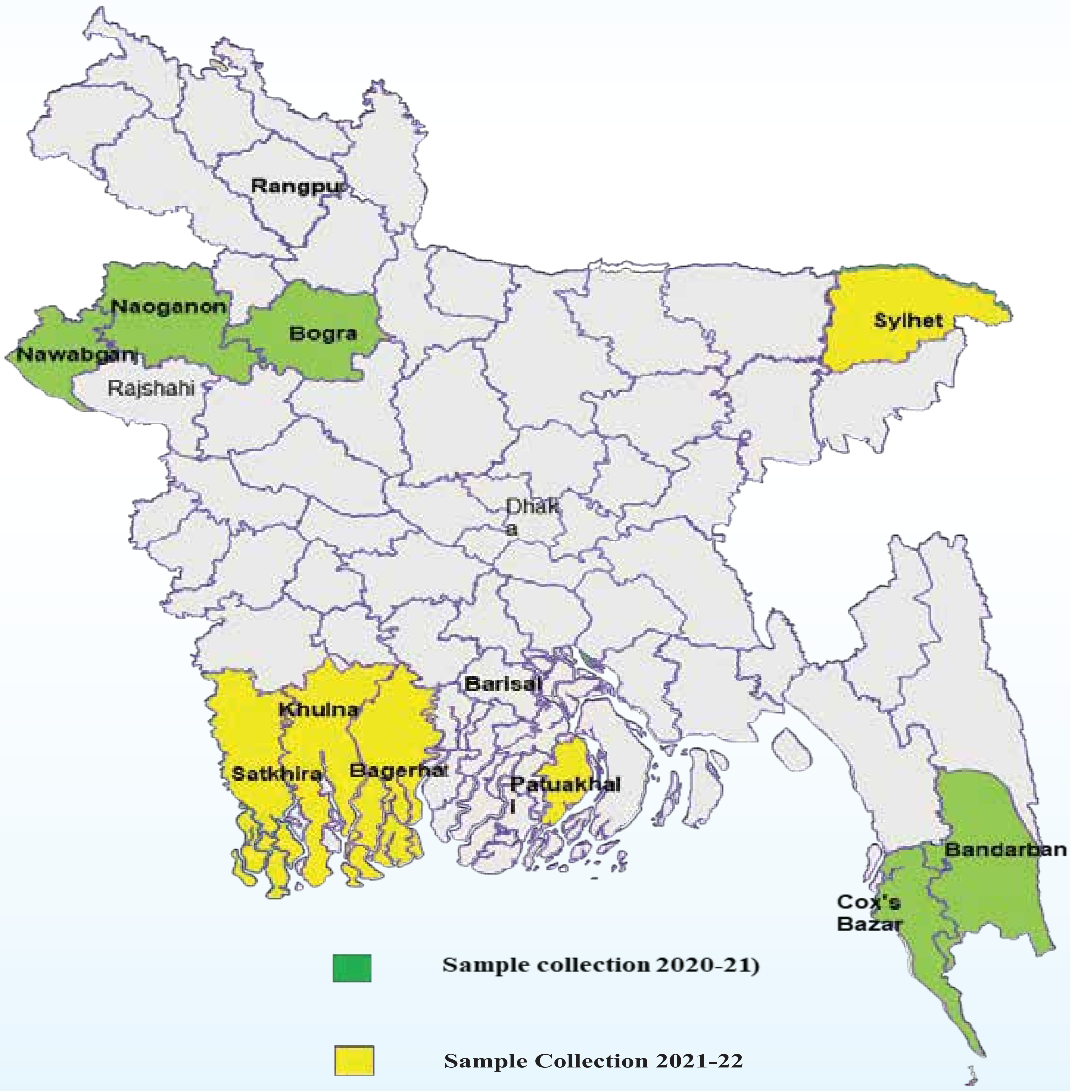


Fig 5. Sample collection sites

11.1.2. Storage of samples and isolation of microbes

Samples were stored at 4^0 C for isolation of microbes. Total 520 microbes were isolated as pure isolates based on the colony morphology (size, shape and color). After isolation, pure isolates were stored at -80 degree Celsius in glycerin stock for enzyme screening.

Plates 21. Isolation of microbes

11.1.3. Screening of microbes

11.1.3.1 Screening of cellulase producing bacteria

Bacteria that produce cellulase enzyme were screened by the clear zone on CMC agar plate. Bacteria were streaked and incubated for 24 hr followed by washing with Congo red to observe the clear zone. About 44 bacteria out of 520 isolates were screened as cellulase producer. (Table 4)

Table 4. Cellulytic bacteria and the origin of that bacterial samples

Sample Code	Origin of the Samples
FB1	Fermented bamboo product, Bandarban
FB2	Fermented bamboo product, Bandarban
FB3	Fermented bamboo product, Bandarban
SB1	Soil, Bandarban
SB2	Soil, Bandarban
SB3	Water, Bandarban
SB4	Soil, Bandarban
SB5	Water, Bandarban
FB1 a	Fermented bamboo product, Bandarban
FB1b	Fermented bamboo product, Bandarban

Sample Code	Origin of the Samples
FB1c	Fermented bamboo product, Bandarban
FB2a	Fermented bamboo product, Bandarban
FB2b	Fermented bamboo product, Bandarban
FB2C	Fermented bamboo product, Bandarban
FB2d	Fermented bamboo product, Bandarban
FB3a	Fermented bamboo product, Bandarban
Fb3b	Fermented bamboo product, Bandarban
FB3c	Fermented bamboo product, Bandarban
FB3d	Fermented bamboo product, Bandarban
SB1a	Soil, Bandarban
SB1b	Soil, Bandarban
SB1c	Soil, Bandarban
SB1d	Soil, Bandarban
SB2a	Soil, Bandarban
SB2b	Soil, Bandarban
SB2c	Soil, Bandarban
SB2d	Soil, Bandarban
SB3a	Water, Bandarban
SB3b	Water, Bandarban
SB3c	Water, Bandarban
SB3d	Water, Bandarban
SB4a	Soil, Bandarban
SB4b	Soil, Bandarban
SB5a	Water, Bandarban
SB5b	Water, Bandarban
SB5c	Water, Bandarban
BSC-2	Soil from Sherpur, Bogura
SPY-1	Yogurt from Patuakhali,Sadar
2JMY-2	Yogurt from Juri, Moulavibazar
GCG-1	Gut from Grass carp
GCG-3	Gut from Grass carp
GCG-5	Gut from Grass carp
GCG-6	Gut from Grass carp
GCG-7	Gut from Grass carp

11.1.3.2. Screening of protease producing bacteria

Protease production of bacterial strain was screened through the clear zone produced by bacteria on casein supplemented agar plate. 55 isolates among 520 were screened as protease enzyme producer (Table 5)

Table 5. Proteolytic bacteria and the origin of that bacterial samples

Sample Code	Origin of the samples
BSC-2	Soil from Sherpur, Bogura
BSC-3	Soil from Shibgonj, Bogura
BSC-3b	Soil from Shibgonj, Bogura
CuCw-1	Water from Curuskul union Cox's bazar (1)
CuCw-1b	Water from Curuskul union Cox's bazar (1)
CuCw-2	Water from Curuskul union Cox's bazar (2)
CuCw-2a	Water from Curuskul union Cox's bazar (2)
UCW-1	Water from Uttor nuniachora Cox bazar (1)
UCW-2	Water from Uttor nuniachora Cox bazar (2)
UCW-2c	Water from Uttor nuniachora Cox bazar (2)
CuCs-1	Soil from Curuskul union Cox's bazar
FB1	Fermented bamboo, Bandarban
FB2	Fermented bamboo, Bandarban
FB3	water, Bandarban
SB2	soil, Bandarban
SB3	water, Bandarban
SB4	soil, Bandarban
KBPY-1	Yogurt from Khalisabazar, Patuakhali
KBPY-2	Yogurt from Khalisabazar, Patuakhali
KBPY-3	Yogurt from Khalisabazar, Patuakhali
SBP-1	Yogurt from Shialibazar,Patuakhali
SBP-2	Yogurt from Shialibazar,Patuakhali
SBP-3	Yogurt from Shialibazar,Patuakhali
1JMY-1	Yogurt from Juri,Moulavibazar
1JMY-2	Yogurt from Juri,Moulavibazar
1JMY-3	Yogurt from Juri,Moulavibazar
2JMY-1	Yogurt from Juri,Moulavibazar
2JMY-2	Yogurt from Juri,Moulavibazar

Sample Code	Origin of the samples
2JMY-3	Yogurt from Juri,Moulavibazar
DPY-1	Yogurt from Dhumki,Patuakhali
DPY-2	Yogurt from Dhumki,Patuakhali
SPY-1/P-1	Yogurt from Patuakhali Sadar
SPY-2/SP-2	Yogurt from Patuakhali Sadar
SV-1	Yogurt from Sreepur,Bhola
SV-2	Yogurt from Sreepur,Bhola
1HG-1	Yogurt from Mymensingh,Haluaghat
1HG-2	Yogurt from Mymensingh,Haluaghat
1HG-3	Yogurt from Mymensingh,Haluaghat
1HG-4	Yogurt from Mymensingh,Haluaghat
2HG-1	Yogurt from Mymensingh,Haluaghat
2HG-2	Yogurt from Mymensingh,Haluaghat
1MT-1/S3-1	Yogurt from Modhupur,Tangail
1MT-2/S3-2	Yogurt from Modhupur,Tangail
1MT-3/S3-3	Yogurt from Modhupur,Tangail
2MT-1	Yogurt from Modhupur,Tangail
2MT-2	Yogurt from Modhupur,Tangail
RS-1	Red soil, RangamatI
RS-2	Red soil, RangamatI
RS-3	Red soil, RangamatI
RS-4	Red soil, RangamatI
SS-1	Sand, Saint Martin
SS-2	Sand, Saint Martin
CSK-1	Clay soil, Kaptai lake
CSK-2	Clay soil, Kaptai lake
CSB-1	Clay soil, Borkol kaptai lake
NR-2	Nga-ppi, Rangamati
NR-3	Nga-ppi, Rangamati
NR-5	Nga-ppi, Rangamati

11.1.3.3. Screening of phytase producing bacteria

For the screening of phytase producing bacteria, isolates were dropped on phytase screening medium (PSM) containing Na-Phytate followed by washing with 2% $CoCl_2$ (Cobalt chloride) and finally with coloring reagent containing equal volumes of 6.25% (w/v) aqueous molybdate and 0.42% (w/v) ammonium vanadate solution. Out of 520 isolates 20 isolates were screened as phytase producer. (Table 6)

Table 6. Phytase producing bacteria and the origin of those bacterial samples

Sample Code	Origin of the Samples
RS-1	Red soil, Rangamati
RS-2	Red soil, Rangamati
NR-1	Nga-pi, Rangamati
NR-2	Nga-pi, Rangamati
NR-3	Nga-pi,Rangamati
CSK-1	Clay soil, kaptai, Rangamati
SKB-1	Sand, kaptai, Borkol upazila, Rangamati
SKB-3	Sand, kaptai, Borkol upazila, Rangamati
SB3a	Fermented Soybean
SB3b	Fermented Soybean
SB4c	Fermented Soybean
GCG-4	Grass Carp Gut
GCG-6	Grass Carp Gut
GCG-7	Grass Carp Gut
GCG-3	Grass Carp Gut
GCG-5	Grass Carp Gut
SV-2	Yogurt Sreepur, Bhola
SV-3	Yogurt Sreepur, Bhola
FB_3a	Fermented bamboo, Bandarban
FB_3d	Fermented bamboo, Bandarban

Common isolates

There were about 21 isolates that singly produce more than one enzyme.

Table 7. Common isolates among cellulytic, proteolytic and phytase bacteria

Sample Code	Cellulytic bacteria	Proteolytic bacteria	Phytase bacteria
FB1	+	+	×
FB2	+	+	×
FB3	+	+	×
SB2	+	+	×
SB3	+	+	×
SB4	+	+	×
SPY-1	+	+	×
2-JMY-2	+	+	×
BSC-2	+	+	×
RS-1	×	+	+
RS-2	×	+	+
NR-2	×	+	+
NR-3	×	+	+
FB3a	+	×	+
FB3d	+	×	+
CSK-1	×	+	+
SB3a	+	×	+
SB3b	+	×	+
GCG-3	+	×	+
GCG-5	+	×	+
GCG-6	+	×	+
GCG-7	+	×	+

11.1.4. Biochemical characterization

11.1.4.1. Hemolytic activity test

Hemolytic activity assay is an important selection criterion to determine either pathogenic or non-pathogenic bacteria. LAB group of bacteria were showed γ-hemolysis that means no breakdown the red blood cell. The hemolytic assay further revealed the suitability of intestinal micro flora for further antibiotic susceptibility assay. The γ-hemolytic isolate

(LAB) revealed no red blood cell lysis activity on the blood agar. This safety precaution is relatively important in the characterization process, as hemolytic bacteria would break down the epithelial layer of host cells and cause malfunction in the defense system. Failure of this defense mechanism would cause invasive diseases in the host (Nurhidayu *et al*., 2012).

a b c

Plates 22. Hemolytic activity te (a) γ hemolysis (no hemolysis) (b) β hemolysis (complete hemolysis), and (c) α hemolysis (partial hemolysis)

11.1.4.2. Gram test

This test is done to identify the presence of peptidoglycan layer and cytoplasmic lipid membrane. *E. coli, Pseudomonas* sp.*, Klebsiella* sp.*,* etc. are considered as gram negative bacteria whereas *Bacillus* sp.*, Lactobacillus* sp.*, Staphylococcus* sp. are considered as gram positive bacteria.

Plate 23. Gram test

11.1.4.3. Catalase test

This test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result. It is well known that LAB is catalase negative; *Enterobacter, Klebsiella* and *Aeromonas* groups are catalase positive.

Plate 24. Catalase Test

11.4.4. Oxidase test

The oxidase test detects the presence of a cytochrome oxidase system that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye- tetramethyl-*p*-phenylene-diamine. The dye is reduced to deep purple color. This test is used to assist in the identification of *Pseudomonas, Neisseria, Alcaligens, Aeromonas, Campylobacter, Vibrio, Brucella* and *Pasteurella,* all of which produce the enzyme cytochrome oxidase.

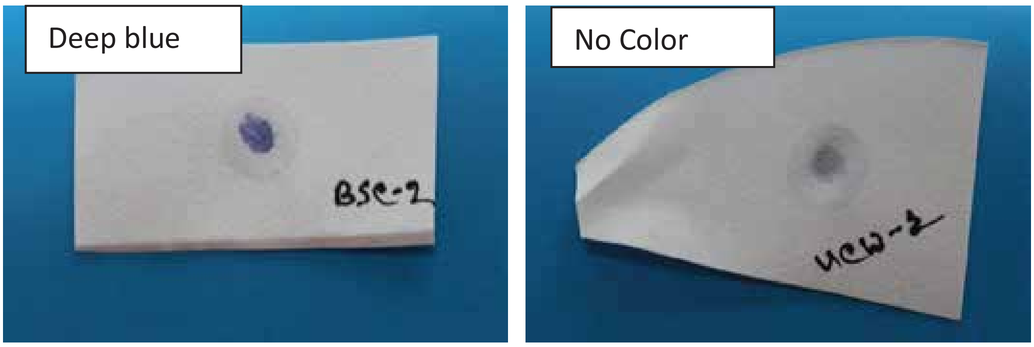


Plate 25. Oxidase test

Table 8. Characterization of cellulase producing bacteria

Code	Gram test	Oxidase test	Catalase test	Hemolytic test	MR test	VP test	H_2S test	Starch Hydrolysis test	Gelatinase test	Urease test	Oxidative test
FB1	+	+	+	Γ	+	-	+ (M)	+	+	-	F
FB2	+	+	+	Γ	-	+	+	-	-	+	NS
FB3	+	-	+	Γ	+	-	- (M)	+	+	-	F
SB1	+	+	-	A	+	+	+	+	+	-	NS
SB2	+	-	+	A	-	-	-	+	+	+	O
SB3	+	+	+	Γ	+	+	- (M)	+	+	-	NS
SB4	+	+	+	Γ	+	-	+	+	+	+	NS
SB5	+	-	+	Γ	-	-	- (M)	-	+	+	O

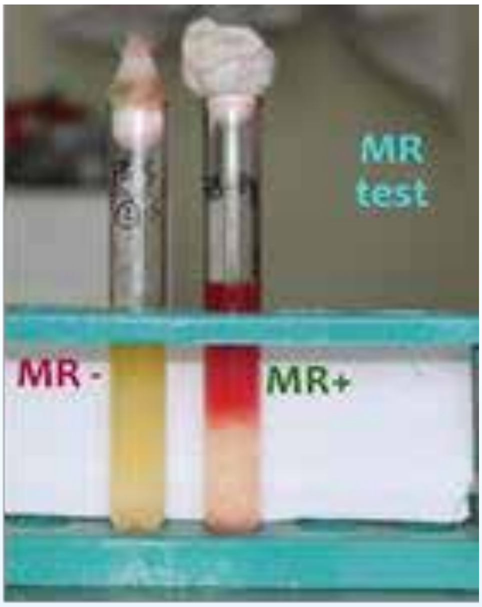
*MR-Methyl Red ; VP-Vogas Proskauer ; M= Motile , *F= Fermentative ; *NS= Non saccharolytic.

Table 9. Sugar fermentation properties of cellulase producing bacteria

Bacteria Code	Citrate Agar Test	Sugar Fermentation			
		Dextrose	Sucrose	Starch	Glucose (acid pro.)
FB1	-	+	+	-	+
FB2	-	-	+	-	+
FB3	-	+	+	+	+
SB1	-	-	+	-	-
SB2	+	+	-	+	+
SB3	-	-	+	+	-
SB4	-	-	+	+	-
SB5	+	+	-	-	+

11.1.4.5. MR-VP test

This test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The stable production of enough acid to overcome the phosphate buffer will result in a pH of below 4.4. In this test, MR positive isolates were suspected to be *Lactococcus* sp. and *Enterococcus* sp. whereas negative were suspected to be *Bacillus* sp., *Klebsiella* sp. VP (Voges-Proskauer) positive bacteria were supposed to be *Lactococcus* sp. and negative were *Bacillus* sp., *Klebsiella* sp. and *Enterococcus* sp.



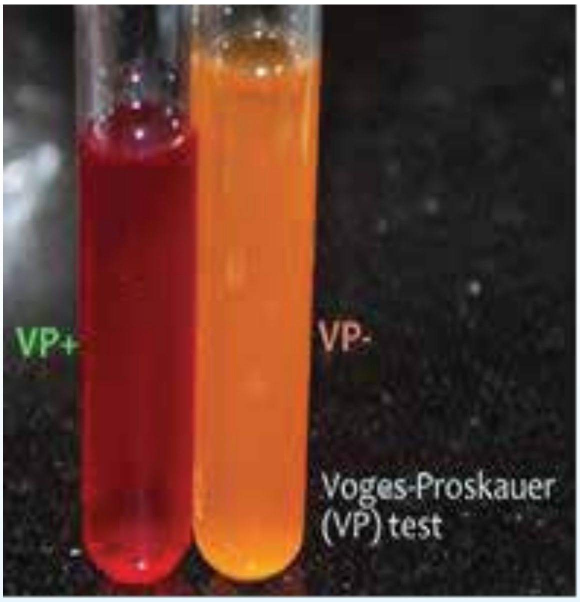


Plate 26. MR-VP test

Table 10. Characterization of protease producing bacteria

Bacteria Code	Oxidase Test	Catalase Test	Hemolytic Test	H_2S	Gelatinase
BSC-2	+	+	Γ	-	+
BSC-3	+	+	Γ	-	+
CuCw-1	+	+	Γ	-	+
CuCw-2	-	+	Γ	-	+
UCW-1	-	+	Γ	-	+
UCW-2	-	+	Γ	-	+
CuCs-1	-	+	Γ	-	+

11.1.4.6. Oxidative fermentative test

Carbohydrates are organic molecules that contain carbon, hydrogen and oxygen in the ratio $(CH_2O)_n$. Organisms use carbohydrate differently depending upon their enzyme complement. This test is done to determine the pattern of fermentation that is characteristics of certain species, genera or groups of organisms and for this reason this property has been extensively used as method for biochemical differentiation of microbes. So, the test is to detect the oxidation or fermentation of carbohydrates by bacteria. It aids in the identification of gram-negative bacteria on the basis of their ability to oxidize or ferment a specific carbohydrate. (Murray *et al.,* 2007)

Normally *Bacillus subtilis, Klebsiella* sp. shows positive in Oxidative test and *Lactococcus* sp., *Enterococcus* sp. Non saccharolytic.

Plate 27. Oxidative fermentative test of bacteria isolates

11.1.4.7. H_2S test

Some microorganisms are able to reduce sulfur (Sulphur) containing compounds to hydrogen sulfide during metabolism which is commonly employed as a test measure for their identification in laboratories. To determine whether the microbe reduces sulfur containing compounds to sulfides to produce hydrogen sulfide gas. H_2S positive bacteria are *Enterobacter aerogenes, Shigella sp., Erwinia* sp. *Enterococcus* sp. and negative are *Bacillus subtilis, Klebsiella* sp. and *Lactococcus lactis.*

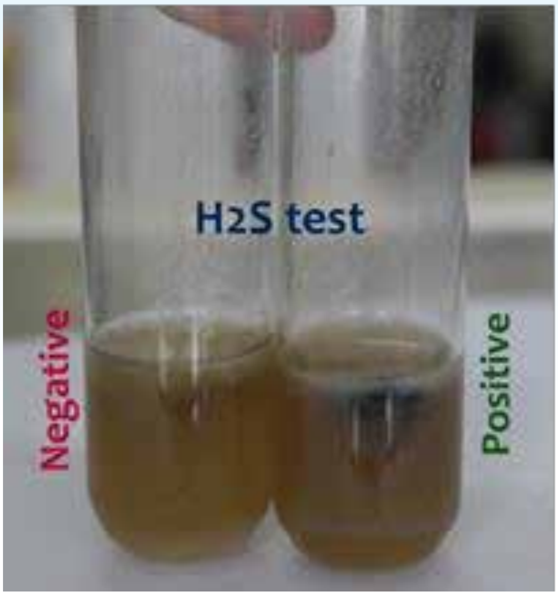


Plate 28. H_2S test

11.1.4.8. Gelatin test

Gelatin is a protein derived from the animal protein collagen– component of vertebrate connective tissue. It has been used as a solidifying agent in food for a long time. Gelatin hydrolysis test is a great way to highlight proteolysis by bacteria. Gelatin hydrolysis test is helpful in identifying and differentiating species of *Bacillus*, *Clostridium*, *Proteus*, *Pseudomona*, and *Serratia*.

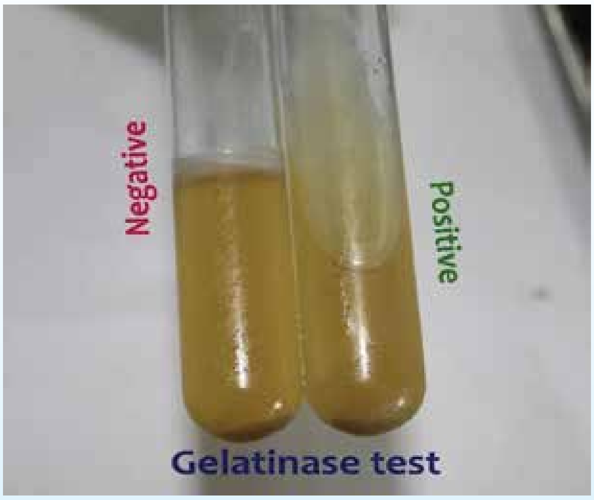


Plate 29. Gelatinase test

11.1.4.9. Urease test

Urea is a diamide of carbonic acid. It is hydrolysed with the release of ammonia and carbon dioxide. Many organisms especially those that infect the urinary tract, have a urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. Urase test is to identify those specific organisms. *Bacillus subtilis, Klebsiella* sp. and *Enterococcus* sp. are *urease* positive bacteria whereas Lactococcus, Enterobacter are urease negative.

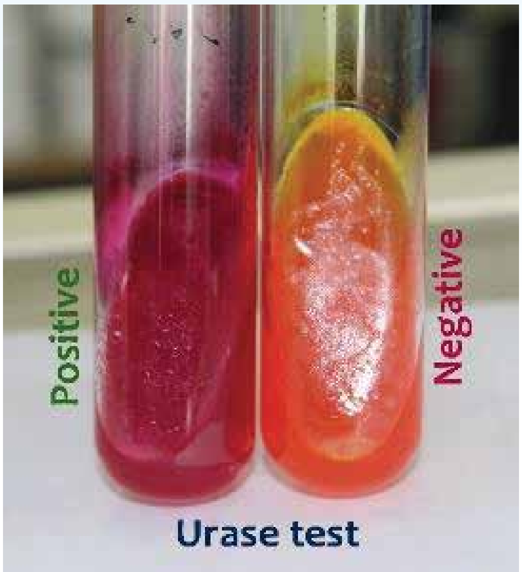


Plate 30. Urease test

11.1.4.10. Starch hydrolysis and citrate agar test

Starch Hydrolysis test is used to identify bacteria that can hydrolyse starch (amylose and amylopectin) using the enzymes a-amylase and oligo-1, 6-glucosidase. Often used to differentiate species from the genera *Clostridium* and *Bacillus*. Because of the large size of amylose and amylopectin molecules, these organisms cannot pass through the bacterial cell wall. In order to use these starches as a carbon source, bacteria must secrete a-amylase and oligo-1,6-glucosidase into the extracellular space. These enzymes break the starch molecules into smaller glucose subunits which can then enter directly into the glycolytic pathway.

Simmons' citrate agar is a selective and differential medium that tests for an organism's ability to use citrate as a sole carbon source and ammonium ions as the sole nitrogen source. It is used for differentiating gram-negative bacteria on the basis of citrate utilization. It is useful for selecting organisms that use citrate as its main carbon and energy source.

(A)

Plate 31. A) Starch hydrolysis

(B)

Plate 31. B) Citrate agar test

11.1.4.11. Indole test

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophane to indole, which accumulates in the medium. Indole production test is important in the identification of enterobacteria. Most strains of *E. coli, P. vulgaris, P. rettgeri, M. morgani* and *Providencia* species break down the amino acid tryptophan with the release of indole. This is performed by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase." In Indole test *Lactococcus lactis subsp. Lactis, Enterococcus* sp.*, Bacillus subtilis, Klebsiella sp.* and *Lactococcus lactis* were negative.

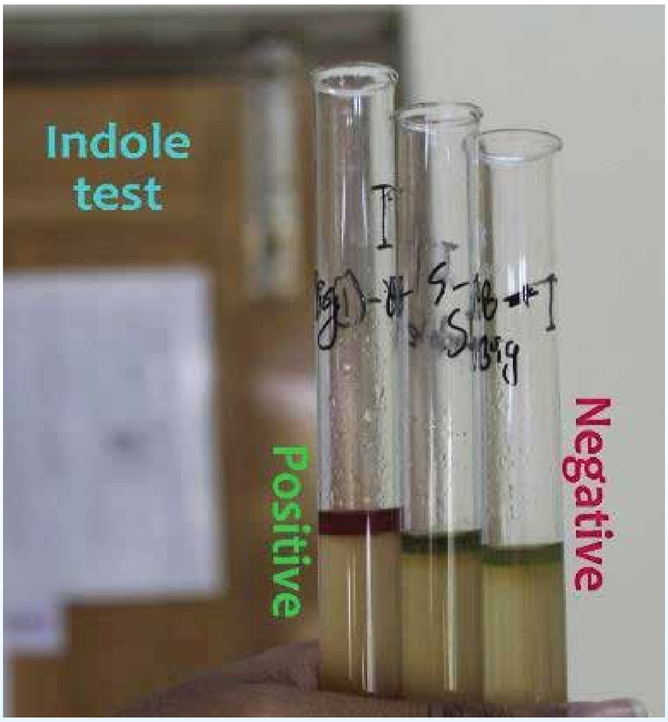


Plate 32. Indole test

11.1.5. Enzyme production, production optimization, activity assay
11.1.5.1. Cellulase

Selection of isolates for crude enzyme production

Eight isolates coded by FB1, FB2, FB3, SB1, SB2, SB3, SB4 and SB5 were selected (based on clear zone) to produce crude cellulase enzyme. Selected media were prepared and enzyme produced in shaker incubator. The cellulase activity was assayed in carboxymethyl cellulose (CMC) solution by 1, 3- dinitro salicylic (DNS) method. (Table 11)

Table 11. Quantification of cellulase activity

Sample no.	Sample name	OD
1	FB1	0.792
2	FB2	0.829
3	FB3	0.880
4	SB1	1.058
5	SB2	1.47
6	SB3	0.795
7	SB4	0.906
8	SB5	0.842

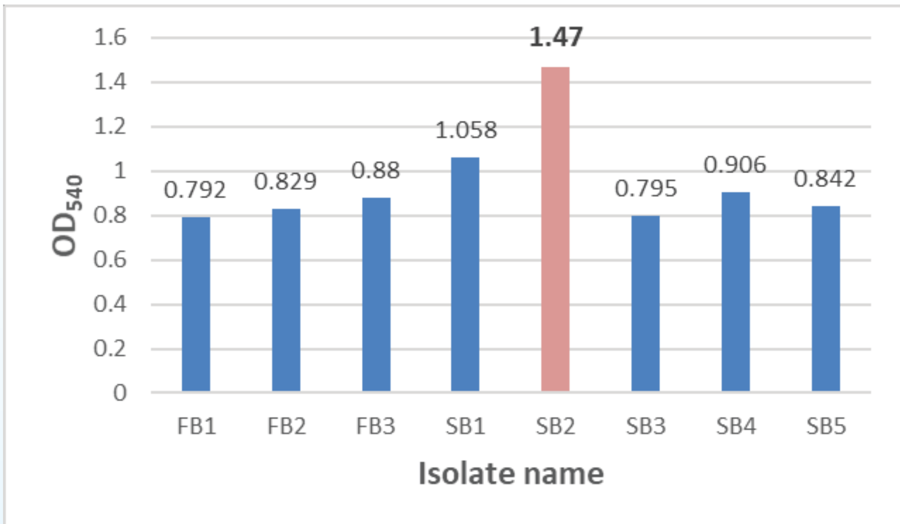


Fig 6. Selection of isolates

From the analysis of assay result it was observed that enzyme from SB2 isolates was highest (1.47) in term of activity and selected for production optimization and pure enzyme production.

Optimization of cellulase production
pH and incubation time

Different enzyme production media having pH range 4-9 were prepared. Sample were withdrawn at 24h, 48h, 72h and 96h and activity were measured through 1, 3- dinitro salicylic (DNS) method (Table 12)

Table 12. Optimization of incubation time and pH for cellulase enzyme production

Serial no.	Time/pH	24 hours	48 hours	72 hours	96 hours
		OD_{540}			
1.	pH 4	0.66	0.69	0.691	0.601
2.	pH 5	0.54	0.685	0.702	0.661
3.	pH 6	0.65	0.782	0.872	0.720
4.	pH 7	0.63	0.723	0.782	0.718
5.	pH 8	0.685	0.705	0.721	0. 685
6.	pH 9	0.66	0.665	0.701	0.663

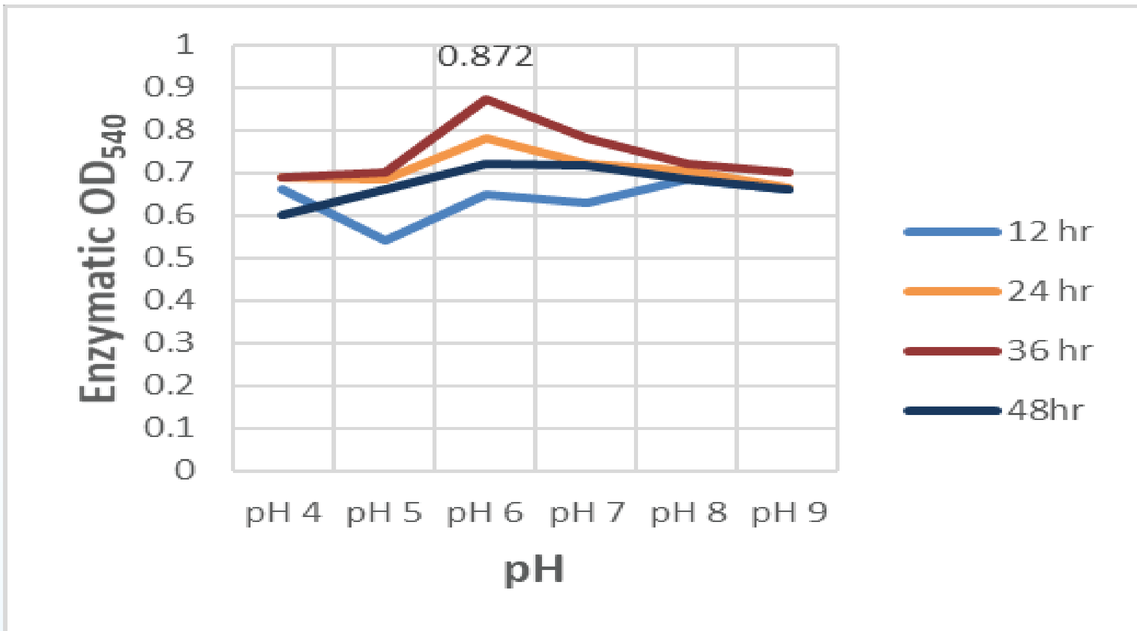


Fig 7. Optimization of pH and incubation time

From the assay result it was stated that pH 6.0 and 72h incubation time was most suited for cellulase production. pH value below or above 7.00 had shown significant reduction in enzymatic activity.

Temperature
A significant difference in cellulase activity was observed with the changes in production temperature. For the analysis of effect of temperature on cellulase enzyme activity, enzymes

are produced at 20° C, 25° C, 30° C, 35° C, 40° C and 45° C, at pH 6 and 72h incubation time and activity was measured through, 3- dinitro salicylic (DNS) method. (Table 13)

Table 13. Optimization of temperature for cellulase production

Temp.	**20^0 C**	**25^0 C**	**30^0 C**	**35^0 C**	**40^0 C**
OD_{600}	0.51	0.67	**0.94**	**0.70**	**0.62**

Pure cellulase assay

The dinitro salicylate (DNS) method detects the reducing sugars liberated by the action of hydrolase enzymes on carbohydrates, under specific pH and temperatures (Bailey, 1988). Enzyme produced in 30^0 C, pH6 and 72h incubation was purified through Ammonium sulphate precipitation and activity was measured in Carboxy methyl cellulose through DNS method. Prior to that a standard curve of glucose was prepared by known concentration of glucose through this DNS method (Fig 8.)

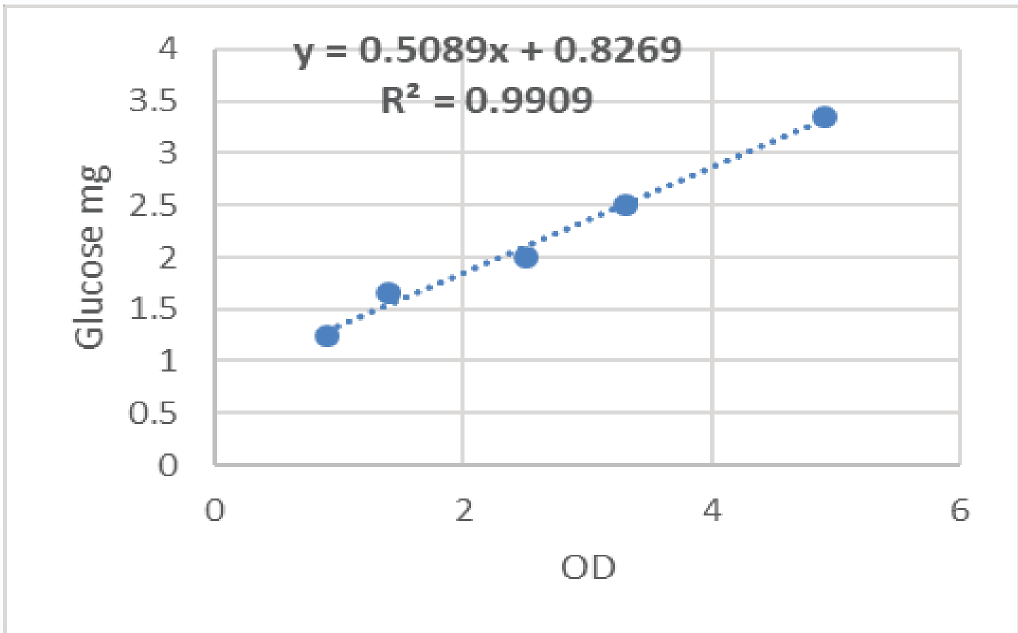


Fig 8. Standard curve of glucose

Units/mL Enzyme =

$$\frac{(\mu\text{mol tyrosine/glucose/phosphate equivalents released}) \times (\text{total volume of assay})}{(\text{Volume of enzyme used}) \times (\text{time of assay}) \times (\text{volume used in colorimetric determination})}$$

Here,

Highest OD= 0.94

Liberated glucose = 1.30 (from St. curve)

Total assay volume= 24 ml

Enzyme volume = 0.5

Time = 30 min

Volume in colorimetric determination = 24 ml

Cellulase Enzyme activity = 0.28 Units/mL

11.1.5.2. Protease

Selection of isolates for crude enzyme production

Seven isolates out of 55 were selected for crude protease production based on clear zone diameter (above 5mm). Crude enzyme was produced in selected media and activity was measured through Lowry's protein estimation. (Table 14)

Table 14. Quantification of protease activity.

Sample no.	Sample name	OD_{750}
1	Protein	1.65
2	BSC-2a	2.53
3	BSC-3b	3.21
4	UCW-1c	2.98
5	UCW-2c	2.73
6	CuCw-1b	3.34
7	CuCw-2a	3.01
8	CuCs-1a	3.12

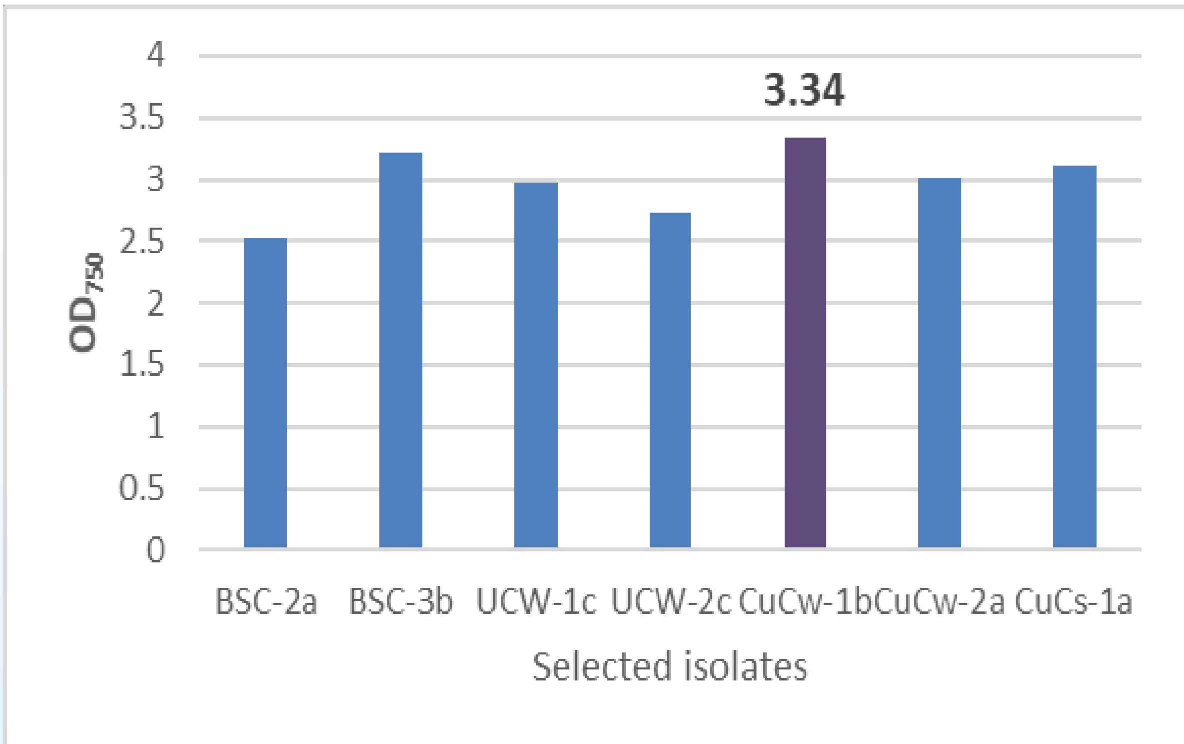


Fig 9. Selection of isolates

Enzyme produced from CuCw-1b bacterial isolates had shown the highest activity 3.34 in lowry assay and selected for optimization of different factors for enzyme production.

Optimization of protease production

pH and incubation time

Different enzyme production media having pH range pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 were prepared. Sample were withdrawn at 12h, 24h, 36h, 48h and 72h and activity were measured through Lowry's protein estimation (Table 15)

Table 15. Optimization of incubation time and pH for protease enzyme production

Serial no.	Time/pH	12 hours	24 hours	36 hours	48 hours	72 hours
		Optical Density (OD_{750} nm)				
1.	Protein	0.792				
2.	pH 4	1.56	1.94	2.85	2.00	1.956
3.	pH 5	1.75	2.14	3.35	3.25	2.934
4.	pH 6	3.31	5.62	7.07	6.73	5.44
5.	pH 7	3.34	3.74	**7.25**	7.15	6.34
6.	pH 8	3.38	5.74	7.00	7.25	7.08
7.	pH 9	3.46	5.54	5.6	7.15	6.88

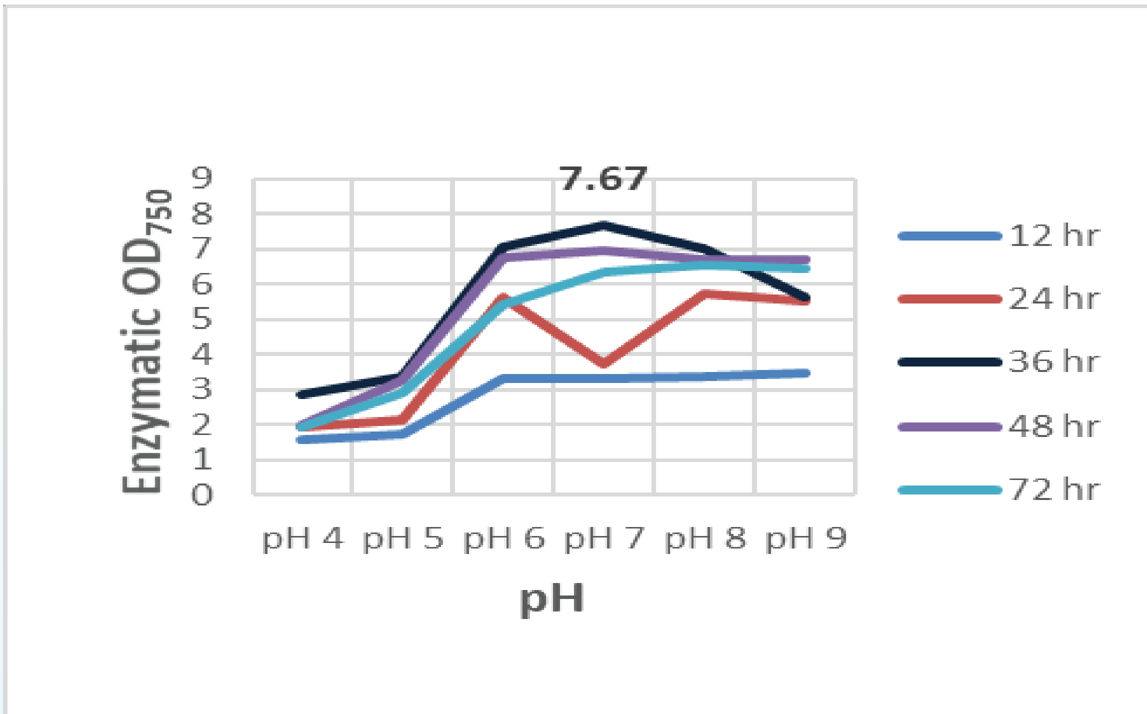


Fig 10. Optimization of pH and incubation time

From the analysis of enzyme activity found at different pH and incubation time it is clearly stated that pH 7 and 36h incubation time is most suited for protease production in case of

CuCw-1b isolates. pH value below or above 7.00 had shown significant reduction in enzymatic activity.

Temperature

For the analysis of effect of temperature on protease enzyme activity, enzymes are produced at 20° C, 25° C, 30° C, 35° C, 40° C and 45° C, at pH 7 and 36h incubation time temperature and activity were measured through Lowry's protein estimation. (Table 16)

Table 16. Optimization of temperature for protease enzyme production

Temp/ sample	20° C	25° C	30° C	35° C	40° C	45° C
pH 7, 36h incubation	6.77	6.25	6.4	**7.25**	6.387	5.96

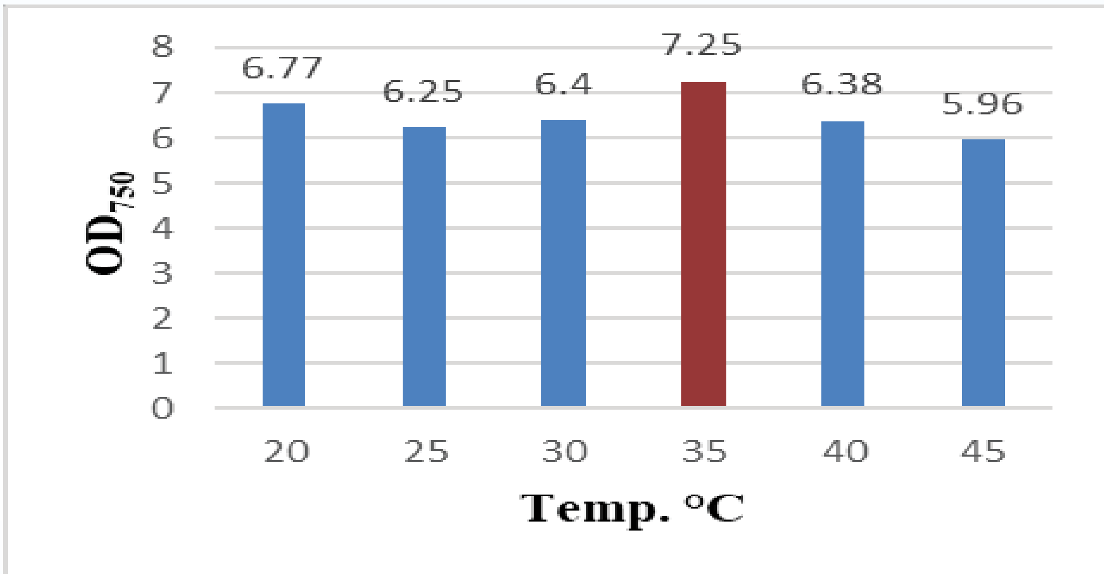


Fig 11. Optimization of temperature

Protease enzyme activity was found highest (7.25) in 35° C temperature. Below or above of this optimized condition there was significant reduction in enzyme activity.

Enzyme purification and pure enzyme assay

The proteolytic activity of pure protease extract (Ammonium Sulphate precipitation) was assayed through Universal protease activity assay: Casein as a Substrate. In this assay, casein acts as a substrate. When the protease we are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve the activity of protease samples can be determined.

Table 17. Standard curve of L-Tyrosine for protease assay

SL No	L-Tyrosine (µmol)	OD
01	0.55	0.179
02	1.11	0.368
03	2.21	0.779
04	4.42	1.415
05	5.53	1.808

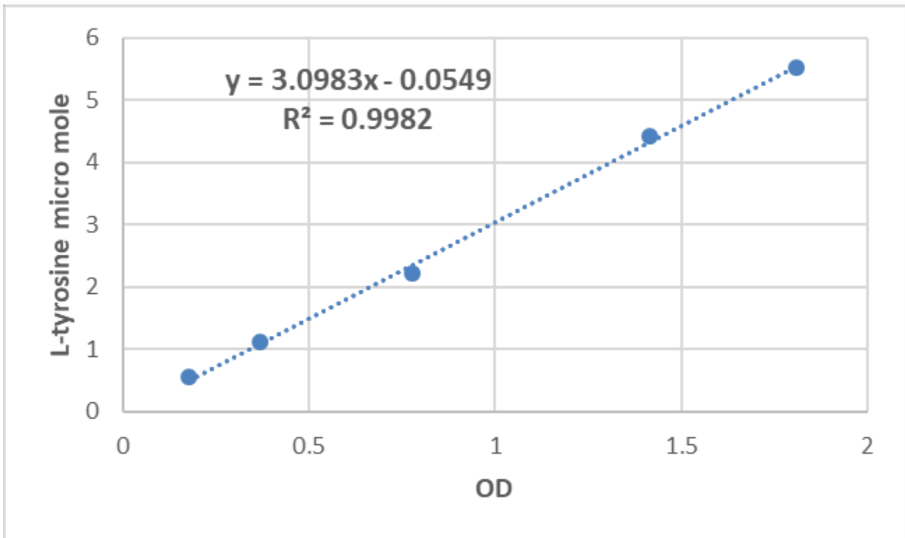


Fig 12. Standard curve of L-tyrosine

$$\text{Units/mL Enzyme} = \frac{(\mu\text{mol tyrosine/glucose/phosphate equivalents released}) \times (\text{total volume of assay})}{(\text{Volume of enzyme used}) \times (\text{time of assay}) \times (\text{volume used in colorimetric Determination})}$$

Here,
Pure enzyme OD= 0.42
Blank sample OD = 0.18
Liberated L tyrosine = 0.6886 µmol (from St. curve)
Total assay volume= 11 ml
Enzyme volume = 1.0 ml
Time = 10 min
Volume in colorimetric determination = 2 ml
Protease Enzyme activity = 0.37 Units/mL

Table 18. Pure protease enzyme assay

Sl no.	Enzyme	10 µg/ml (OD_{600})	20 µg/ml (OD_{600})	30 µg/ml (OD_{600})	Enzyme activity(U/ml)
1.	Sigma enzyme	0.12	0.36	0.53	0.566
2.	Bacterial enzyme		0.16	0.42	0.379
3.	Blank sample	0.18			

11.1.5.3. Phytase

Optimization of phytase

Phytase production media of different pH like pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 were prepared and crude enzyme was assayed by the liberation of inorganic phosphate (Pi) from the breakdown of phytic acid in 2 mM Na-phytate solution. (Table 19).

Table 19. Optimization of pH and incubation time for phytase enzyme production

pH	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7
pH 4	0.37	0.39	0.41	0.40	0.37	0.35
pH 5	0.53	0.58	0.54	0.46	0.42	0.43
pH 6	0.61	0.66	o.67	0.71	0.58	0.48
pH 7	0.67	0.76	**0.81**	0.76	0.63	0.61
pH 8	0.48	0.51	o.63	0.65	0.53	0.47
pH 9	0.41	0.47	0.46	0.40	0.42	0.31

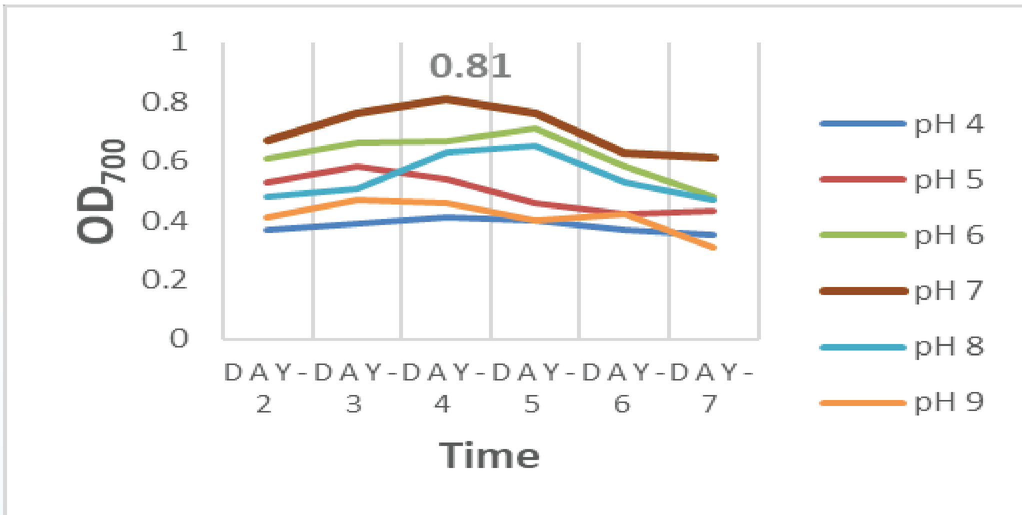


Fig 13. Optimization of pH and incubation time for phytase enzyme production

From the analysis of enzyme activity found at different pH and incubation time it is clearly stated that pH 7 and Day-4 incubation time is most suited for phytase production. pH value below or above 7.00 had shown significant reduction in enzymatic activity.

Temperature optimization

For the analysis of effect of temperature on protease enzyme activity, enzymes are produced at 20° C, 25° C, 30° C, 35° C, 40° C and 45° C, at pH 7 and 4-day incubation time temperature and activity were measured. (Table 20).

Table 20. Optimization of temperature for phytase production

Temp.	20^0 C	25^0 C	30^0 C	35^0 C	40^0 C
OD_{700}	0.41	0.56	0.72	**0.87**	0.71

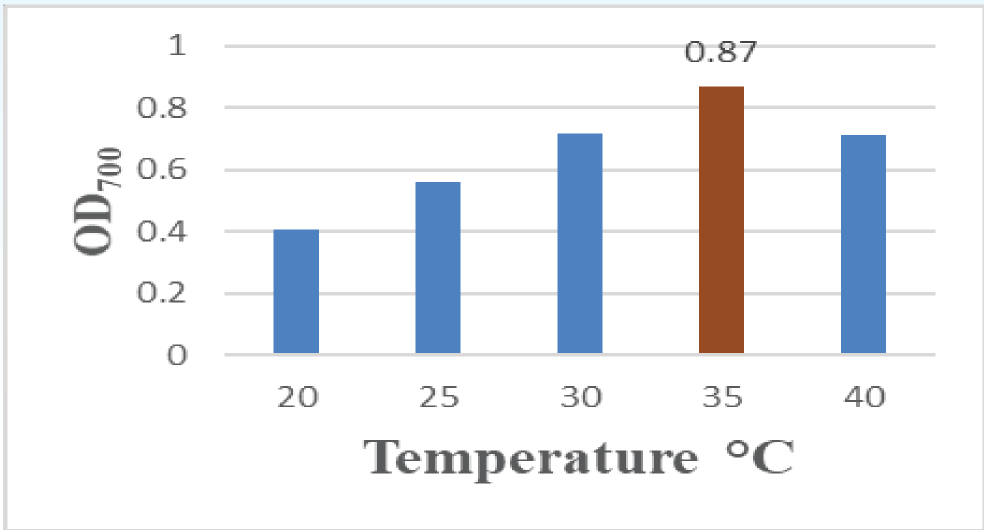


Fig 14. Temperature optimization

Phytase enzyme activity was found highest (0.87) in 35° C temperature. Below or above of this optimized condition there was significant reduction in enzyme activity.

Pure phytase enzyme activity

Enzyme produced in 35^0 C, pH 7 and 4-day incubation was purified through Ammonium sulphate precipitation and activity was measured in Na-phytate solution by detetcting the liberation of Pi. Prior to that a standard curve of inorganic phosphate (Pi) was prepared by known concentration through this method. (Fig 15)

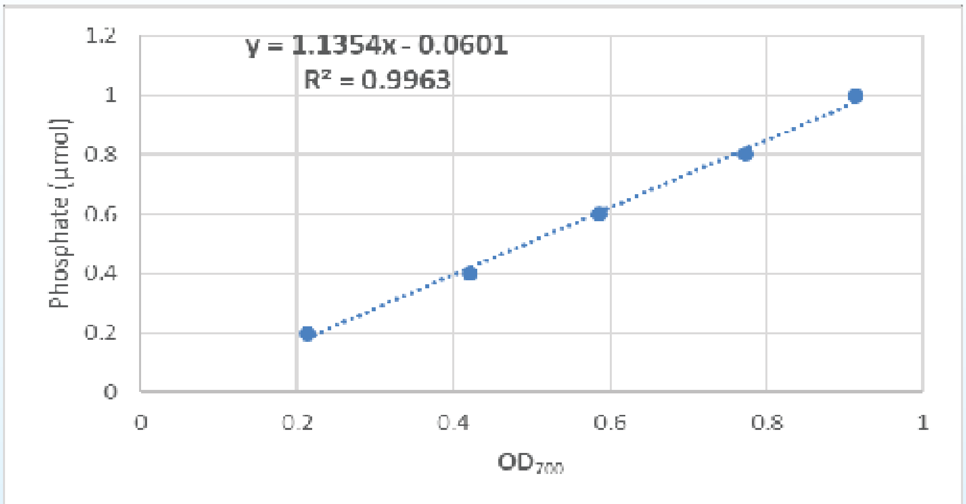


Fig 15. Standard curve of phosphate

$$\text{Units/mL Enzyme} = \frac{(\mu\text{mol tyrosine/glucose/phosphate equivalents released}) \times (\text{total volume of assay})}{(\text{Volume of enzyme used}) \times (\text{time of assay}) \times (\text{volume used in colorimetric Determination})}$$

Here,
Highest OD= 0.87
Liberated glucose = 0.9276 (from St. curve)
Total assay volume= 3 ml
Enzyme volume = 0.15 ml
Time = 30 min
Volume in colorimetric determination = 3 ml
Phytase Enzyme activity = 0.20 Units/mL

Molecular identification

Different enzymes producer bacteria were identified at molecular level through Bruker MALDI Bio type and sequencing analysis. About 78 isolates are identified through Bruker MALDI-TOF and gene sequencing (Fig 16). Different species like *Bacillus* sp., *Enterobacter* sp., *Xenorabdus* sp., *Acinetobacter* etc. Beside all these helpful bacteria we found some pathogenic strain in our isolation as isolation of beneficial microbes from natural samples is a very difficult job though we have done the hemolytic test to detect pathogenicity.

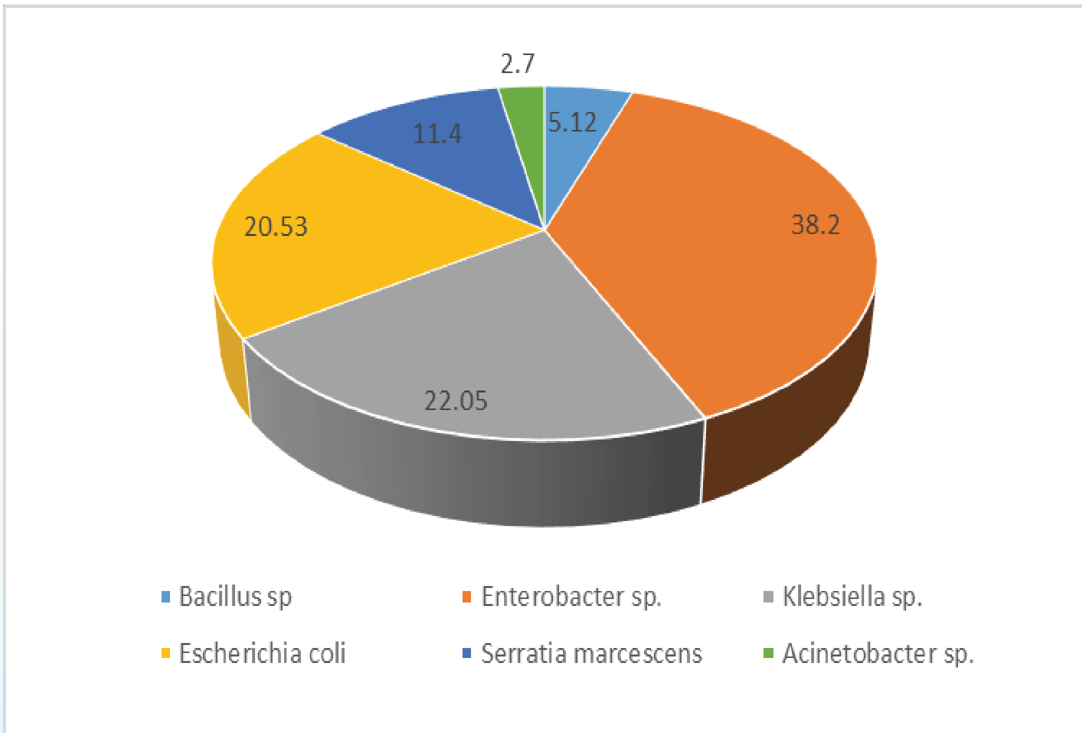


Fig 16. Identified different enzymes producing bacteria at molecular level through Bruker MALDI Bio type and sequencing analysis

11.2. Component 2(NSTU)

11.2.1. Identification and distribution of polychaete worms in the coastal and/marine waters

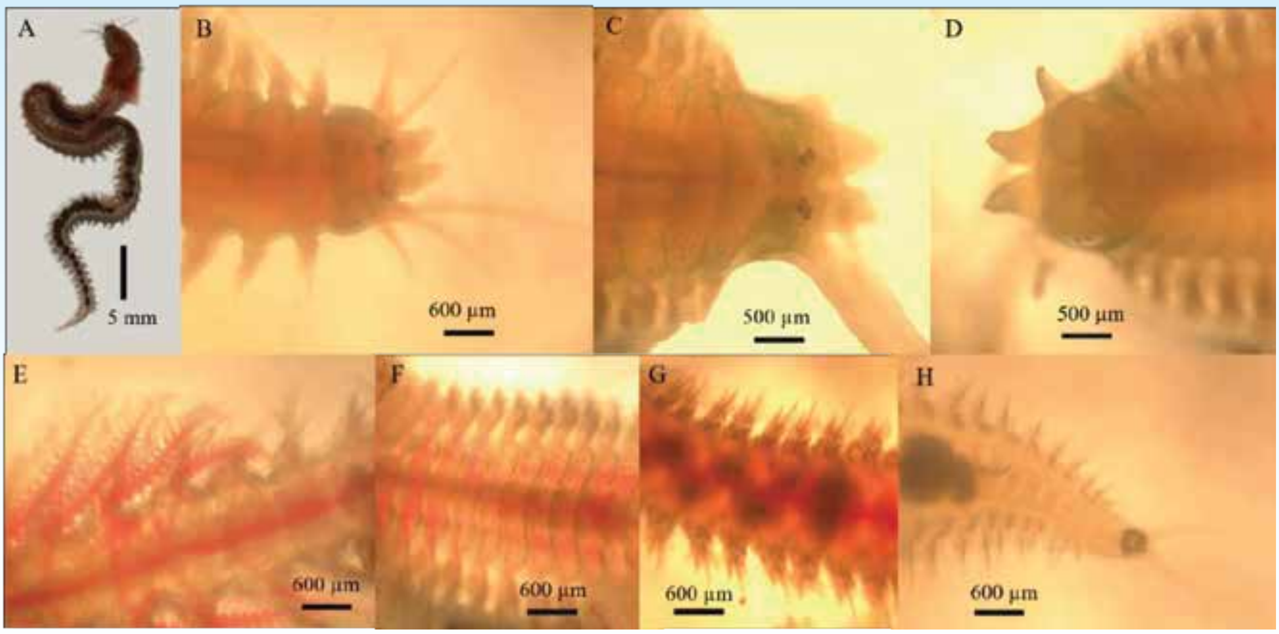
Based on the morphological features and taxonomic keys, the collected polychaete specimens were identified as *Dendronereis dayi, Glycera* cf. *sheikhmujibi*, *Glycera* sp., *Lycastonereis indica*, *Namalycastis fauveli*, *Namalycastis* sp., *Neanthes* sp., and *Composetia* sp., respectively. Description of each species presented as follows:

Dendronereis dayi

Dendronereis Peters, 1854 is a small genus comprising four species: *D. aestuarina* Southern, 1921 from Chilka Lake, India, *D. arborifera* Peters, 1854 from Mozambique, *D. dayi* Misra, 1999 from West Bengal, India and *D. pinnaticirris* Grube, 1878 from the Philippines. Specimens of *Dendronereis* sp. was collected from the Sundarbans mangrove and Mosheshkhali and Sonadia mangrove channel of the Cox's Bazar coast. The specimens were identified as *D. dayi* Misra, 1999, based on the presence of papillae on both rings of pharynx, dorsal cirrus develops into bipinnate branchia on some middle segments and the chaetae include homogomph and heterogomph spinigers.

Plate 33. Anterior end in dorsal view of *Dendronereis dayi*.

Diagnosis: Prostomium has two sets of antennae and biarticulate palps, and is deeply divided anteriorly. Four pairs of tentacular cirri on Peristomium. Smooth or with papillae eversible pharynx Chaetigers 1 and 2 have sub-biramous parapodia, notopodia with just notopodial dorsal ligules, and succeeding chaetigers have biramous parapodia. There is a notopodial prechaetal lobe. Branchiae are bipinnate or pinnate subdivisions of the dorsal cirri. In anterior chaetigers, neuropodia have multifid acicular lobes. Spinigers of the Notopodial Chaetae homogomph and hemigomph (=sesquigomph). With or without blunt-tipped short blades, neuropodial chaetae homogomph spinigers.



Plates 34. ***Dendronereis dayi*** A. Specimens of *D. dayi* immediately after killed. B. Anterior end of the dorsal view. C. Two pairs of eyes present closely in the prostomium. D. Posterior view of the pharynx with palps and antennae. E. Cluster of bipinnate branchiae start from 10-12 chaetigers. F. Branchial view after death of a specimens. G. Posterior view of parapodia and heterogomph spinigers. H. Posterior view of the ventral end with anal cirri.

Namalycastis fauveli

Diagnosis: The body is broadest in the middle, tapering anteriorly and posteriorly. The anterior small cleft of the cleft of the prostomium is missing. Antennae are quite small. There are notosetae present. Type A Neurosetae. Setae of Heterogomph with a very lengthy boss. Setiger 10 has supra-neuroacicular falcigers with blades that are slightly curved, 8.0–9.5 times the width of the shaft head, coarsely serrated, 20–40 teeth (rarely to 12), and teeth that are roughly the same length. Dorsum convex, although more strongly arched in the middle anteriorly. In alcohol, the color ranges from yellow-white to yellow-brown (rarely). Epidermal pigment is normally lacking; however, one specimen's head has some irregular orange pigment. Absent or shallow anterior cleft, with narrow, shallow longitudinal groove running from tip to mid-posterior prostomium (occasionally) (dorsal groove scarcely absent).

Prostomium form is generally trapezoidal, notched or slightly depressed on the side, and 1.5–2.4 times longer than broad. Antennae are small and positioned above the mid-palps. Two sets of black eyes, positioned transversely, with lenses. Tentacular cirri with indistinct cirrophores and smooth cirrostyles. Anteroventral anterodorsal tentacular cirri 1.5–2.0 in. 0.9–1.1 length posterodorsal tentacular cirri anterodorsal Posterodorsal tentacular cirri, length 1.1–2.0, posteroventral. Tentacular cirri extending from the posterior margin of setiger 1 to the anterior edge of setiger 2. Brown jaws with a single strong terminal tooth, 2–3 subterminal teeth, and 2–4 proximally ensheathed teeth. Parapodia with weakly bilobed acicular neuropodial ligule.

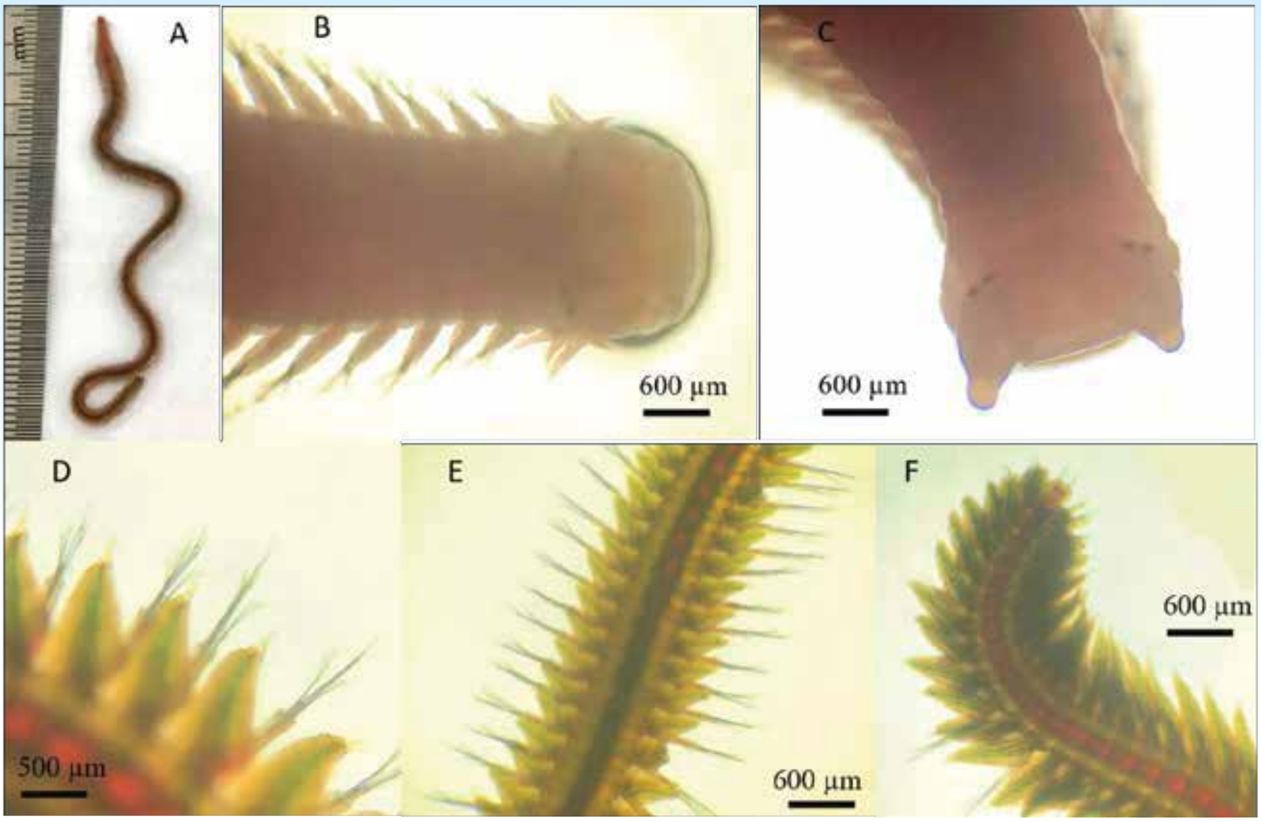
Plate 35. Anterior end in dorsal view of *Namalycastis fauveli*.

Dorsal cirri lengthens slightly (or dramatically) posteriorly; 0.69–1.3 length of podium at setiger 3, 1.1–2.4 length of podium in mid-body, 1.8–6.0 length of podium posteriorly; 1.9–3.6 length of podium posteriorly; 1.9–3.6 length of podium posteriorly; 1.9–3.6 length of podium posteriorly; 1.9–3.6 length of podium posteriorly; 1.9–3.6 length of podium posteriorly; 1.9–3.6 length of podium posteriorly; 1.9– At setiger 3, the ventral cirri is 0.30–0.63 length of podium, while the posterior cirri is 0.18–0.33 length of podium.

***Namalycastis* sp.**

This specimen was placed into the *Namalycastis* sp. genus based on the small antennae, subconical; dorsal cirri anteriorly with cylindrical cirrophores, posteriorly cirrophores are flattened (leaf-like).

Plate 36. Anterior end in dorsal view of *Namalycastis* sp.



Plates 37. A. Fresh specimen of ***Namanlycastis* sp**. **B**. Prostomium, tentacular cirri, dorsal view. **C**. Two pairs of eyes moderately close to each other at the prostomium. **D**. Dorsal view the parapodia with a bunch of sesquigomph spinigers. **E**. Posterior view of the urinamous parapodia and spinigers arranged in the both side of the segments (almost bi-pinnate arrangement). **F**. Anal cirri arise ventro-laterally.

The notosetae are usually present and pygidium is button-shaped, multi-incised. The mature individuals having numerous spherical oocytes in each segment.

Lycastonereis indica

Diagnosis: Pigmentation present in all specimens, consisting in brown irregular spots along body. Prostomium with two longitudinal, central lines, extending from the anterior margin to middle of prostomium, some additional rounded spots at the base of the cirrophores. One row of irregular spots in the anterior dorsal margin of the achaetous ring. One pair of antennae and two pairs of eyes. Three pairs of anterior cirri. Pharynx with rounded papillae present in both oral and maxillary ring, in all areas except area V. Chaetigers 1 and 2 with neuroaciculae only. Notopodial dorsal ligules, notopodial prechaetal lobes, and neuroacicular ligules bilobed present in anterior chaetigers; notopodial dorsal ligules, notopodial prechaetal lobes and neuropodial postchaetal lobes absent in posterior chaetigers. Neuropodial ventral ligules absent throughout body. Notochaetae homogomph spinigers; neurochaetae homogomph spinigers and falcigers in supra-acicular fascicles, homogomph and sesquigomph spinigers and homogomph falcigers in sub-acicular fascicles.

Additional features included in the current diagnosis are the lack of neuropodial ventral ligules along the body, the lack of several parapodial processes toward posterior end, and homogomph falcigers deprived of dentition. The presence of papillae in both oral and maxillary rings is a unique feature in this genus.

Plate 38. Anterior end in dorsal view of *Lycastonereis indica*.

***Neanthes* sp.**

Diagnosis of *Neanthes* sp.: In total 83 (61-76) chaetigers, measuring 40 (22-37) mm long and 1.6 (1.5-2.4) mm broad. Antennae 0.7 (0.5-0.8) times prostomial length; clear patch on posterior half of prostomium narrowly produced towards anterior margin; longest tentacular cirri (posterodorsal) reaching chaetiger 4 (3-6); longest tentacular cirri (posterodorsal) reaching chaetiger 4 (3-6); longest tentacular cirri (posterodorsal) reaching chaetiger 4 (3-6); longest tentacular c (patch discoloured in some specimens). Brown pigment on both the prostomium and peristomium's anterior sides. Peristomium is around 1.5 (1.4-1.9) times the length of chaetiger 1 and the chaetigers after it. Large, conical, melted type paragnaths emerge from a plate-like foundation. Paragnath is counted in the following way: 2 (1-2) in longitudinal row; 9 (5-13), 11 (5-14) in a single curving row, progressing toward 2 rows in lectotype; 3 (2-4) in longitudinal line (no lateral ones). Jaws with four (4-5) teeth.

Plate 39. Anterior end in dorsal view of *Neanthes* sp.

Transition from thick, terminally rounded parapodial lobes to narrower, more elongate and pointed parapodial lobes occurs after chaetiger 10 (11-12). On anterior chaetigers, the dorsal cirri are 1.5 (1.2-1.6) times longer than the notoacicular lobe, and about 1.4 (1.3-1.8) times longer in the mid and posterior body. From chaetiger 3 to 27, the prechaetal notopodial lobe is present (16-18). From chaetiger 1 to 29, the neuropodial postchaetal lobe is present (18-28). Ventral cirrus inserted basally, around 0.6 (0.5-0.7) times anteriorly, about 0.7 (0.6-0.9) times posteriorly than ventral neuropodial lobe. Simple neuropodial falcigers, partially or totally united, with several 2-3 in the dorsal fascicle and 2-4 in the ventral fascicle; present from chaetiger 22 (15-28) in the dorsal fascicle and chaetiger 55 (45) in the ventral fascicle.
Anal cirri are present (but missing in some species), ventrally connected, and reach about 15 chaetigers forwards. Around chaetiger 11, a distinct shift occurs from thick, terminally rounded parapodial lobes to thinner, more elongate, and pointed parapodial lobes. On the first seven chaetigers, the dorsal and ventral cirri are enlarged with an abruptly tapering tip, indicating epitokal modification. Chaetiger 3 has a prechaetal notopodial lobe. Chaetiger 1 has a neuropodial postchaetal lobe. At least from chaetiger 8, there are partially or entirely united simple neuropodial falcigers in the dorsal fascicle, numbers 2-3.

***Composetia* sp.**

Diagnosis *Composetia* sp.: Prostomium with entire anterior margin, one pair of antennae, one pair of palps, and two pairs of eyes. Eversible proboscis with conical paragnaths only on maxillary ring, without any paragnath and papilla (or soft cushion) on oral ring. Four pairs of tentacular cirri. Parapodia of first two chaetigers sub-biramous, all following parapodia biramous. Sub-biramous parapodia with or without notoacicula. Notopodial prechaetal lobe present or absent. Notochaetae all homogomph spinigers. Neurochaetae all compound with homogomph, sesquigomph or heterogomph articulations, simple chaetae absent.

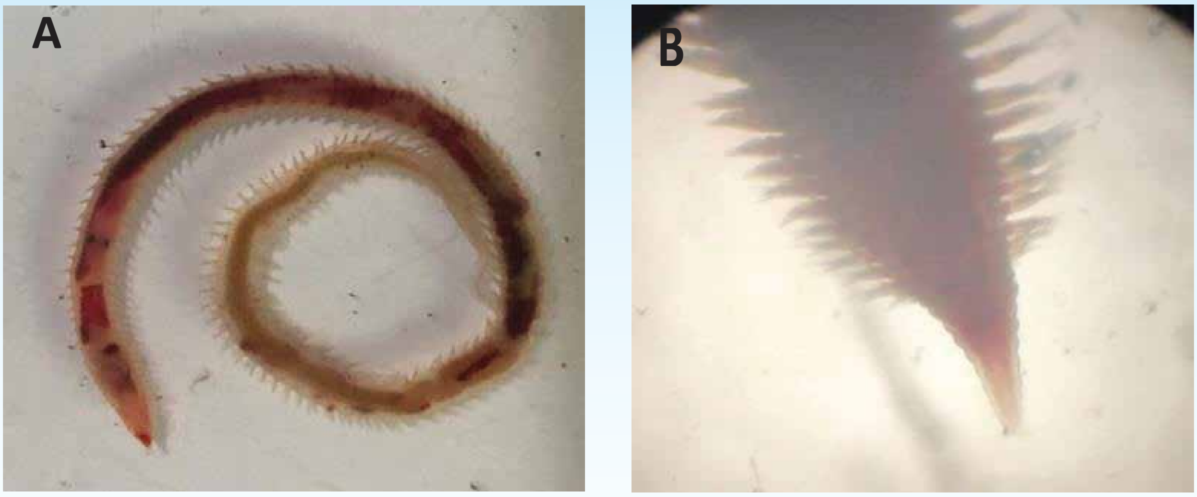
***Composetia* sp.**

Plate 40. Anterior end in dorsal view of *Composetia* sp.

Glycera* cf. *Sheikhmujibi

Diagnosis

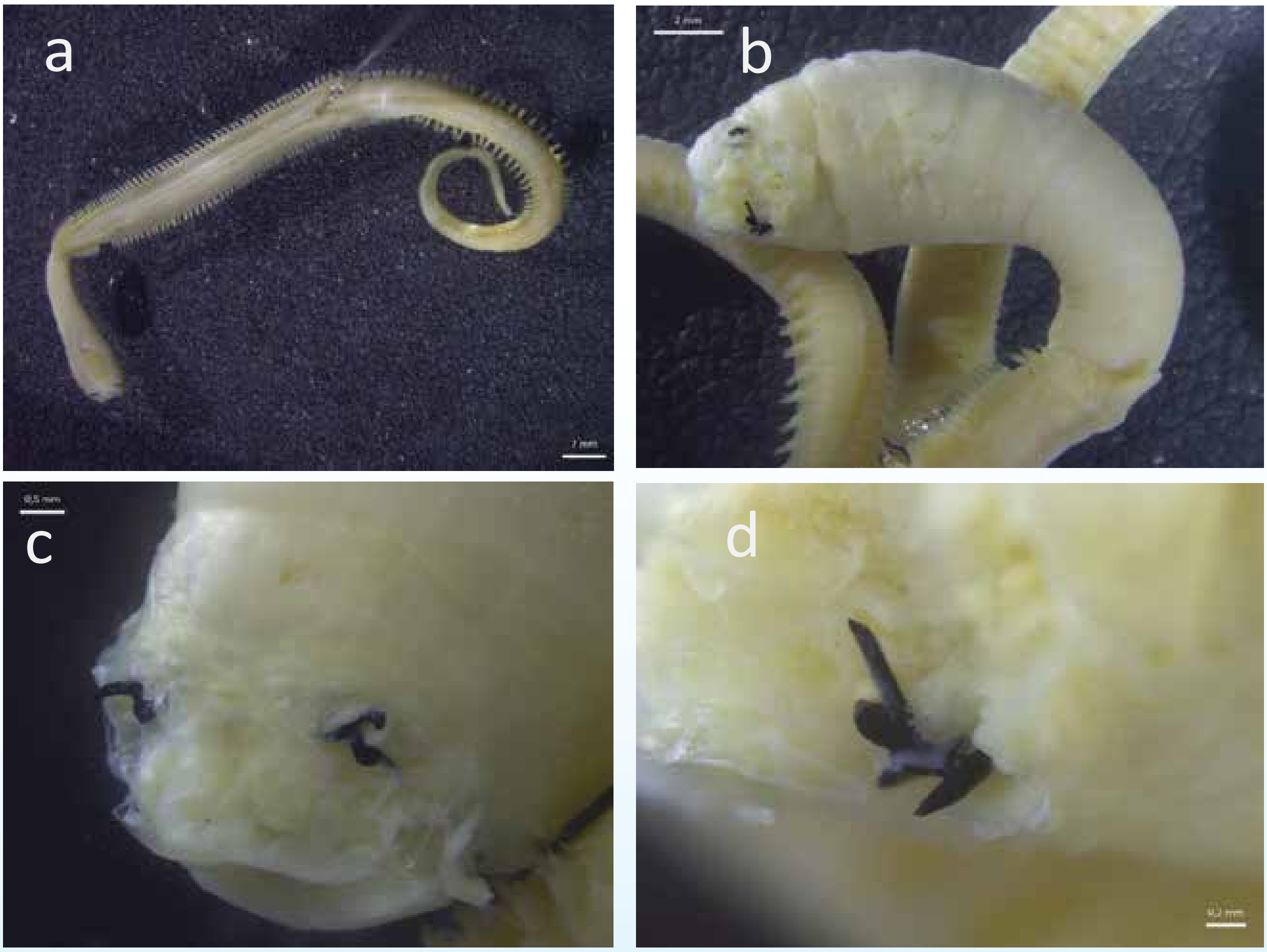
Because of its general physical similarities with other *Glycera* species, particularly the presence of distinct types of thick papillae on its proboscis, this species has been assigned to the genus *Glycera*. The following distinguishes the genus *Glycera* Lamarck, 1818 from other genera: a sharply pointed, typically ringed prostomium with four terminal tentacles; and a long, eversible, club-like proboscis with four hooked horny jaws and auxiliary lateral ailerons. Ailerons have a more complex structure with exterior and inner rami, as well as an interramal plate on occasion. The ventral chaetae compound and dorsal capillary chaetae, as well as the ventral chaetae compound and dorsal capillary chaetae, feature two anterior lobes with cirri and one or two posterior lobes. This species has three types of proboscideal papillae: type 1 papillae (main type), which are conical with three transverse ridges; type 2 papillae, which are conical with straight, median, longitudinal ridges; and type 3 papillae, which are round, shorter, and broader, with straight, median, longitudinal ridges; (ii) Y-shaped ailerons with gently incised triangular.



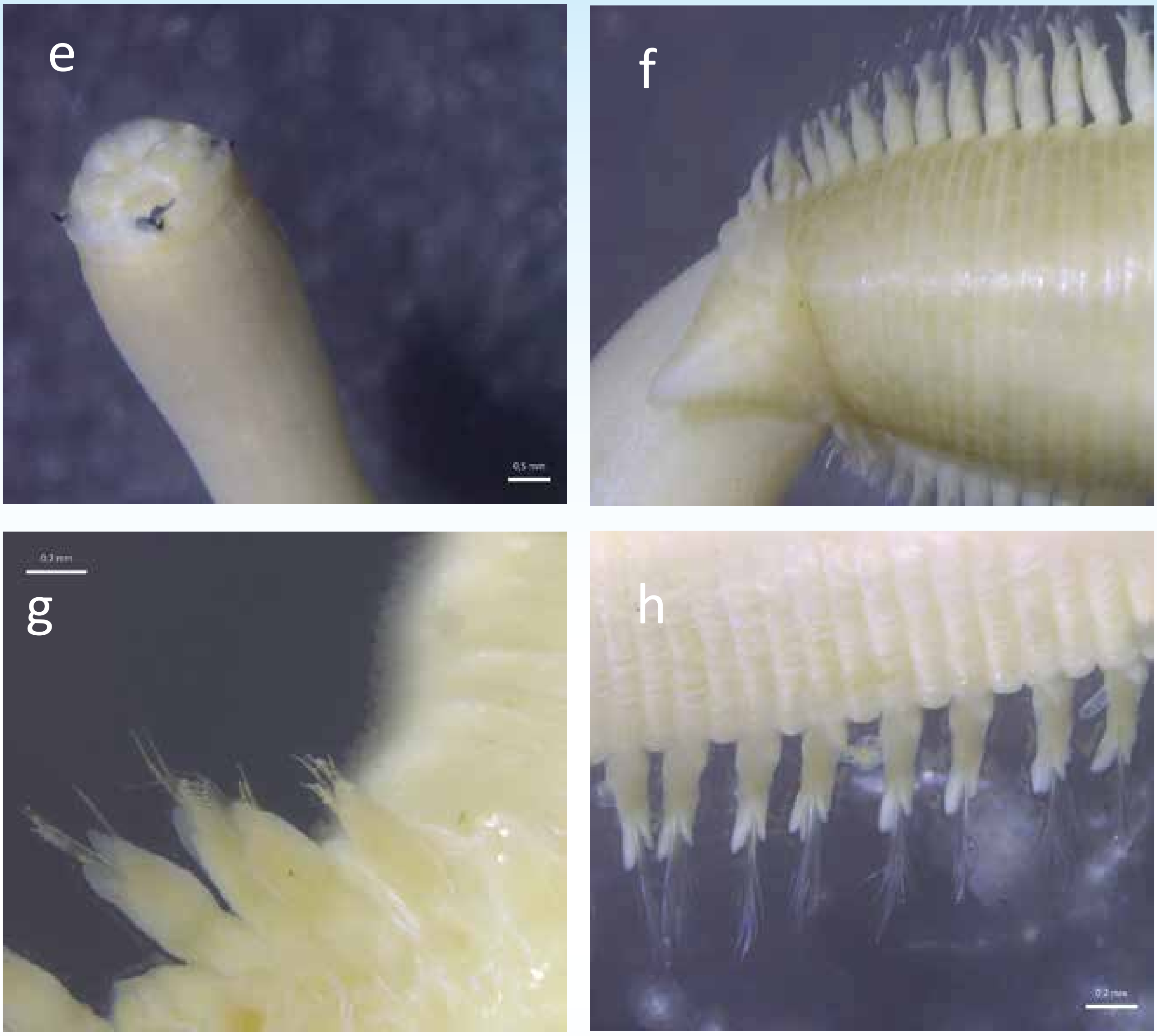
Plates 41. A. Specimen of *Glycera* cf. *sheikhmujibi.* B. Anterior view with the sharp head.

***Glycera* sp**

A few specimens of a glycerid were found in this collection, but it is unidentifiable to species. The segmented body (Plate-42 a-d) reaches lengths up to 12.0 cm, is tapered at both ends and is circular in cross section.



Plates 42. Glycera sp. a). Dorsal view of complete specimen of *Glycera* sp. b). Anterior end with everted proboscids. c). Mandibular jaw with aileron. d). Close view of mandibular jaw with aileron.



Plates 43. *Glycera* sp. e) Proboscis. f). Prostomium and anterior chaetigers, dorsal view. g). Anterior-most parapodia. h). Parapodia, dorsal view.

The number of segments varies with length, averaging about 130-195 in a 10.0 cm worm. The parapodia along the sides of the body are of uniform size throughout. There are two slender naudal cirri (tail filaments). The anterior (head) end is pointed and equipped with four small tentacles (feelers). There are no eyes. The anterior end of the digestive tract has powerful muscles which enable the worm to evert it as a large club-shaped proboscis (Plate-43 e-h). It bears four sharps, curved, terminal jaws. There is no true blood or vascular system. The coelomic (body cavity) fluid takes the place of blood and its circulation is accomplished by contractions of the body wall and ciliated tracts in the gills on the parapodia. The living specimens possess hemoglobin in corpuscles in the coelomic fluid. This gives the worm its ordinary reddish color and makes it appear to "bleed" more than other worms when it is damaged. Sexes are separate and eggs and sperms are found free in the coelomic fluid. As the worms mature, these germ cells fill the coelomic cavity, showing through the body wall and changing the color of the worm. Mature females become pale brown and males cream colored.

11.2.2. Length-weight models and population dynamics of *Namalycastis* sp. (Nereididae, Polychaetaes) from the Cox's Bazar coast of Bangladesh

Length-weight (L-W) relationship of *Namalycastis* sp.

The length of individuals ranged from 4.3 to 21.5 cm and weight from 0.100 to 1.980 g. The monthly L-W relationships of *Namalycastis* sp. with the co-efficient of determination (r^2) in different functions, regression coefficient/slope (b), standard error (SE) etc. have been depicted in Table 21a & 21b. A significant ($p < 0.001$) L-W relationship of *Namalycastis* sp. was determined in the annual (August-2020 to July-2021) samples (n = 1300, $r^2 = 0.73$). However, the estimated b was < 3 for *Namalycastis* sp. in the analyzed samples, which, indicated a negative allometric growth. The highest b was measured 2.12 in March (n = 104), whereas the lowest b was 1.18 in June (n = 129). The highest $r^2 = 0.94$ was estimated in April-2021 (n = 104) both in linear (l), power (pw) and polynomial (p) model, while no correlations were found in logarithmic (log) ($r^2 = 0.12$), l ($r^2 = 0.19$), pw ($r^2 = 0.20$), exponential (e) ($r^2 = 0.37$), but moderate correlation in p ($r^2 = 0.59$) models in June-2021 (Table 21a). Moreover, the curvilinear polynomial and exponential are better fitted models than others. Similarly, the curvilinear regressions (e.g., p, pw, e) were fitted well than the linear model for *Armandia maculata* (Opheliidae) and *Aglaophamus macroura* (Nephtyidae) specimens collected in the Otago shelf, New Zealand (Paavo et al., 2008). The estimated intercepts (a) in the present study are > 0 which indicates that the individuals hatch at a definite size, whereas an intercept ≤ 0 is biologically meaningless (Paavo et al., 2008). The estimated b value remained < 3, which generally indicated a negative allometric growth of *Namalycastis* sp. Rosati et al., (2012) calculated b =1.11 and 2.20 in Nereididae, Phyllodocida ($r^2 = 0.54$, n = 5) and *Dediste diversicolor* (Nereididae) ($r^2 = 0.90$, n = 207), respectively in the Mediterranean and Black Sea. Stoffels et al. (2003) estimated b = 1.875 ± 0.124) in Oligochaete ($r^2 = 0.72$, n = 90) in New Zealand. Although b was measured 3.52 in *Armandia maculata* (Opheliidae) ($r^2 = 0.88$, n = 69), it was 0.16 in *Aglaophamus macroura* (Nephtyidae) ($r^2 = 0.92$, n = 56), accordingly in New Zealand (Paavo et al. 2008, Table 21a & 21b). The b value was measured 4.27 and 2.36 in mesogastropoda species *Bithynia tentaculate* (Bithyniidae) and Naticidae, respectively (Rosati et al., 2012).

Table 21a. L-W relationship of *Namalycastis* sp. with regression parameters. (L = total length, W = weight, , AFW eFW = estimated fresh weight, DW = dry weight = ash-free weight, A = area, TA = total area, n = total number, SE = standard error, r^2 = correlation coefficient, linear (1), logarithmic (log), polynomial (p), power (pw), and exponential (e) regressions. '–' = not found. P-value <0.001.

References & Group	Months	Equation	N	Intercept (a)	Slope (b)	SE	P-value	log r^2	1 r^2	pw r^2	e r^2	p r^2
Present Study	August-20	$W=aL^{1.50}$	87	0.741	1.50	0.047	< 0.001	0.82	0.89	0.90	0.92	0.92
	September	$W=aL^{1.53}$	77	0.732	1.53	0.068	< 0.001	0.79	0.80	0.82	0.80	0.80
	October	$W=aL^{1.36}$	122	0.820	1.36	0.148	< 0.001	0.43	0.44	0.48	0.47	0.45
	November	$W=aL^{1.80}$	109	0.599	1.80	0.086	< 0.001	0.70	0.75	0.76	0.77	0.77
	December	$W=aL^{1.72}$	111	0.779	1.72	0.254	< 0.001	0.69	0.64	0.69	0.70	0.66
Namalycastis sp., Nereididae	January-21	$W=aL^{1.52}$	130	0.742	1.52	0.062	< 0.001	0.70	0.78	0.85	0.88	0.87
	February	$W=aL^{1.63}$	105	0.824	1.63	0.058	< 0.001	0.74	0.83	0.86	0.89	0.89
	March	$W=aL^{2.12}$	104	0.720	2.12	0.039	< 0.001	0.88	0.87	0.90	0.87	0.87
	April	$W=aL^{2.03}$	104	0.698	2.03	0.030	< 0.001	0.93	0.94	0.94	0.92	0.94
	May	$W=aL^{1.53}$	106	0.715	1.53	0.890	< 0.001	0.80	0.87	0.90	0.90	0.91
	June	$W=aL^{1.18}$	129	0.372	1.18	0.740	< 0.001	0.12	0.19	0.20	0.37	0.59
	July	$W=aL^{1.72}$	101	0.657	1.72	0.103	< 0.001	0.80	0.81	0.82	0.81	0.82
	Overall	$W=aL^{1.65}$	1300	0.72	1.65	0.138	< 0.001	0.63	0.70	0.72	0.71	0.73

Table 21b. L-W relationship of *Namalycastis* sp. with regression parameters. (L = total length, W = weight, eFW = estimated fresh weight, DW = dry weight, AFW = ash-free weight, A = area, TA = total area, n = total number, SE = standard error, r^2 = correlation coefficient, linear (1), logarithmic (log), polynomial (p), power (pw), and exponential (e) regressions. '–' = not found, P-value <0.05.

References	Groups	Months	Equation	N	Intercept (a)	Slope (b)	SE	log r^2	1 r^2	pw r^2	e r^2	p r^2
Paavo et al., 2008	Armandia maculata Opheliidae	March-2003	eFW=L3.52	69	0.0014	3.52	–	0.69	0.77	0.88	0.80	0.79
		March-2003	DW=L3.24	69	0.0006	3.24	–	0.69	0.77	0.88	0.80	0.79
		March-2003	AFW=W2.39	58	0.3102	2.39	–	0.55	0.60	0.76	0.73	0.61
Paavo et al., 2008	Aglaophamus macroura, Nephtyidae	March-2003	W7=e0.04L	56	0.2867	0.04	–	0.62	0.78	0.70	0.77	0.82
		March-2003	eFW=e 0.16L	56	0.1871	0.16	–	0.50	0.73	0.89	0.90	0.92
		March-2003	DW= e 0.15L	56	0.0359	0.15	–	0.50	0.72	0.90	0.91	0.90
		March-2003	AFW=e 0.14L		0.0312	0.14	–	0.48	0.69	0.87	0.91	0.86
		March-2003	eFW =TA 1.5		0.1847	1.5	–	0.60	0.93	0.97	0.75	0.97
Rosati et al., 2012	Phyllodocida, Nereididae	Nov. 2004 and May 2005	W=aL1.11	5	0.0841	1.11	–	–	0.54	–	–	–
Rosati et al., 2012	Hediste diversicolor, Nereididae	Nov. 2004 and May 2005	W=aL2.20	207	0.0023	2.20	–	–	0.90	–	–	–

In addition, b was calculated 3.05 and 2.61 in male and female mud crab (*Scylla olivacea*), respectively collected from the Sundarbans mangrove, Bangladesh (Sakib et al., 2022). The fluctuation of b might be depended on the species gravity, habitats, body dimensions (either 2 or 3), and other factors. For example, a taxon's spatial distribution can differ in the L-W relationships due to natural variation of the local environmental populations (Benke et al., 1999; Johnston and Cunjak 1999). Therefore, the variation of L-W values among different models for the same species may vary in different habitats. Secondly, the shape of the polychaetes and their specific gravity must remain unaltered throughout the larval development, and the desired b would be exactly 3. However, polychaetes shape and all the macrobenthos changes somewhat as they grow; it becomes proportionately narrower as body length increases and the specific gravity declines with size (Benke et al., 1999). The b value is supposed to be nearly 2 for organisms that are more likely to be 2-dimensional (relatively flattened) rather than 3-dimensional (Wenzel et al., 1990; Towers et al., 1994). This recommendation would only be applied if the flattened dimension remains unaltered or only slightly changed (Benke et al. 1999). The estimated average b = 1.65 in the present study of *Namalycastis* sp. remained closely 2, which implies the more 2-dimensional matrix of the species, and there were no major changes in heights. The precision of L-W equations is crucial in determining if and how they may be applied to different ecosystems and species that are linked. In addition, a fundamental concern in aquatic ecology as applied to the monitoring and protection of aquatic ecosystem health is the search for simple, rapid, and accurate methods for assessing biological features of populations and communities (Rosati et al., 2012). Another important factor is to compute the L-W models in different seasons, species populations, diverse aquatic ecosystems and various ecoregional areas that are depended on individual weight and body conditions of several environmental niche axes of transitional water ecosystems (Basset et al., 2008). The existing information implies that L-W models can be employed for the same species in different habitats if the environmental circumstances and intensity of anthropogenic pressures are consistent (Basset, 1993) and bias is decreased by expanding the spatial scale examined (Rosati et al., 2012). Further research is necessary to adjust these equations based on the regional biology or available information on the life history of the selected species. When using these equations in different areas, several factors affecting body size should be considered (Paavo et al., 2008). For example, the reproduction between *Armandia brevis* population with variable length was independent of photoperiod or food quality in the Washington samples but the California specimens were at the same developmental stage with similarly sized (Hampton 1997). In addition, seasons, sediment quality and environmental conditions have measurable impact on the growth and body mass of polychaetes (Meador and Rice 2001; Paavo et al., 2008).

Population dynamics of *Namalycastis* sp.

Growth parameters

Asymptotic length (L_∞) of the von Bertalenffy growth function (VBGF) was 21.5cm and the growth coefficient (K) was 1.5 year^{-1} for *Namalycastis sp.* The calculated growth curve using these parameters are shown over the restructured length distribution in Fig 17.

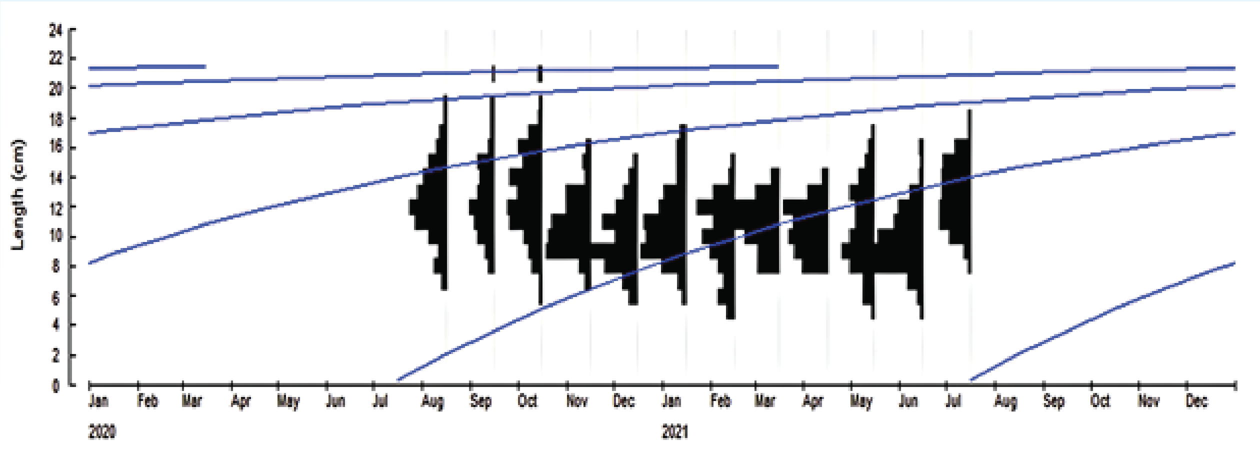


Fig 17. Von Bertalanffy growth curves of *Namalycastis* sp. drawn over their restructured length-frequency distribution using ELEFAN-1 ((L_∞ = 21.5 cm; K = 1.5 year^{-1}).

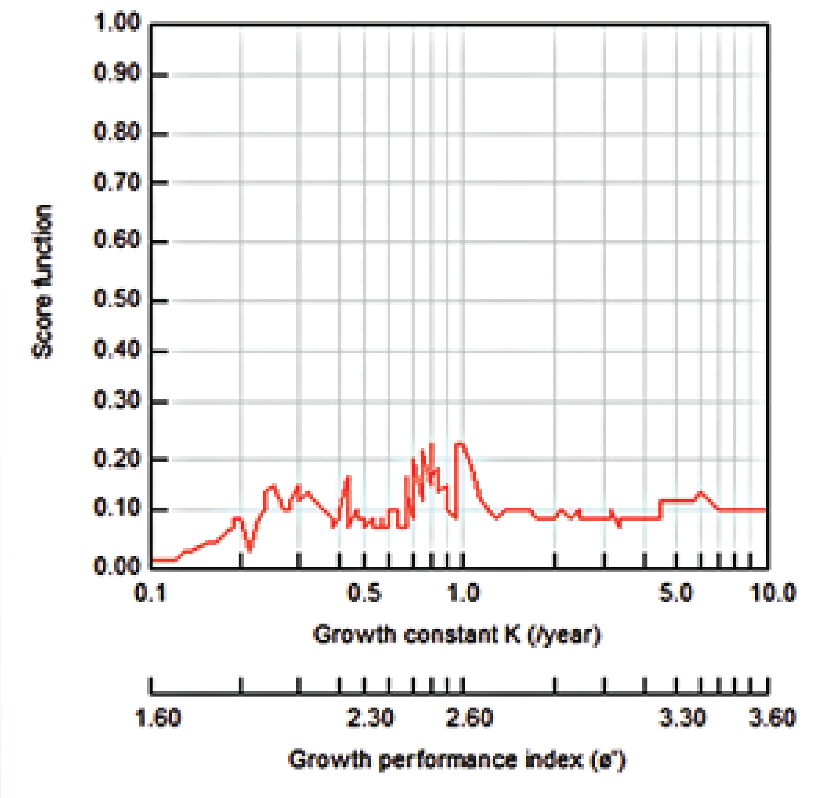


Fig 18. Estimation of growth coefficient (K) and growth performance index (ϕ) for *Namalycastis* sp. in the Cox's Bazar coast.

The best estimated value of K and growth performance index (ϕ) were 1.5 $year^{-1}$ and 2.60, respectively (Fig. 18). Growth parameter 21.5 cm indicates that *Namalycastis* sp. can grow up to 21.5cm unless any fishing mortality occurs. Five cohorts or size-groups in the *Namalycastis* sp. population has been observed through the indicator of five growth curve in Fig. 17.

Mortality and exploitation

Figure 6 represents the catch curve utilized in the estimation of total mortality (Z) and exploitation rate (E). The darkened circles were used in calculating the Z through the least square linear regression. The blank circles represent the points not fully recruited. Length converted catch curve analysis produce total mortality estimated value for *Namalycastis sp.* was Z = 4.56 $year^{-1}$ (Fig. 19). The estimated natural mortality (M) and fishing mortality (F) were 1.96 $year^{-1}$ and 2.60 $year^{-1}$, respectively According to Gulland (1971), when the F and M are equal, the yield is optimized; however, the stock would be over exploited when E is > 0.5. In this study, Exploitation level (E) was calculated 0.57 for *Namalycastis* sp.

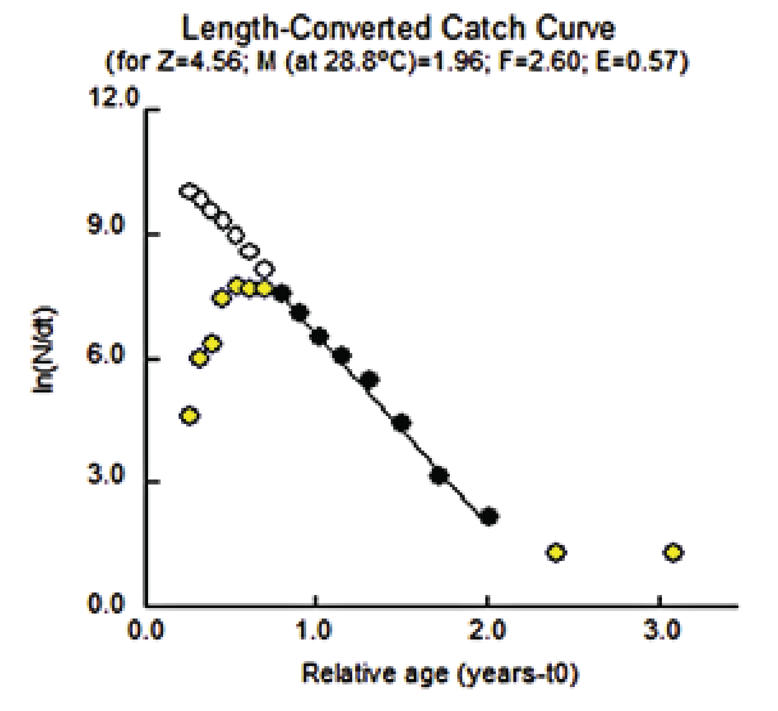


Fig 19. Length converted catch curves for *Namalycastis* sp. of Cox's Bazar coast of Bangladesh (The filled dots represent the points used in least square linear regression and the open dots indicate points that have not been recruited fully).

Recruitment pattern and Virtual Population Analysis (VPA)

The recruitment pattern of *Namalycastis* sp was continuous throughout the year and two major peaks observed from October–November and February-April (Fig. 20). In, Oct-Nov, the peak pulse produced around 16.0 % of the observed recruitment, while it was 13.0 % in Feb to April during the study period (Fig. 20).

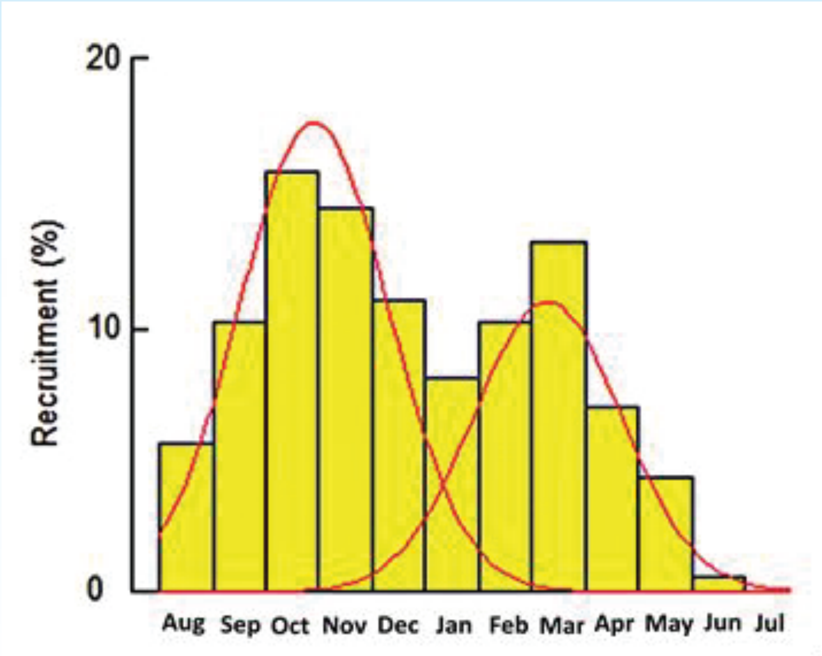


Fig 20. Annual recruitment patterns of *Namalycastis* sp. sampled from the Cox's Bazar coast.

The length-structured VPA is a powerful tool for stock assessment by which the size of each cohort is estimated along with the annual mortality caused by fishing. The results of length based virtual population analysis (VPA) was depicted in Fig 21. It shows the fishing mortality in relation to mean length. The VPA results indicated that fishing pressure is high on 10-18 cm length (Fig 21).

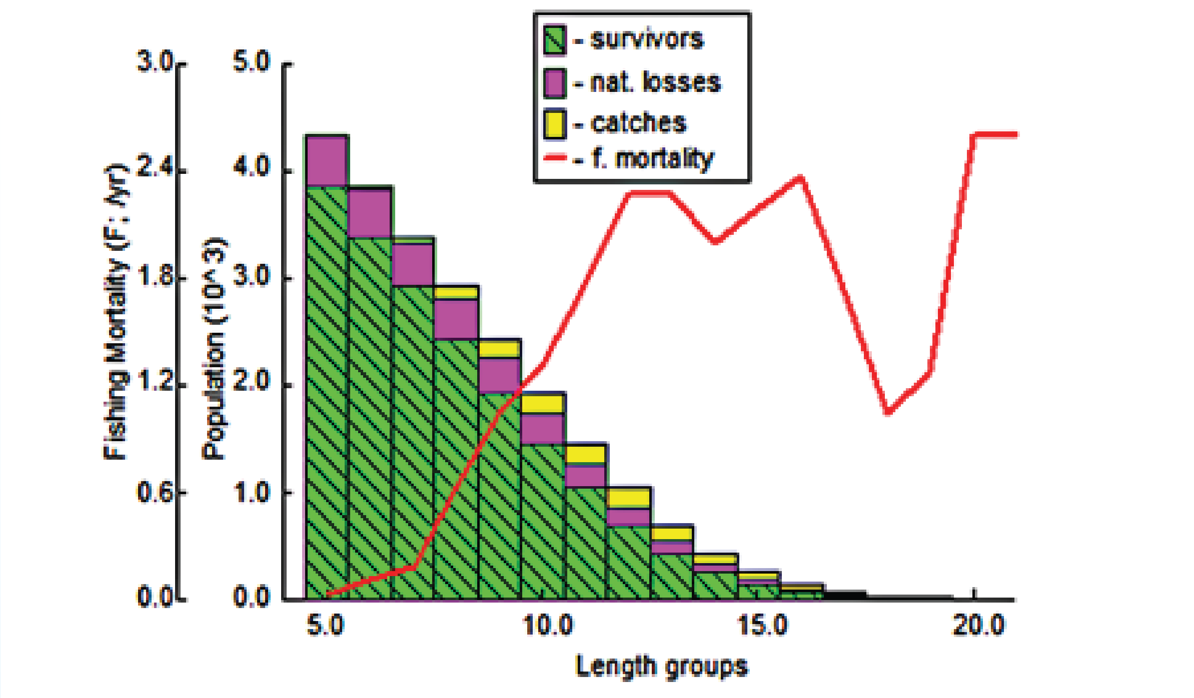


Fig 21. Length based virtual population analysis for *Namalycastis* sp. collected from Cox's Bazar coast of Bangladesh.

As no studies have been reported on the L-W relationship and population dynamics on *Namalycastis* sp. to date, comparison was made based on the Nereditae species. Several factors should be cautiously considered while comparing the population parameters. For examples, the study areas, habitat, seasons, etc. play a pivotal role in population structure. Due to higher temperatures and greater abundance of food sources, northern hemisphere species usually have reproductive seasons limited to the Spring and/or Summer, and, most crucially, show seasonal maturation, with substantially varied growth rates throughout the life cycle (Fischer 1999; Peixoto et al., 2018). Seasonal growing peaks, shown in temperate species, may still not appear in tropical and subtropical environments, and breeding may occur year-round (Florêncio 2000, Sette et al. 2013). Moreover, the population studies of polychaetes are often interrupted due lack of hard structures, and body flexibility and susceptibility (Omena and Amaral 2000). The present study was conducted in the tropical muddy mangrove areas, where yearly temperature was recorded at 28.58 °C (Fig 22).

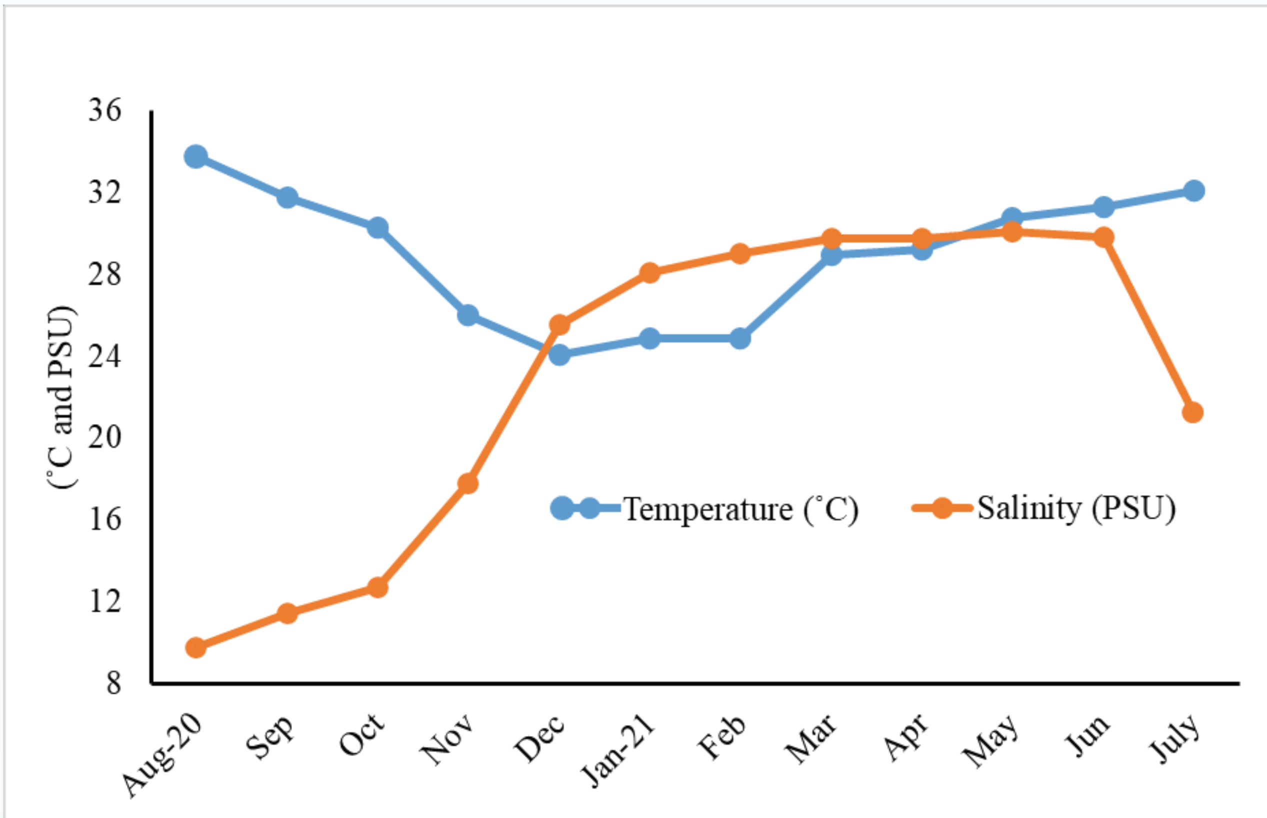


Fig 22. Annual (Aug-2020 to July-2021) temperature and salinity patterns in the sampling areas of Cox's Bazar, Bangladesh.

The asymptotic length (L_∞) and highest recruitment were recorded in October-2021 (Fig. 17, 20), where, temperature and salinity were measured 30.30 ˚C and 12.71 PSU, respectively (Fig. 22). The first highest recruitment occurred in October and November, where the average temperature and salinity were 28.15 ˚C and 15.25 PSU, accordingly. Moreover, the second highest recruitment was calculated in March-2021 (Fig. 20), where the mean temperature and salinity belonged to 28.93 ˚C and 29.75 PSU, respectively (Fig. 22). Thus, the recruitment was highly relied on the temperature fluctuations. Probably, the recruitment and growth of *Namalycastis* sp. is rarely depended on salinity, due to its euryhaline (0.5 to 73 PSU) nature (Komendantov et al., 1989; Ezhova 2011). However, the tolerant turned out to be greater stenohaline during the pre-recruitment states such as, ontogenesis gametes and early ontogenetic stages of development (Ezhova 2011). There was a significant decline in bigger size-classes of *Namalycastis* sp, between December 2020 and January 2021, which could be associated to the end of reproductive peaks, like other Nereididae species, may be semelparous (Wilson 2000), dying after releasing of gametes (Fischer and Dorresteijn 2004). Likewise, the larger sized *Namalycastis* sp. sharply declined from May to July-2021. During the reproductive peaks, growth was largely decreased because most nutrients and energy are consumed for gametes maturation and growth of epitokal modifications (Fischer 1999, Olive et al. 2000). This similar phenomenon was also reported by Scaps et al. (2000) for *Perinereis cultrifera* and Daas et al. (2011) for *Nereis falsa* in the Northern hemisphere.

Other factors such as food availability (Basset and Glazier, 1995), competitive interactions among species (Basset and Rossi, 1990), and pollutions (Basset, 1993) are reported to stimulate individual body size and characteristic, causing potential variations in population size (e.g. L-W). However, L-W relationships likely to be resilient to these sources of variation, especially when ecological variations are predicted to be minor because the studied ecosystems are spatially adjacent and belonging to the same aquatic ecosystem group.

In our study, the estimated growth co-efficient (K), growth performance index (ϕ) and total mortality (Z) were 1.5 year^{-1}, 2.60 and 4.56 year^{-1}, respectively. Overall, the K and ϕ were almost similar to other literatures reported from the tropical to subtropical regions belonged to the Nereididae family. For example, the K and ϕ were ranged from 1.4 – 2.2 and 0.485 – 0.872 in *Laeonereis acuta* in Southeast coast of Brazil, respectively (Omena & Amaral, 2000). In addition, the K and ϕ were recorded 1.68 – 2.72 and 2.86 in *Alitta succinea* in a tropical estuary of Brazil, respectively (Sette et al., 2013). Furthermore, the K, ϕ and Z value was calculated 2.36 year^{-1}, 1.83 and 9.99 year^{-1} in *Perinereis anderssoni* in a subtropical Atlantic beach of Southeast Brazil, accordingly (Peixoto et al., 2018). These comparative data are subjected to alter a bit in different regions, species and some other conditions that influence growth and survival year-round, like photoperiod and temperature (Fischer 1999). In addition, our estimated fishing mortality (F) was higher than natural mortality (M) and the population was over exploited (Fig. 19). This wild populations might be affected by overfishing, habitat disturbances, seasonality, and environmental factors (Scaps 2003, Garcia-Alonso et al., 2008). As no farms and hatcheries do not produce polychaetes in Bangladesh, local fishers and anglers catch polychaetes in the mangrove channel. Even some shrimp post larvae (PL) producing hatcheries collect wild polychaetes from local fishers at a high price (150 USD/Kg) (Personal contact). Hatchery owners feed polychaetes to the brood shrimp for gonadal maturity and good quality PL. Besides, natural mortality might be occurred due to temperature and salinity fluctuations, inter and intra-species competition for food and habitats.

11.2.3. Effect on growth, survival and fatty acid profile of *Namalycastis* sp. (Nereididae, Polychaetaes) in three variable diets

Daily growth rate (DGR), specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), feed efficiency ratio (FER) and survival rate (SR) of *Namalycastis* sp. following triplicate fashions (T1, T2 and T3) were calculated and presented in Table 22. The principal component analysis (PCA) of growth and survival have also documented in the Fig 23 A & B). Both T1 (37.619 %), T2 (44.960%) and T3 (39.697%) gained weight during the culture period, however, there were no significant ($p > 0.05$) variations calculated from the single factor ANOVA. The daily growth rate and specific growth rate were 1.395, 1.734, 1.497 and 5.396, 5.612 5.448 for T1, T2 and T3, respectively (Table 22). Although all the treatments gained final weight, there was no significant ($p > 0.005$) differences measured from single factor ANOVA. The calculated FCR and FER were 0.349, 0.304, 0.343 and 2.865, 2.287, 2.917 for the treatments T1, T2 and T3, respectively. The highest survival rate was measured for T3 (83%) and the lowest survival was for T1 (70.333%), however there were no significant ($p > 0.05$) changes recorded among the treatments (Table 22).

Table 22. Growth and survival of *Namalycastis* sp. in different diets along with other calculated values.

Species	diets	Initial avg. wt. (mg)	Final avg. wt. (mg)	Growth rate (mg/Day)	Specific growth rate (%/Day)	Weight gain (%)	Feed conversion ratio	Feed efficiency ratio	Survival rate (%)	References
Namalycastis sp.	A (T1)	333.68 ± 11.10	459.20 ± 37.40	1.395 ± 0.29	5.369 ± 1.34	37.62	0.35	2.86	70.33	**Present study**
	B (T2)	347.16 ± 26.78	503.20 ± 32.20	1.734 ± 0.06	5.612 ± 0.20	44.96	0.30	3.28	77.16	
	C (T3)	339.60 ± 31.20	474.32 ± 76.46	1.497 ± 0.50	5.448 ± 0.99	39.66	0.34	2.91	83.0	
Hediste Diversi color	D	-	-	13.90	6.80	-	-	-	100.00	Fidalgo e Costa et al., 2000
	E	-	-	9.40	6.10	-	-	-	100.00	
	F	-	-	9.40	6.80	-	-	-	88.80	
	G	-		8.80	5.80	-	-	-	100.00	
	H	-	-	6.90	5.30	-	-	-	94.40	
	I	-	-	6.20	5.40	-	-	-	77.70	
M. sanguinea	J	450.00 ± 30.0	3500 ± 230.0	-	0.750 ± 0.03	672.2 ± 59.0	-	-	62 ± 5.00	Parandavar et al., 2015

A= code:155-FF-01; B= code:155-FF-02; C= code:55-FF-03; D= code:155-FF-10; E code:155-FF-11; F=code:155-FF-14; G=code:155-FF-12; H= code:155-FF-16; I=Artemia; J= Commercial fish food pellets.

An almost reverse trends noticed from the PCA scatter plots and the line graph between growth and survival. Nearly no or negative growth was recorded during the first month (1-3) of *Namalycastis sp.,* however the growth rate transition (4) occurred after the first months and was increasing until the harvesting period (5-7) (Fig. 23A). On the other hand, the survival rate was gradually decreasing at the first half the months after stocking (Fig. 23B and 24).

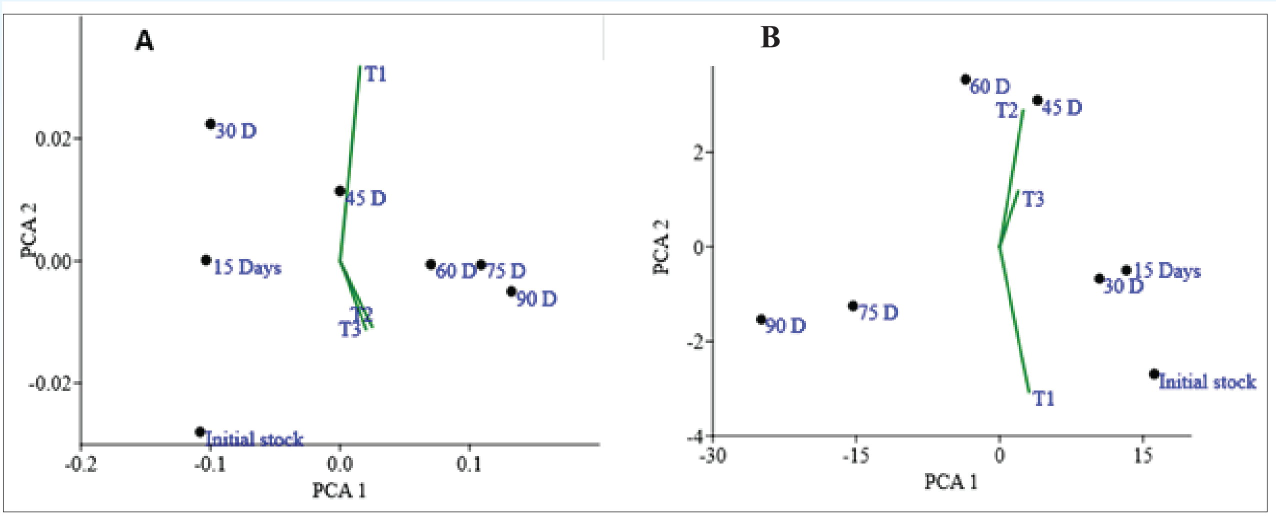


Fig 23A-B. Principal component analysis (PCA) shows the growth (A) and survival (B) rate of *Namalycastis sp*

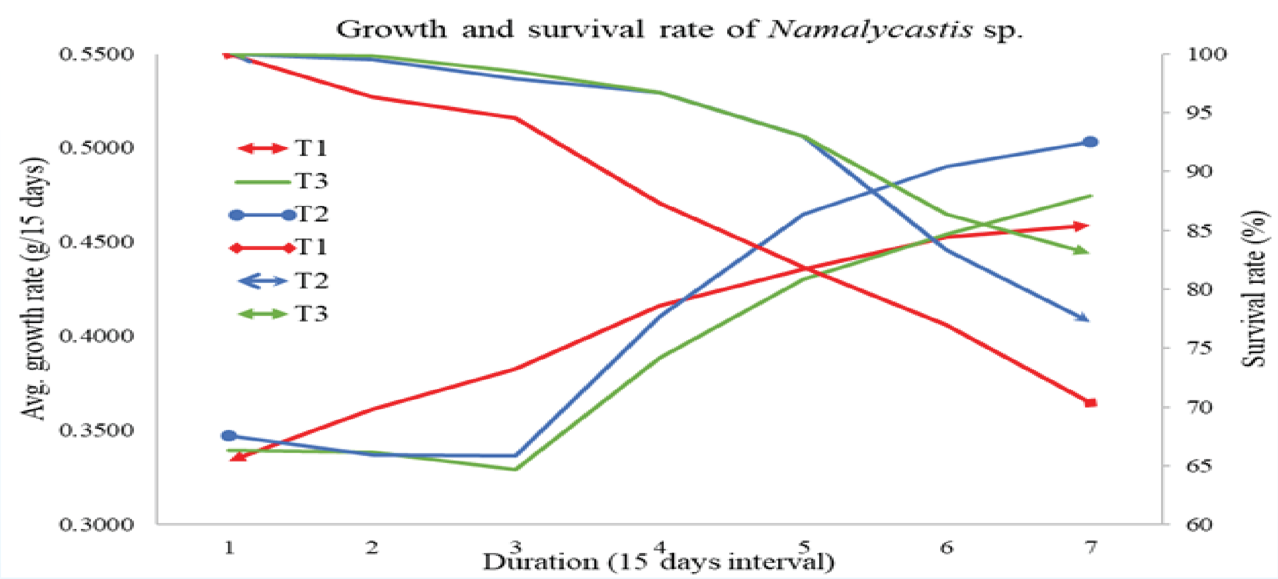


Fig 24. Growth and survival curves of *Namalycastis sp*. T2 showed the maximum growth, while T3 showed the highest survival.

The fatty acid was analysed from the wild and cultured specimens of *Namalycastis sp*. Although T1 and T2 possessed both saturated and unsaturated fatty acids, T3 and wild *Namalycastis sp.* were below the detectable limits. The total fatty acid recoveries were higher in T2 (95.28%) than T1 (92.35%). The paired two sample means were 13.571 and 13.611 for T1 and T2, respectively which was non-significant (p>0.05). The unsaturated vaccenic acid (C18:1) contents were 34.61 ± 2.32%, whereas arachidic acid (C20:0) acid was the lowest amount (1.67 ± 0.08%) for T1. One the other hand the saturated palmitic acid (C16:0) contained the highest amount (34.32 ± 2.87%), while, arachidic acid (C20:0) acid possessed the lowest amount (2.68 ± 0.13%) for T2 (Table 23).

Table 23. Fatty acids content in *Namalycastis sp.* analyzed from wild and culture. [- Below detection limit; NA -Not analyzed]

		myristic acid (C14:0)	**palmitic acid (C16:0)**	**stearic acid (C18:0)**	**vaccenic acid (C18:1 (total))**	**linoleic acid C18:2 (n-6)**	**arachidic acid (C20:0)**	**behenic acid (C22:0)**	**References**
Wild	*Namalycastis sp.*	-	-	-	-	-	-	-	Present study
Cultured *Namalycastis sp.*	code:155-FF-01 (T1)	8.2 ± 0.63	34.1 ± 2.31	6.43 ± 0.81	34.61 ± 2.32	7.34 ± 0.88	1.67 ± 0.08	2.65 ± 0.15	
	code:155-FF-02 (T2)	10.92 ± 1.62	34.32 ± 2.87	9.11 ± 1.08	28.61 ± 2.07	9.64 ± 1.03	2.68 ± 0.13	-	
	code:155-FF-03 (T3)	-	-	-	-	-	-	-	
Cultured	Commercial fish feed (code:155-FF-11)	2.02 ± 0.55	19.15 ± 0.33	7.21 ± 1.14	13.25 ± 3.06	4.50 ± 0.58	NA	NA	
Hediste diversicolor	Commercial fish feed (Moist sole)	1.6 ± 0.31	16.63 ± 1.71	6.04 ± 0.72	15.90 ± 4.33	8.03 ± 1.45	NA	NA	Santos et al., 2016
	Fish pellets (*T. trachurus*)	0.97 ± 0.49	22.40 ± 0.11	8.35 ± 0.90	9.2 ± 5.57	5.21 ± 4.72	NA	NA	
Cultured	Feed containing vegetable protein	1.073	8.833	3.496	2.443	NA	NA	0.636	Wibowo et al., 2020
Dendronereis pinnaticirris	Feed containing animal protein	0.91	7.776	3.063	2.065	NA	NA	0.516	

The PCA analysis showed that the saturated palmitic acid (C16:0) and unsaturated vaccenic acid (C18:1) were more dominated for both treatments, while rest of the acids were non-significant those of other acids (Fig. 25). Almost all the assessed fatty acids were higher in T2 than T1 except for the unsaturated vaccenic acid (C18:1) and behenic acid (C22:0). Vaccenic acid was 28.61 ± 2.07% in T2, however, it was 34.61 ± 2.32% in T1. In addition, behenic acid (C22:0) was 2.65 ±0.15% in T1, while it was below the detection limit for T2 (Table 23).

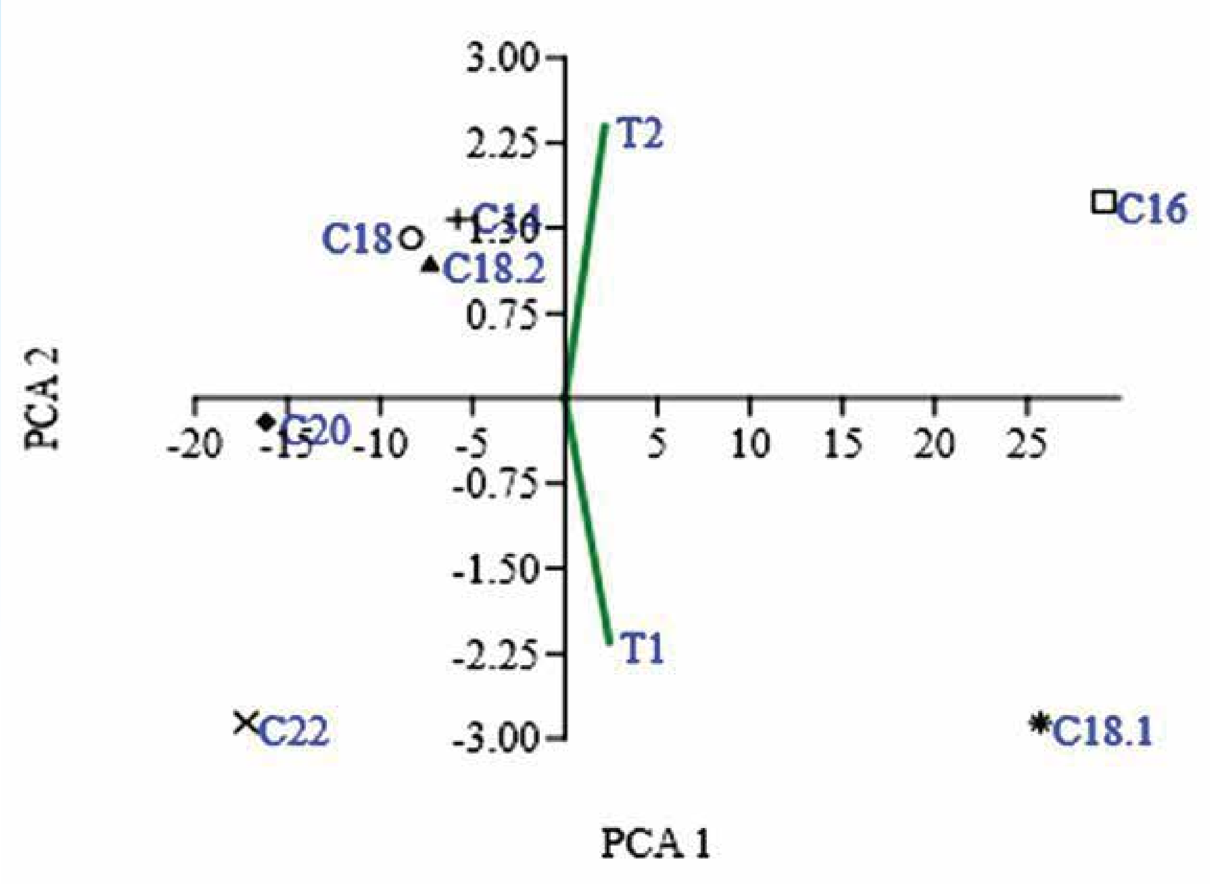


Fig 25. PCA (Principal Component Analysis) of the fatty acid. C16 and C18:1 was positively dominated while others were negatively dominated.

Namalycastis sp. gained weight from both commercial shrimp (PL 1-10) feeds. Although the protein and fat contents were higher in feed code:155-FF-01, the GR, SRR, WG, FER were higher in the code:155-FF-02 feed (Table 22). However, the highest survival (83%) was recorded in CP feeds. We did not measure the proximate composition and other additives of the feeds. All the specifications were provided by the feed company. The GR and SGR were between 1.395-1.734 mg Day^{-1} m^{-2} and 5.369-5.612 % Day^{-1}, respectively which was not significant ($p>0.05$) (Table 23). The slight alteration of GR, SGR WG, FER etc. maybe due to the different feed composition and intra-species competition. Moreover, the commercial shrimp feed was fed with the T1, T2 and T3. These might be the reasons to be non-significant. It would be clearer if another natural diets such as macroalgae, spinach etc. were fed and compared the growth and survival of *Namalycastis sp*. For example, the GR was highest in *Nereis diversicolor* when fed with code:155-FF-10 feed (13.9 $mgDay^{-1}$) followed by dry food code:155-FF-11(9.4), code:155-FF-14 (fish dry food) (9.4), code:155-FF-12 (8.8), code:155-FF-16 (6.9) and decapsulated *Artemia* cysts (6.2) (Fidalgo E Costa et al., 2000). In addition, the SGR (% Day^{-1}) was greatest 6.8 in code:155-FF-10 and code:155-FF-14 feed, while it was between 5.3-6.1 for rest of feeds. Moreover, the survival rate was 100% in case of code:155-FF-10 and code:155-FF-11 feed and code:155-FF-12 feeds whereas the survival rate was 77.7-94.4% in Tetramin, pollen and *Artemia* feeds (Fidalgo e Costa et al., 2000). High protein food (Larviva-Biomar®) showed better growth rate with commercial minimum weight (500.0 mg) after 6 weeks, recording the daily biomass production of 12,000.0 mg m^{-2} day^{-1}, while the *Hediste diversicolor* was fed with low-protein food (Classic C22-Hendrix®) reached the threshold to 9100.0 mg m^{-2} day^{-1} two weeks later (Nesto et al., 2012). The mean weight of *H. diversicolor* was significantly higher in code:155-FF-13 (920.0 ± 70.0 mg) than the in code:155-FF-15 (650 ± 90 mg) and brown macroalgae (*Sargassum muticum*) (220 ± 50 mg) after culturing them eight weeks (Nesto et al., 2012). The survival rate greatly reduced to 78%, 63% and 58% with code:155-FF-15, code:155-FF-13 and *S. muticum*, respectively from the eight weeks culture periods. Similarly, *H. diversicolor* was fed 3 different diets (two commercial diets, dry feed (code:155-FF-11) and semi-wet pellets for reared sole (Moist Sole), and a non-processed diet consisting on mackerel's fillets (*Trachurus trachurus*) and the highest final weight, SGR and DGR were highest in the commercial diets (Santos et al., 2016). Likewise, our selected 3 commercial diets possessed 38.0 - 42.0 % protein and there were no significant variations ($p > 0.05$) measured. Therefore, the commercial feeds can elevate higher growth and fecundity of polychaetes than feeding with natural feeds. For example, the commercial feed with code:155-FF-14 had a 2- or 3-fold higher fecundity of *Dinophilus gyrociliatus* than those fed with spinach (Prevedelli and Simonini 2000). In addition, the polychaetes were fed with animal protein showed the highest body weight gain than was fed with plant protein (Wibowo et al., 2020). It can be occurred due to higher absorption of animal feeds and containing indispensable amino acids methionin and lysin than the plant protein (Millamena et al., 2002; Yuwono 2008; Wibowo et al., 2020).

Both diets and salinity were statistically significant for the growth and survival of *D. gyrociliatus*. It showed better salinity tolerance below 35 PSU than those were cultured above 35 PSU (Prevedelli and Simonini 2000). In addition, *Marphysa sanguinea* juveniles and adults' polychaetes showed the highest weight gain and survival in 30 and 25 PSU, respectively, whereas the lowest survival was recorded at 35 PSU. Thus, the salinity has a significant effect on the growth and survival of polychaetes (Thu et al., 2019). The effect of temperature may also influence the growth rates of the nereidid polychaete. For example, there was a much slower growth measured in *Perinereis rullieri* at 15° C than at 21° C or 27° C ($P<0.05$) (Prevedelli, 1992). Even, the commercial feed Tetramin had a gradual growth at 15° C. However, culturing *P. rullieri* at 27° C showed better growth fed with tetramin or algae (Prevedelli, 1992).

The growth and survival of polychaetes also largely depends on the stocking density. For example, *M. sanguinea* was cultured in a semi-circulating system with 500 indv. m^{-2} , 1000 indv. m^{-2} and 2000 indv. m^{-2} stocking density. The results revealed that the highest growth was recorded in the lowest density, while the highest density (2000 specimens m^{-2}) was measured the lowest growth (Parandavar et al., 2015). Similarly, the growth and survival of *Hediste diversicolor* was the highest at the lowest densities (300 ind. m^{-2}) than the density stocked at 1000 and 3000 ind. m^{-2} (Nesto et al., 2012). It might be due to the negative influence of increasing intra-specific competition in the polychaetes (Nesto et al., 2012; Parandavar et al., 2015). We stocked at 1225 ind. m^{-2} for *Namalycastis sp.* in our culture systems. The SGRs of polychaetes may fluctuate by the culture periods too. Such as the SGRs of *M. sanguinea* was higher during the first culture period (0-3 months) than the second (3-6 months) and third periods (6-9 months) (Parandavar et al., 2015). Feeding ratio can also influence the growth and survival of polychaetes. Such as *M. sanguinea* was fed with 3%, 6%, 9%, 12% and 15% of total body weight. The result showed that 15% feeding rate had the highest growth rate with 81.9% survival, however, 3% and 9% feeding rates showed lower growth with the highest survival rates (100%) (Phoo, 2017). For *Namalycastis sp.*, we gave 3% feeds per total body weight which gained moderate weight (avg. 40.745 %) and comparatively lower survival rate (76.833%). These might be species specific, density and diets effect or any other factors that fluctuates growth and survival *Namalycastis sp*.

Growth performance of worms also influenced by the substrates type and depth. We provided 10.0 cm sand depth for the growth and survival of *Namalycastis sp*. *D. pinnaticirris* was cultured with 10.0 cm sand depth and it showed better worm growth (Mustofa et al., 2012). Kaba'ah (2015) used different artificial substrates such as white polyethylene beads, recycled polyethylene beads, corrugated polypropylene sheet and sand for Nereis virens culture. The results revealed that white polyethylene beads and recycled polyethylene beads were not statistically significant, while sand bed showed better growth than other substrates. In addition, an experiment designed with 7.0, 10.0 and 15.0 cm sand depth for the culture of *N. virens* culture. The results showed that both 10.0 and 7.0 cm were not statistically significant, however, the average worm's weight in 15.0 cm sand depth was significant than two of them (Kaba'ah 2015). Therefore, probably the growth and survival of polychaetes are depth specific in relation to species. However, the natural sand can be the best substrate than others.

The fatty acid composition in *Namalycastis sp.* greatly varies with habitats and types of diet. We analyzed the fatty acid contents both in cultured and wild *Namalycastis sp*. Although T1 and T2 had the higher fatty acid contents, the wild individuals and T3 were below the detection limit. The variation of fatty acid contents in wild *Namalycastis sp.* and cultured individuals depends on their respective diets and habitats (Limsuwatthanathamrong et al., 2012). Although there were no details on the food and feeding habits of *Namalycastis sp.,* Ezhova (2011) mentioned that *N. littoralis* feeds on organic debris and algae. However, ample study on the food and feeding habits of *Namalycastis sp.* would provide more information. Although there were no significant ($p > 0.05$) variations found between T1 and T2, surprisingly the fatty acid contents were below the detection limits in T3 (Table 22). It might have happened that the proximate crude fat composition was not accurate in T3 or other fatty acids were present that was not detected. The total fatty acid recoveries were higher in T2 (95.28 %) than T1 (92.35 %). The paired two sample means were 13.571 and 13.611 for T1 and T2, respectively which was non-significant ($p > 0.05$). In T1, the unsaturated vaccenic acid [C18:1 (total)] contents were highest (34.61 ± 2.32 %), whereas the saturated arachidic acid (C20:0) had the lowest (1.67 ± 0.08 %) concentration.

However, in T2, the saturated palmitic acid (C16:0) had the highest (34.32 ± 2.87 %) percentages, while arachidic acid (C20:0) possessed the lowest percentages (2.68 ± 0.13 %) (Table 22). The total unsaturated fatty acid was higher 41.95 ± 3.20 % in T1 and 38.25 ± 3.10 % in T2, accordingly (Table 22). The recovered myristic acid (C14:0) composition was higher in T2 (10.92 ± 1.62) than T1 (8.2 ± 0.63). In *H. diversicolor* juveniles, the myristic acid (C14:0) was higher in code:155-FF-11 feed (2.02 ± 0.55) than Moist Sole (1.56 ± 0.31) and mackerel's fillets (*T. trachurus*) (0.97 ± 0.49) (Santos et al., 2016). Wibowo et al., (2020) measured fatty acid contents in cultured *Dendronereis pinnaticirris* fed with vegetable and animal protein. They calculated myristic acid (C14:0) compositions between 1.073 % and 0.91 % fed with vegetable protein and animal protein, respectively. The palmitic acid (C16:0) composition was 34.1 ± 2.31 % in T1 and 34.32 ± 2.87 % in T2 that did not vary so much from both treatments. The palmitic acid (C16:0) was measured higher in *H. diversicolor* juveniles fed with 3 different diets and mackerel's fillets (*T. trachurus*) (22.40 ± 0.11) was higher than the commercial feed code:155-FF-11 (19.15 ± 0.33) and Moist Sole (16.63 ± 1.71) (Santos et al., 2016). Wibowo et al., (2020) calculated the palmitic acid (C16:0) compositions between 8.833 % and 7.776 % in *D. pinnaticirris* fed with plant and animal protein, respectively. The stearic acid (C18:0) concentration was 6.43 ± 0.81 % in T1 and 9.11 ± 1.08 % in T2, respectively. The stearic acid (C18:0) composition in cultured *H. diversicolor* was calculated 8.35 ± 0.90 %, 7.21 ± 1.14 and 6.04 ± 0.72 % fed with Fish pellets (*T. trachurus*), code:155-FF-11feed and Moist Sole, respectively (Santos et al., 2016). In addition, the stearic acid (C18:0) composition in *D. pinnaticirris* was between 3.496 % and 3.063 % fed with plant and animal protein, respectively (Wibowo et al., 2020). In addition, the vaccenic acid [C18:1 (total)] was higher 34.61 ± 2.32% in T1 than T2 (28.61 ± 2.07%). The total vaccenic acid (C18:1) in *H. diversicolor* was measured 15.90 ± 4.33 %, 13.25 ± 3.06 % and 9.2 ± 5.57 % fed with commercial feed Moist Sole and Aquagold, and T. *trachurus* Pellets, respectively (Santos et al., 2016). The vaccenic acid [C18:1 (total)] in *D. pinnaticirris* was measured 2.443 % and 2.065 %, fed with plant and animal-based feed, respectively (Table 23) (Wibowo et al., 2020). The estimated arachidic acid (C20:0) percentages were higher 2.68 ± 0.13 % in T2 than T1 (1.67 ± 0.08 %). However, the behenic acid (C22:0) content was 2.65 ±0.15 in T1 although it was below the detectable limit for T2. (Table 23). The calculated behenic acid (C22:0) composition in *D. pinnaticirris* was between 0.636% and 0.516 %, fed with plant and animal protein, respectively (Wibowo et al., 2020). The PCA showed that the saturated palmitic acid (C16:0) and unsaturated vaccenic acid (C18:1) were more dominated in both treatments, while rest of the acids were non-significant ($p > 0.05$). Almost all the assessed fatty acids were higher in T2 than T1 except for the unsaturated vaccenic acid [C18:1(total)] and behenic acid (C22:0).

Although the *H. diversicolor* was fed with high protein and fat containing commercial feed [code:155-FF-11(protein: 46.00%; Lipids: 18.00%), Moist Sole (protein: 52.12%; Lipids: 20.03%)] (Santos et al., 2016), the assessed saturated and unsaturated fatty acid contents were comparatively lower than our estimated fatty acids in *Namalycastis sp.* fed with moderate protein and fat containing commercial feeds code:155-FF-01 (protein 42.00 %, fat 7.00 %, code:155-FF-02 (42.00 %, fat 6.50 %) and code:155-FF-03 with protein 38.00%, fat 5.00 %)]. In addition, *D. pinnaticirris* was fed with Vegetables (protein 37.313, fat 4.676 %) Animal (protein 39.403, fat 7.123 %) based feed, however the measured fatty acids percentages were a bit less than our recovered value (Table 23; Wibowo et al., 2020). It may be due to the higher feed conversion and feed efficiency ratio of *Namalycastis sp.* than *H. diversicolor* and *D. pinnaticirris* (Tables 22 and 23).

11.2.4. Effect of growth, survival and fatty acid profile of *Namalycastis* sp. (Nereididae, Polychaetaes) in different salinity gradients

The daily growth rate (DGR), specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), feed efficiency ratio (FER) and final survival rate (FSR) of ***Namalycastis* sp** were calculated and presented in Table 24. Only 30.0 PSU gained highest biomass, survival and feed efficiency ratio, while rest of the salinities decreased biomass (Table 24). A total of 36 fatty acids (FA) were determined, where the number of saturated fatty acid (SA) was 17, monounsaturated fatty acid (MUFA) was 9 and poly unsaturated fatty acid (PUFA) was 10, respectively (Table 25). The FA composition between the wild and cultured worms were not statistically significant ($p > 0.05$), while the ∑SFA, ∑MUFA and ∑PUFA were significant ($p < 0.05$) to each other.

Table 24. The growth and survival parameters of *Namalycastis* sp. in different salinities (sal). Initial biomass (IBM), final biomass (FBM), daily growth rate (DGR), specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), feed efficiency ratio (FER) and final survival rate (FSR)

Salinity (PSU)	IBM (g)	FBM (g)	DGR (g day^{-1})	SGR (% day^{-1})	WG (%)	FCR	FER	FSR (%)
Sal-15	31.953	4.134	-0.309	-2.272	-672.932	-3.266	-0.348	15.7
Sal-20	28.554	25.487	-0.034	-0.126	-12.032	-47.649	-0.024	92.8
Sal-25	33.694	26.122	-0.084	-0.283	-28.987	-21.872	-0.052	89.2
Sal-30	33.694	39.748	0.067	0.184	15.231	28.497	0.040	91.6
Sal-35	28.066	28.359	0.003	0.012	1.035	470.022	0.002	83.1
Sal-40	30.014	25.680	-0.048	-0.173	-16.877	-31.672	-0.036	72.3

Although *Namalycastis* sp. showed the highest survival in 20.0 PSU, this treatment had a negative weight gain (WG %). Salinity was statistically significant for the growth and survival of *D. gyrociliatus*. It showed better salinity tolerance below 35.0 PSU than those were cultured above 35.0 PSU (Prevedelli and Simonini 2000). In addition, *Marphysa sanguinea* juveniles and adults' polychaetes showed the highest weight gain and survival in 30.0 and 25.0 PSU, respectively, whereas the lowest survival was recorded at 35.0 PSU. Thus, the salinity has a significant effect on the growth and survival of polychaetes (Thu et al., 2019). The effect of temperature may also influence the growth rates of the nereidid polychaete. For example, there was a much slower growth measured in *Perinereis rullieri* at 15° C than at 21° C or 27° C ($P < 0.05$) (Prevedelli, 1992). Even, the commercial feed Tetramin had a gradual growth at 15° C.

However, culturing *P. rullieri* at 27° C showed better growth fed with tetramin or algae (Prevedelli, 1992).

A total of 37 fatty acids were detected from the *Namalytiscas* Sp. during the salinity treatments. Salinity treatments showed the highest omega-3 fatty acid (Table 25) contents in cultured species (7.66 g/100g) at 30.0 PSU than the wild (6.69g/100g). Among the assessed fatty acid, Undecanoic acid (C11:0), Butyric acid (C4:0), Tridecanoic acid (C13:0), cis-10-Pentadecenoic acid (C15:1), cis-11,14,17-Eicosatrienoic acid (C20:3n-3) were absent in the *Namalycastis* sp. The saturated Myristic acid (C14:0), palmitic acid (C16:0) and Stearic acid (C18:0) were higher in worms than other SFA (Table 25). Comparatively, the wild worms contained higher amount SFAs and MUFAs than the cultured worms (Table 25). However, the PUFAs were higher in the cultured worms than the wild worms. The Linoleic acid (LA) (C18:2n-6c), Arachidonic acid (AA) (C20:4n-6), cis-5,8,11,14,17- Eicosapentaenoic acid (EPA) (C20:5n-3) and cis-4,7,10,13,16,19- Docosahexaenoic acid (DHA) (C22:6n-3) were higher in the cultured *Namalycastis* sp. than the wild species (Table 25).

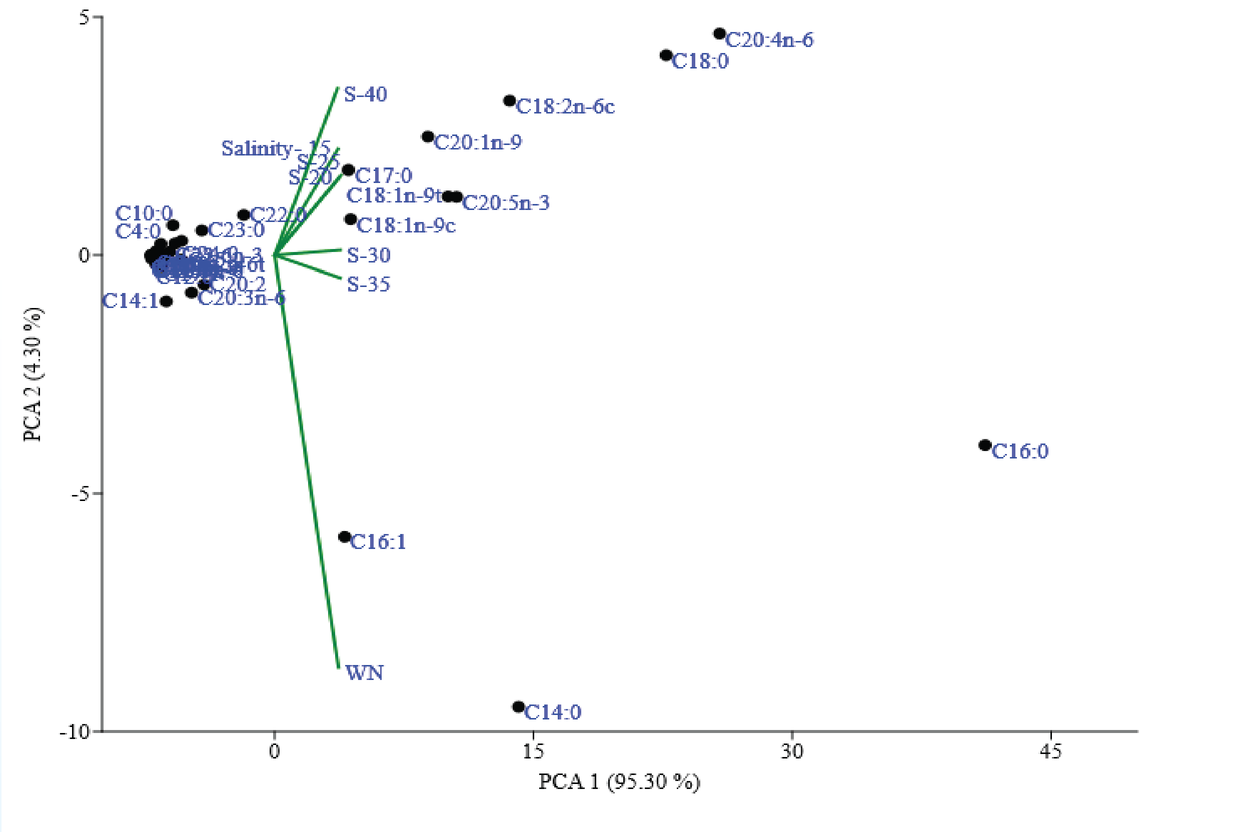


Fig 26. Principal component analysis (PCA) of fatty acids (e.g., C14:0) among the wild (WN) and cultured *Namalycastis* sp. in different salinities (e.g., S-15= cultured in salinity 15 PSU) showed the variance between two components

The PCA showed C14:0, C16:0, C16:1, C18:0 and C20:4n-6 fatty acids composition was higher than other FA. The growth and survival of polychaetes also largely depends on the stocking density. For example, *M. sanguinea* was cultured a semi-circulating system with 500 indv. m^{-2} 1000 indv. m^{-2} and 2000 indv. m^{-2} stocking density. The results revealed that the highest growth was recorded in the lowest density, while the highest density (2000 specimens m^{-2}) was measured the lowest growth (Parandavar et al., 2015). Similarly, the growth and survival of *Hediste diversicolor* was highest at the lowest densities (300 indv. m^{-2}) than the density stocked at 1000 and 3000 ind. m^{-2} (Nesto et al., 2012). It might be due to the negative influence of increasing intra-specific competition in the polychaetes (Nesto et al., 2012; Parandavar et al., 2015). We stocked at 500 indv. m^{-2} for *Namalycastis* sp. in our culture systems. The SGRs of polychaetes may fluctuate by the culture periods too. Such as the SGRs of *M. sanguinea* was higher during the first culture period (0-3 months) than the second (3-6 months) and third periods (6-9 months) (Parandavar et al., 2015). Feeding ratio can also influence the growth and survival of polychaetes. Such as *M. sanguinea* was fed with 3%, 6%, 9%, 12% and 15% of total body weight.

Table 25. Total fat (g/100 g) and fatty acid composition (%) of freeze-dried wild (W) and cultured *Namalycastis* sp. in different salinities (Sal).

Fatty acid	Fatty acid shorthand	Wild W	Sal-15	Sal-20	Cultured Sal-25	Sal-30	Sal-35	Sal-40
Total fat (g/100g)	–	10.35	7.59	6.86	6.00	7.10	6.73	6.43
Undecanoic acid	C11:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Butyric acid	C4:0	0.00	0.00	0.88	0.56	0.00	0.00	0.00
Caproic acid	C6:0	0.00	0.00	0.31	0.26	0.04	0.18	0.00
Caprylic acid	C8:0	0.15	0.15	0.14	0.13	0.12	0.13	0.36
Capric acid	C10:0	0.05	0.48	1.24	0.06	0.31	0.20	1.02
Lauric acid	C12:0	0.27	0.09	0.09	0.08	0.09	0.10	0.00
Tridecanoic acid	C13:0	0.06	0.00	0.00	0.00	0.00	0.00	0.00
Myristic acid	C14:0	16.58	6.21	6.30	6.49	7.96	7.75	5.04
Pentadecanoic acid	C15:0	0.75	0.55	0.63	0.65	0.56	0.72	0.72
Palmitic acid	C16:0	21.19	16.06	17.89	18.64	18.52	19.68	15.77
Heptadecanoic acid	C17:0	2.58	4.80	4.24	4.45	4.21	5.09	4.85
Stearic acid	C18:0	7.35	11.84	11.50	12.15	11.08	12.35	12.67
Arachidic acid	C20:0	0.24	0.19	0.23	0.24	0.24	0.28	0.26
Heneicosanoic acid	C21:0	0.12	0.30	0.16	0.16	0.17	0.16	0.17
Benhenic acid	C22:0	1.28	2.16	2.13	2.15	1.98	2.09	2.39
Tricosanoic acid	C23:0	0.61	1.09	1.14	1.22	1.08	1.28	1.32
Lignoceric acid	C24:0	0.38	0.71	0.74	0.68	0.62	0.76	0.76
∑SFA	–	51.61	44.63	47.62	47.92	46.98	50.77	45.33
Myristoleic acid	C14:1	1.25	0.21	0.15	0.15	0.23	0.20	0.11
cis-10-Pentadecenoic acid	C15:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Palmitoleic acid	C16:1	9.41	3.49	3.10	2.60	4.22	4.70	2.17
cis-10-Heptadecenoic	C17:1	0.44	0.14	0.14	0.39	0.15	0.17	0.41

Fatty acid	Fatty acid shorthand	Wild W	Sal-15	Sal-20	Cultured Sal-25	Sal-30	Sal-35	Sal-40
acid								
Elaidic acid	C18:1n-9t	5.05	6.77	6.26	6.67	6.98	7.38	6.36
Oleic acid (OA)	C18:1n-9c	3.81	4.37	4.36	4.55	4.35	4.14	5.01
cis-11-Eicosenoic acid	C20:1n-9	3.79	6.57	6.35	6.27	6.12	6.38	6.95
Erucic acid	C22:1n-9	0.08	0.00	0.12	0.12	0.18	0.15	0.00
Nervonic acid	C24:1n-9	0.00	0.00	0.00	0.00	0.00	0.00	0.00
∑MUFA	–	23.83	21.55	20.48	20.75	22.23	23.12	21.01
Linolelaidic acid	C18:2n-6t	0.32	0.44	0.44	0.49	0.32	0.50	0.35
Linoleic acid (LA)	C18:2n-6c	5.11	8.42	8.63	8.50	7.90	7.28	9.12
g-Linolenic acid	C18:3n-6	0.13	0.15	0.11	0.09	0.09	0.08	0.00
α-Linolenic acid (ALA)-n-3	**C18:3n-3**	**0.45**	**0.17**	**0.15**	**0.15**	**0.18**	**0.14**	**0.19**
cis-11,14-Eicosadienoic acid	C20:2	1.84	1.28	0.94	1.01	0.98	0.90	1.18
cis-8,11,14-Eicosatrienoic acid	C20:3n-6	1.63	0.78	0.72	0.70	0.92	0.72	0.73
cis-11,14,17-Eicosatrienoic acid	C20:3n-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Arachidonic acid (AA)	C20:4n-6	8.71	14.10	13.37	13.62	12.91	10.47	14.00
cis-13,16-Decosadienoic acid,	C22:2n-6	0.08	0.00	0.00	0.00	0.00	0.00	0.00
cis-5,8,11,14,17-Eicosapentaenoic acid (EPA), n-3	**C20:5n-3**	**5.91**	**7.89**	**6.45**	**6.40**	**7.04**	**5.64**	**7.55**
cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA), n-3	**C22:6n-3**	**0.33**	**0.59**	**1.07**	**0.37**	**0.44**	**0.36**	**0.51**
∑PUFA	–	24.51	33.82	31.88	31.33	30.78	26.09	33.63
∑n-6	–	15.98	23.89	23.27	23.40	22.14	19.05	24.20
∑n-3	–	6.69	8.65	7.67	6.92	7.66	6.14	8.25

The result showed that 15% feeding rate had the highest growth rate with 81.9% survival, however, 3% and 9% feeding rates showed lower growth with the highest survival rates (100%) (Phoo, 2017). For *Namalycastis* sp., we gave 3% feeds per total body weight which gained moderate weight gain. This might be species-specific, density and diets effect or any other factors that fluctuates growth and survival *Namalycastis* sp.

Growth performance of worms also influenced by the substrates type and depth. We provided 10.0 cm sand depth for the growth and survival of *Namalycastis* sp. *D. pinnaticirris* was cultured with 10.0 cm sand depth and it showed better worm growth (Mustofa et al., 2012). Kaba'ah (2015) used different artificial substrates such as white polyethylene beads, recycled polyethylene beads, corrugated polypropylene sheet and sand for Nereis virens culture. The results revealed that white polyethylene beads and recycled polyethylene beads were not statistically significant, while sand bed showed better growth than other substrates. In addition, an experiment designed with 7.0, 10.0 and 15.0 cm sand depth for the culture of *N. virens* culture. The results showed that both 10.0 and 7.0 cm were not statistically significant, however, the average worm's weight in 15.0 cm sand depth was significant than two of them (Kaba'ah 2015). Therefore, probably the growth and survival of polychaetes are depth specific in relation to species. However, the natural sand can be the best substrate than others.

The fatty acid composition in *Namalycastis* sp. greatly varies with habitats and types of diet. We analyzed the fatty acid contents both in cultured and wild *Namalycastis* sp. The variation of fatty acid contents in wild *Namalycastis* sp. and cultured individuals depends on their respective diets and habitats (Limsuwatthanathamrong et al., 2012). Although there were no details on the food and feeding habits of *Namalycastis* sp., Ezhova (2011) mentioned that *N. littoralis* feeds on organic debris and algae. However, ample study on the food and feeding habits of *Namalycastis* sp. would provide more information.

In *H. diversicolor* juveniles, the myristic acid (C14:0) was higher in Sea Bream Dry Feed (Aquagold) (2.02 ± 0.55) than Moist Sole (1.56 ± 0.31) and mackerel's fillets (*T. trachurus*) (0.97 ± 0.49) (Santos et al., 2016). Wibowo et al. (2020) measured fatty acid contents in cultured *Dendronereis pinnaticirris* fed with vegetable and animal protein. They calculated myristic acid (C14:0) compositions between 1.073 % and 0.91 % fed with vegetable protein and animal protein, respectively. The palmitic acid (C16:0) was measured higher in *H. diversicolor* juveniles fed with 3 different diets and mackerel's fillets (*T. trachurus*) (22.40 ± 0.11) was higher than the commercial feed code:155-FF-11 (19.15 ± 0.33) and Moist Sole (16.63 ± 1.71) (Santos et al., 2016). Wibowo et al., (2020) calculated the palmitic acid (C16:0) compositions between 8.833 % and 7.776 % in *D. pinnaticirris* fed with plant and animal protein, respectively. The stearic acid (C18:0) composition in cultured *H. diversicolor* was calculated 8.35 ± 0.90 %, 7.21 ± 1.14 and 6.04 ± 0.72 % fed with Fish pellets (*T. trachurus*), feed code:155-FF-11 and Moist Sole, respectively (Santos et al., 2016). In addition, the stearic acid (C18:0) composition in *D. pinnaticirris* was between 3.496 % and 3.063 % fed with plant and animal protein, respectively (Wibowo et al., 2020). The total vaccenic acid (C18:1) in *H. diversicolor* was measured 15.90 ± 4.33 %, 13.25 ± 3.06 % and 9.2 ± 5.57 % fed with commercial feed Moist Sole and code:155-FF-11, and *T. trachurus* Pellets, respectively (Santos et al., 2016). The vaccenic acid [C18:1 (total)] in *D. pinnaticirris* was measured 2.443 % and 2.065 %, fed with plant and animal-based feed, respectively (Wibowo et al., 2020). The calculated behenic acid (C22:0) composition in *D. pinnaticirris* was between 0.636% and 0.516 %, fed with plant and animal protein, respectively (Wibowo et al., 2020). The PCA showed that the saturated palmitic acid (C16:0) and unsaturated vaccenic acid (C18:1) were more dominated in both treatments, while rest of the acids were non-significant ($p > 0.05$) (Fig. 26).

Although the *H. diversicolor* was fed with high protein and fat containing commercial feed [code:155-FF-11with (protein: 46.00%; Lipids: 18.00%), Moist Sole (protein: 52.12%; Lipids: 20.03%)] (Santos et al., 2016), the assessed saturated and unsaturated fatty acid contents were comparatively lower than our estimated fatty acids in *Namalycastis* sp. fed with moderate protein and fat containing commercial feeds [code:155-FF-09 with protein 40.0 %, fat 9.0 %)]. In addition, *D. pinnaticirris* was fed with Vegetables (protein 37.313, fat 4.676 %) Animal (protein 39.403, fat 7.123 %) based feed, however the measured fatty acids percentages were a bit less than our recovered value (Wibowo et al., 2020). Furthermore, the *Namalycastis* sp. fed with fish culture waste contained lower PUFA than our cultured worms (Lim et al., 2021).

11.2.5. Habitat preferences and density-dependent growth of *Namalycastis* sp. (Polychaetes, Nereididae) in semi-circulatory culture systems

Daily growth rate (DGR), specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), feed efficiency ratio (FER) and survival rate (SR) were calculated for each treatment and presented in Table 26. The principal component analysis (PCA) of the substrate's biomass has documented in the Fig 29. Only the T2 (18.061 %) T3 (27.109 %) gained biomass, while rest of the treatment's loss biomass (Table 26). In addition, DT-1 (77.209 %) and DT-2 (18.835 %) gained biomass, while DT-3 (Density dependent treatment 3) lost biomass after the final harvest (Table 26). The Pearson correlation of the survival in all substrate's treatments were significant (Table 27). However, only T2–T3 ($r = 0.98$, $p < 0.05$) and T4 – T6 ($r = 0.95$, $p < 0.05$) were significant in the growth performances. In density experiment, a significant ($p < 0.05$) variations were calculated for survival to all the treatments (DT-1 to DT-3); whereas the growth of DT1 – DT3 ($r = -0.91411$, $p < 0.05$) was negatively significant (Table 27). The highest survival rate was measured for T3 (92.68 %) and the lowest survival was for T4 (67.07 %), respectively. In density experiment, the highest survival was recorded in DT-1 (97.56 %), while, DT-2 (72.34 %) had the lowest survival, respectively (Table 26). The DGR, SGR, WG and FCR were higher in T2 and T3 for the substrates treatment, and DT-1 and DT-2 for the density treatment. However, lower FCR indicates higher biomass gain which was much lower for T2, T3, DT-1 and DT-2.

Although the individual mean weight of T1 and T5 increased at final harvest, the biomass was much lower due to the survival of *Namalycastis* sp. (Fig. 27). However, both survival rate and the individual mean weight greatly reduced for T4 and T6.

Growth performance of *Namalycastis* sp. significantly influenced by the substrates type and depth (Table 26 & 27). Among the variable substrates, 10.0 cm beach sand (T3) showed the highest growth and survival rates (Table 26). *D. pinnaticirris* was cultured with 10.0 cm sand depth and it showed better worm growth (Mustofa et al., 2012). Kaba'ah (2015) used different artificial substrates such as white polyethylene beads, recycled polyethylene beads, corrugated polypropylene sheet and sand for Nereis virens culture. The results revealed that white polyethylene beads and recycled polyethylene beads were not statistically significant, while sand bed showed better growth than other substrates. Other studies designed with 7.0, 10.0 and 15.0 cm sand depth for the culture of *N. virens* culture showed that both 10.0 and 7.0 cm were not statistically significant, however, the average worms' weight in 15.0 cm sand depth was significant than two of them (Kaba'ah 2015). Therefore, probably the growth and survival of polychaetes are depth specific in relation to species. However, the natural sand can be the best substrate than others.

Table 26. The growth, survival (mean ± standard deviation) and other parameters for the habitat preferences and density dependent growth treatments of *Namalycastis* sp.

Treatments	Initial biomass (g)	Final biomass (g)	Daily growth rate (g/day)	Specific growth rate (%/day)	Weight gain (%)	Feed conversion ratio	Feed efficiency ratio	Survival rate (%)
Beach sand 3.0 cm depth (T1)	54.038 ± 4.140	52.123 ± 3.447	-0.021 ± 0.003	-0.040 ± 0.002	-3.543 ± 0.032	-467.206 ± 7.43	-0.002 ± 0.000	80.49 ± 4.43
Beach sand 7.0 cm depth (T2)	52.972 ± 3.782	62.539 ± 2.199	0.106 ± 0.002	0.184 ± 0.005	18.061 ± 2.141	99.471 ± 5.110	0.011 ± 0.001	87.8 ± 3.41
Beach sand 10.0 cm depth (T3)	52.0782 ± 2.236	66.196 ± 2.461	0.157 ± 0.002	0.267 ± 0.007	27.109 ± 2.783	68.538 ± 3.552	0.016 ± 0.001	92.68 ± 4.55
Silty sand (4.0 cm) and gravel (7.0 mm diam.) (2.0 cm) mix (T4)	51.824 ± 2.854	23.144 ± 3.452	-0.319 ± 0.005	-0.896 ± 0.013	-55.341 ± 4.521	-24.489 ± 2.042	-0.046 ± 0.003	67.07 ± 3.52
Silty sand 6.0 cm depth (T5)	53.464 ± 3.341	49.595 ± 2.234	-0.043 ± 0.002	-0.083 ± 0.002	-7.237 ± 0.004	-214.989 ± 7.943	-0.005 ± 0.000	79.27 ± 4.41
Unmodified mangrove mud (8.0 cm) (T6)	52.48 ± 2.54	31.248 ± 3.216	-0.236 ± 0.005	-0.576 ± 0.004	-40.457 ± 2.173	-37.630 ± 2.410	-0.030 ± 0.001	68.3 ± 2.33
500 individuals (indv.)/m^2 (DT1)	17.2 ± 1.461	30.48 ± 2.126	0.148 ± 0.032	0.636 ± 0.004	77.209 ± 4.652	28.655 ± 2.043	0.039 ± 0.003	97.56 ± 3.52
1000 indv./ m^2 (DT2)	29.88 ± 2.642	35.508 ± 2.482	0.063 ± 0.002	0.192 ± 0.002	18.835 ± 2.210	84.368 ± 3.521	0.013 ± 0.001	80.98 ± 3.07
2000 indv./ m^2 (DT3)	56.56 ± 2.854	43.35 ± 2.581	-0.147 ± 0.004	-0.296 ± 0.004	-23.356 ± 2.221	-60.703 ± 3.031	-0.019 ± 0.002	72.34 ± 3.12

DT= Density dependent treatment

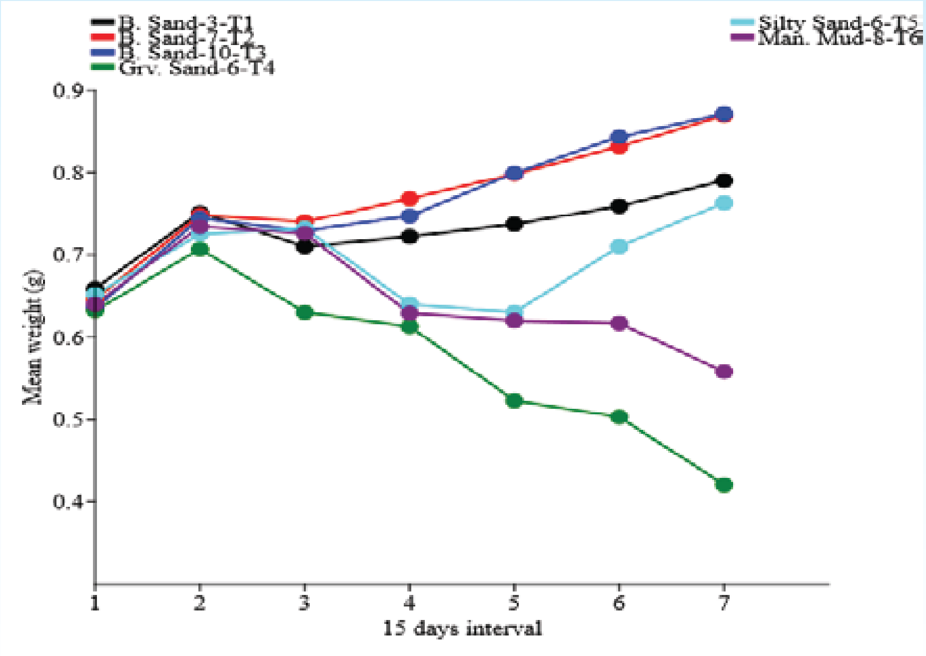


Fig 27. Individual mean weight (g) of *Namalycastis* sp. in six different substrate treatments. (B. Sand-3-T1 = Beach sand at 3.0 cm depth for treatment 1; Grv. Sand-6-T4 = Small gravel and sand mix at 6.0 cm depth for treatment 4; Man. Mud-8-T6= Natural mangrove mud at 8.0 cm depth for treatment 6).

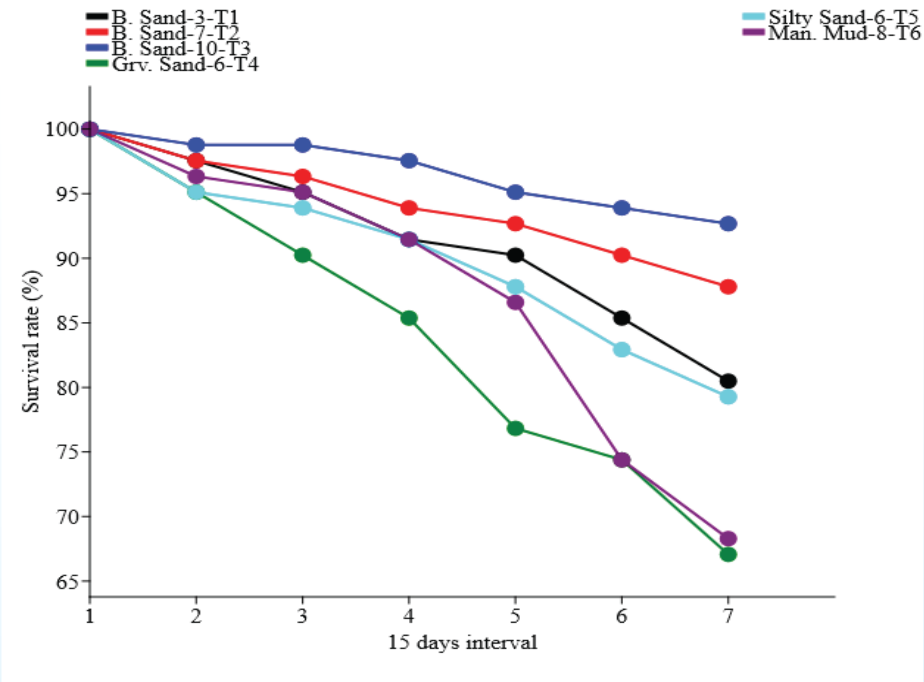


Fig 28. Survival rates (%) of *Namalycastis* sp. in six different substrate treatments.

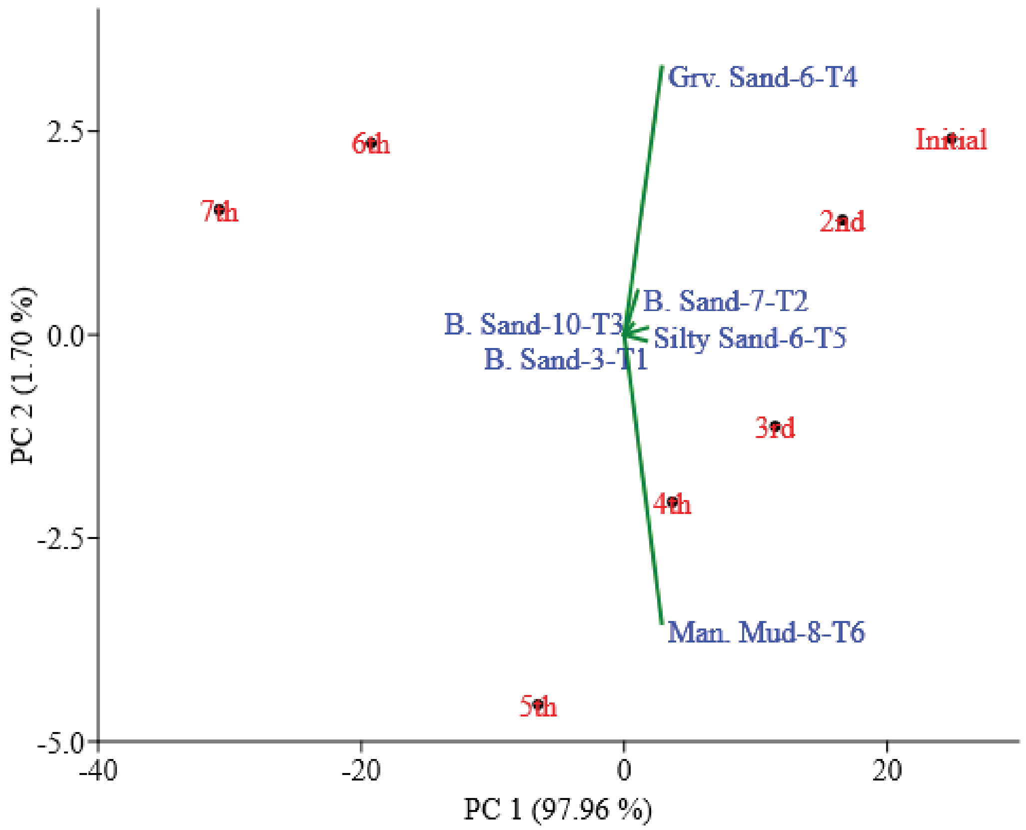


Fig 29. Principal component analysis of six substrate treatment in 90 days culture period dividing into 7th quartiles at 15 days interval.

The growth and survival of *Namalycastis* sp. also significantly ($p < 0.05$) depended on the stocking density. The hierarchy of density performances followed as: 500 > 1000 > 2000 indv. /m^2, respectively in three different treatments. *M. sanguinea* was cultured a semi-circulating system with 500 indv. $m^{-2,}$ 1000 indv. m^{-2} and 2000 indv. m^{-2} stocking density. The results revealed that the highest growth was recorded in the lowest density, while the highest density (2000 specimens m^{-2}) was measured the lowest growth (Parandavar et al., 2015). Similarly, the growth and survival of Hediste *diversicolor* was highest at the lowest densities (300 ind. m^{-2}) than the density stocked at 1000 and 3000 ind. m^{-2} (Nesto et al., 2012). The highest survival (92.68 %) was recorded in T1 (Fig 28) and T4 and T6 show the highest significant variances among the six treatments (Fig 29).

Table 27. Pearson correlation of the survival rates and growth performances of *Namalycastis* sp. at six treatments.

Survival rates						
Treatments	**B. Sand-3-T1**	**B. Sand-7-T2**	**B. Sand-10-T3**	**Grv. Sand-6-T4**	**Silty Sand-6-T5**	**Man. Mud-8-T6**
B. Sand-3-T1		0.000	0.004	0.002	0.000	0.002
B. Sand-7-T2	0.994		0.002	0.000	0.000	0.006
B. Sand-10-T3	0.971	0.976		0.001	0.001	0.003
Grv. Sand-6-T4	0.980	0.990	0.985		0.001	0.015
Silty Sand-6-T5	0.989	0.994	0.986	0.985		0.001
Man. Mud-8-T6	0.980	0.967	0.974	0.952	0.981	

Growth performances

B. Sand-3-T1		1.000	1.000	0.803	1.000	0.596	
B. Sand-7-T2	-0.118		0.001	1.000	1.000	1.000	
B. Sand-10-T3	-0.258	0.984		0.425	1.000	0.991	
Grv. Sand-6-T4	0.747	-0.713	-0.807		0.910	0.013	
Silty Sand-6-T5	0.628	-0.424	-0.475	0.733		0.938	
Man. Mud-8-T6	0.777	-0.610	-0.723	0.953	0.730		

B-sand-3-T1= Beach sand 3.0 cm substrate treatment-1, B-sand-7-T2= Beach sand 7.0 cm substrate treatment-2, B-sand-10-T3= Beach sand 10.0 cm substrate treatment-3, Grv. Sand-6-T4= Gravels 6.0 cm substrate treatment-4, Silty Sand-6-T5= Silty sand 6.0cm substrate treatment-5, Man. Mud-8-T6= Mangrove mud 8.0 cm treatment-6.

It might be due to the negative influence of increasing intra-specific competition in the polychaetes (Nesto et al., 2012; Parandavar et al., 2015). We stocked at 500 ind. m^{-2} for the variable substrate treatments. The SGRs of polychaetes may fluctuate by the culture periods too. Such as the SGRs of *M. sanguinea* was higher during the first culture period (0-3 months) than the second (3-6 months) and third periods (6-9 months) (Parandavar et al., 2015). Feeding ratio can also influence the growth and survival of polychaetes. Such as *M. sanguinea* was fed with 3%, 6%, 9%, 12% and 15% of total body weight. The result showed that 15% feeding rate had the highest growth rate with 81.9% survival, however, 3% and 9% feeding rates showed lower growth with the highest survival rates (100%) (Phoo, 2017). For *Namalycastis*, we gave 3% feeds per total body weight which gained moderate weight survival. We started feeding at 5% and 7 % body weight/day, however, a major proportion of feed remained unbeaten and fungal growth was noticed in feeds deposited on the bottom. Besides, higher feeding rates deteriorated the water quality. The feeding ratio might be species specific, dietary preferences or other factors might fluctuate the growth and survival of *Namalycastis* sp.

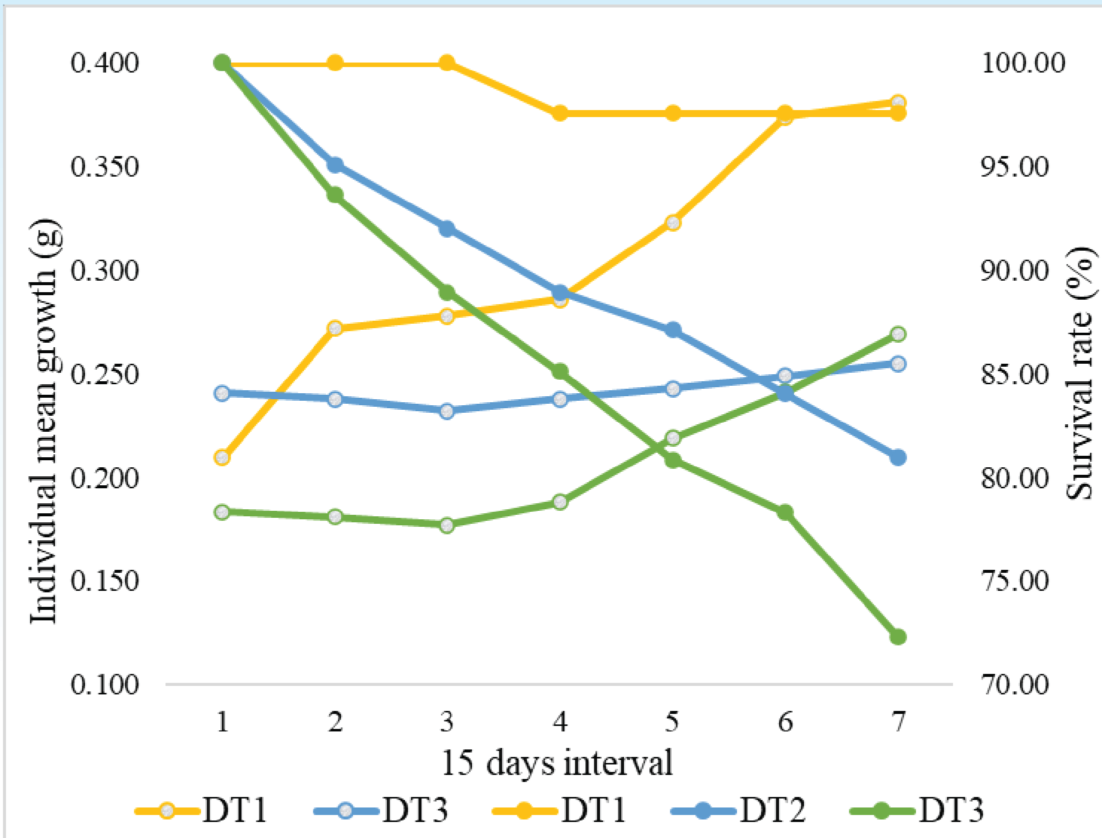


Fig 30. Individual mean growth (g) and survival (%) of density dependent treatments. DT1 showed the highest growth and survival. (DT- Density dependent treatment).

The density treatment (DT1) showed the highest weight gain (77.20 %) growth and survival (97.56 %), and followed as DT1 > DT2 > DT3, respectively (Fig 30). Furthermore, final biomass, the specific growth rate, feed efficiency ratio was higher in DT1 (Fig 30). The outcomes of our study would aid to intensify the mass polychaetes culture as a nutritive feed supplement to shrimp and fish in the aquaculture farms and hatcheries of Bangladesh.

11.2.6. Optimizing the growth and survival of *Namalycastis* sp. (Polychaetes, Nereididae) in variable temperature

Almost the whole planet has seen surface warming in terms of temperature (IPCC, 2022). Observations suggest that the average global temperature risen by 0.78 ˚C over the previous century, and that global warming has accelerated since the mid-20th century (IPCC, 2022). Global mean surface temperature is anticipated to rise 1.5 ˚C by the end of the twenty-first century, with best estimates of ocean warming in the top one hundred meters ranging from 0.6 ˚C to 2.0 ˚C (IPCC, 2022). In the recent decade, climate data has showed an increase in the frequency of heat waves, as well as an increase in the frequency of severe drought periods (Grilo et al., 2011). Temperature shifts have been linked to species expansion and retreat in some studies, with implications for life cycle, establishment, and range expansion (Berke et al., 2010; David and Simon, 2014).

Polychaete growth, maturation timing, and oocyte development rates have all been shown to be influenced by environmental factors such as temperature (Nesto et al., 2012; Wang et al., 2020). Temperature regulates polychaete metabolism, which kicks off the transition from somatic growth to gamete formation and was therefore, linked to age-at-maturity (Olive et al., 1997). Polychaetes maintained under constant high temperatures spawned asynchronously (Batista et al., 2003), but decreasing temperature in winter and increasing temperature from winter to early spring encourages synchronization of gamete maturation and spawning (Bartels-Hardege and Zeeck, 1990; Lawrence and Soame, 2004). Temperature may have a synergistic impact that affects brood development and oogenesis. Chu and Levin (1989) discovered that increasing temperature in conjunction with increasing or constant long photoperiods resulted in a faster brood development rate than falling temperature and temperature during the winter–spring transition. Our previous research suggested 30.0 PSU salinity showed optimum the growth and survival of *Namalycastis* sp. However, no studies focused on the optimization of *Namalycastis* sp. in the Bangladesh coast.

Namalycastis sp. was stocked at density 500 indv/m^2 at five treatments ranging from 26 – 34 ^{0}C and supplied 4 % commercial feeds body weight/day. The commercial feed (with code:155-FF-09) contained 40 % protein, 10 % moisture, 9% lipid, 14 ash % and 5 % fiber, respectively. The stocked was cultured for 40 days and sampled at five days interval.

Table 28. Growth and survival parameters of *Namalycastis* sp. in variable temperature treatment (TT) [Survival rate (SR), Daily growth rate (g/day), Specific growth rate (SGR), Weight gain (WG), Feed conversion ratio (FCR), Feed efficiency ratio (FER)].

TT	Initial biomass (g)	Final biomass (g)	SR (%)	SGR (%/day)	WG (%)	DGR (g/day)	FCR	FER
T-26	3.212	3.052	63.63	-0.128	-4.981	-0.004	-32.120	-0.035
T-28	3.157	4.716	81.82	1.003	49.382	0.039	3.240	0.343
T-30	3.091	6.831	100.00	1.982	120.996	0.094	1.322	0.840
T-32	3.421	2.080	45.45	-1.244	-39.199	-0.034	-4.082	-0.272
T-34	3.333	0.682	18.18	-3.966	-79.538	-0.066	-2.012	-0.552

The highest biomass, SR, SGR, and WG, were calculated at 30 ^{0}C (T-30) (Table 28). Lower FCR indicates the higher biomass gain by this treatment. In addition, higher efficiency denoted the provided feeds was efficient and converted into higher biomass (Table 28). The second highest WG was measured at T-28. The overall SGR, SR and WG ranked as: T-30 > T-28 > T-26 > T-32 > T-34, respectively (Table 28). The highest survival (100 %) was recorded at T30 treatment means no *Nmalycastis* sp. died during the culture period. The estimation of relative growth rate (RGR) also showed the highest growth rate of *Namalycastis* sp.

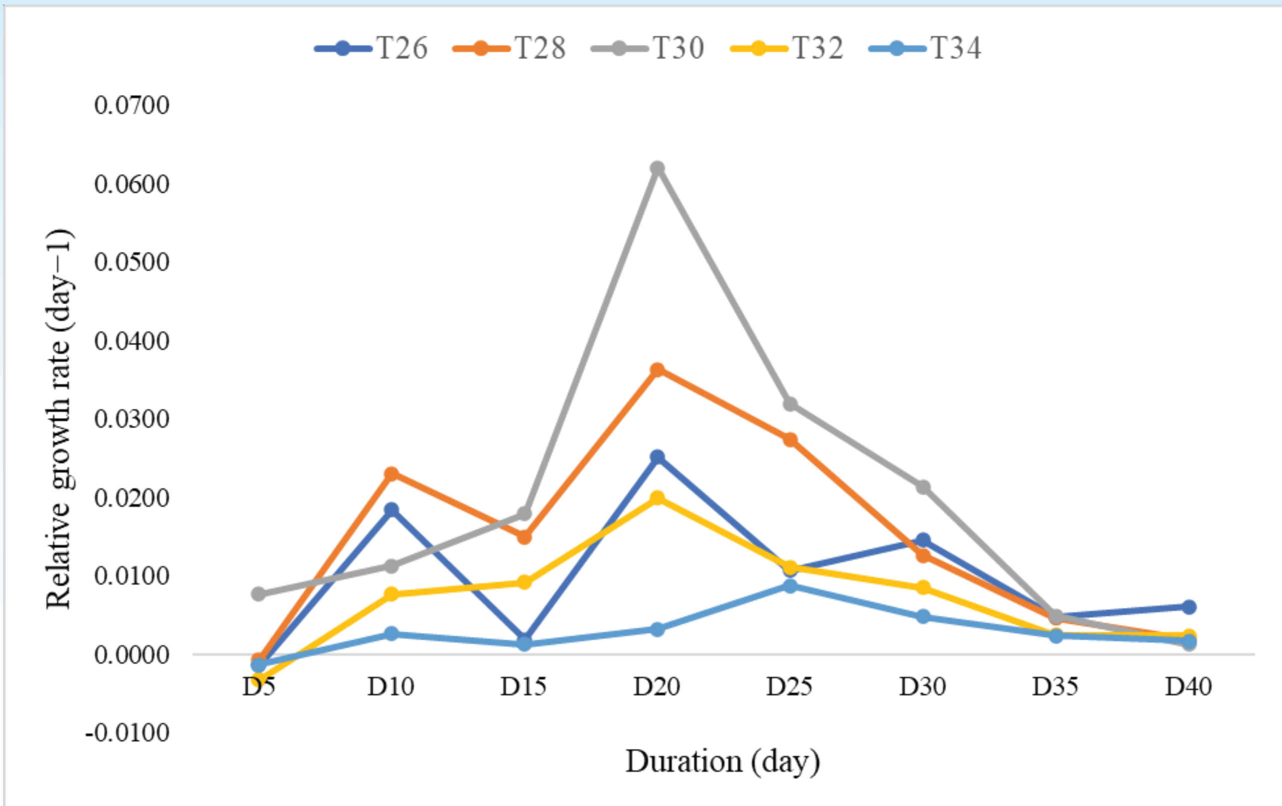


Fig 31. Relative growth rate (day $^{-1}$) of the individual mean of *Namalycastis* sp. in five treatments under 40 days culture.

Fig 31 showed that the RGR (day $^{-1}$) of *Namalycastis* sp. was highest at 20 days of culture period, while it was minus or steady growth during the first five days of the culture period.

Table 29. Single factor ANOVA of relative growth rate (RGR) among the treatments.

ANOVA: Single Factor SUMMARY						
Groups	***Count***	***Sum***	***Average***	***Variance***		
T26	8	0.080178	0.010022	8E-05		
T28	8	0.120402	0.01505	0.000172		
T30	8	0.158595	0.019824	0.000389		
T32	8	0.058178	0.007272	4.85E-05		
T34	8	0.02363	0.002954	8.64E-06		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	**0.001391**	**4**	**0.000348**	**2.488762**	**0.061033**	**2.641465**
Within Groups	**0.00489**	**35**	**0.00014**			
Total	**0.006281**	**39**				

The growth rate was slowly increased in the following five days. However, the RGR sharply decreased at 26 ˚C temperature at the 15 days culture period, although rest of the treatments were slightly increasing. A steady *Namalycastis* sp. RGR (g g^{-1} day^{-1}) was recorded in 34 ˚C temperature while, it largely fluctuated in 30 ˚C temperature, respectively (Fig 31).

Single factor analysis of variance (ANOVA) showed that there were no significant ($p > 0.05$) variations of temperature between the initial and final biomass (Table 29). In addition, RGR among the treatments were also non-significant to each other. However, the survival rate significantly ($p < 0.05$) decreased among the temperature treatments. The temperature influences the growth rates of the nereidid polychaete. For example, there was a much slower growth measured in *Perinereis rullieri* at 15° C than at 21° C or 27° C ($P < 0.05$) (Prevedelli, 1992). Even, the commercial feed Tetramin had a gradual growth at 15° C. However, culturing *P. rullieri* at 27° C showed better growth fed with tetramin or algae (Prevedelli, 1992).

11.2.7. Effect of growth and survival of *Namalycastis* sp. (Polychaetes, Nereididae) in different formulated diets in a semi-circulatory culture system.

Over the previous several decades, global aquaculture output has grown at an annual rate of 8%, and the industry is predicted to develop more in the future (FAO 2017). This type of sustainable growth necessitates the use of alternate feeds and feed additives. Polychaete cultivation can assist the aquaculture sector in producing new feed supplies for shrimp and fish culture as well as the recreational fishing industry. As 57.0 % of the wild stock of *Namalycastis* sp. has already been exploited in Bangladesh, artificial intensification is necessary to restore the wild population. Culture parameters such as optimum temperature and salinity have already been investigated in the previous study. During *Namalycastis* sp. culture, artificial commercial diets are supplied for biomass intensification. Quality diets are expensive and increase the production cost of *Namalycastis* sp. Therefore, we aimed to formulate some available and reasonable feeds for *Namalycastis* sp. to reduce the production cost. Feeds were formulated from available Tilapia fish and marine fish wastes as protein source; wheat flour and potato peels were used as carbohydrates binders of the feeds. A total of four feeds were prepared as T1 (code:155-FF-04), T2 (code:155-FF-05), T3 (code:155-FF-06) and T4 (code:155-FF-07), respectively. *Namlycastis* sp. was stocked at density 500 ind./m^2 with 3% feeds body weight once in a day. Specimens were sampled fortnightly and culture duration was 75 days.

Table 30. Growth and survival parameters of Namalycastis sp. in different formulated feeds. (IB-Initial biomass, FB-Final biomass, DGR-Daily growth rate, SR-Survival rate, SGR-Specific growth rate, FCR-Feed conversion ratio, FER-Feed efficiency ratio).

Treatments	IB (g)	FB (g)	DGR (g/day)	SR (%)	SGR (%/day)	FCR	FER	WG (%)
T1	31.156	50.400	0.257	90.36	0.641	4.491	0.253	61.767
T2	28.890	27.480	-0.019	72.29	-0.067	-45.089	-0.025	-4.881
T3	29.833	47.190	0.231	83.13	0.611	4.726	0.24	58.179
T4	26.066	44.610	0.247	81.93	0.716	3.837	0.296	71.14

The culture parameters such as DGR (g/day), SGR (%/day), FCR, FER and WG (%), of four treatments (T1 to T4) have been supplied in the Table 11.

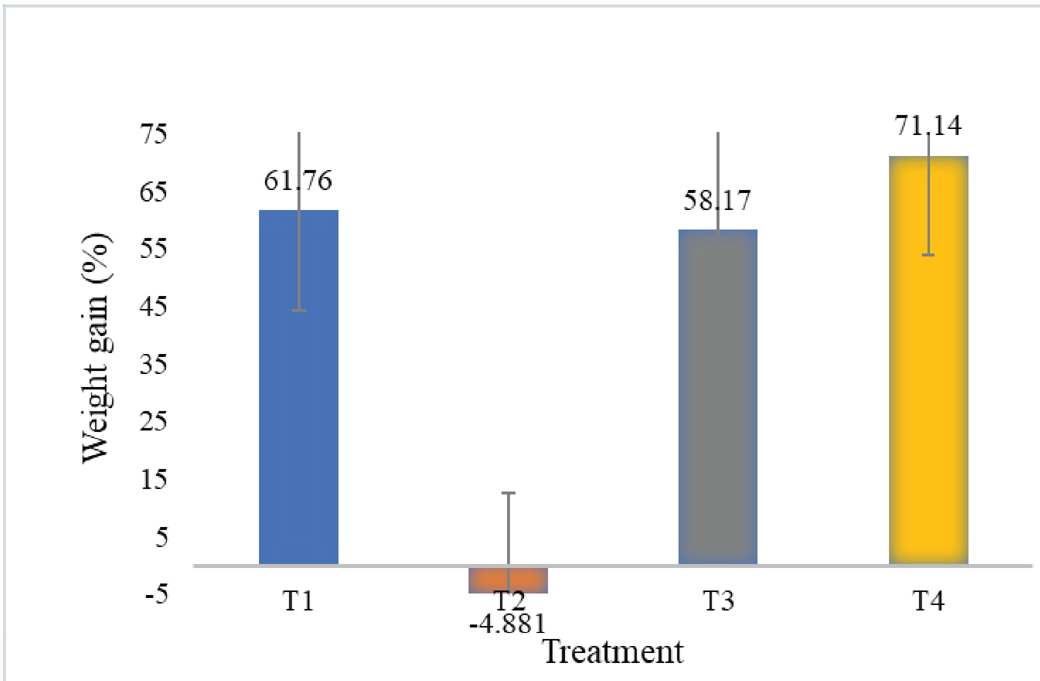


Fig 32. The weight gain (%) of *Namalycastis* sp. under four treatments of a 75 days culture period.

The WG of *Namalycastis* sp. was significant ($p < 0.05$) and the highest WG was recorded fed with code:155-FF-07 in T4 (71.14 %), while it was decreased fed with code:155-FF-04 in T1 (61.76 %), code:155-FF-06 in T3 (58.17 %) and code:155-FF-05 in T2 (-4.88 %), respectively (Fig 32). Although Treatment-2 showed the 72% survival rate (Table 30) yet the weight gain (WG) was negative. Supplied formulated feed with tilapia fins, intestine and wheat flour might not be ingested by the *Namalycastis* sp. That resulted (Fig 32) negative value of feed conversion ratio (FCR) and Feed Efficiency Ratio (FER) which ultimately resulted in negative weight gain (WG). The highest SR and DGR were measured in T1, whereas, T2 had the lowest SR and minus DGR, accordingly (Table 30).

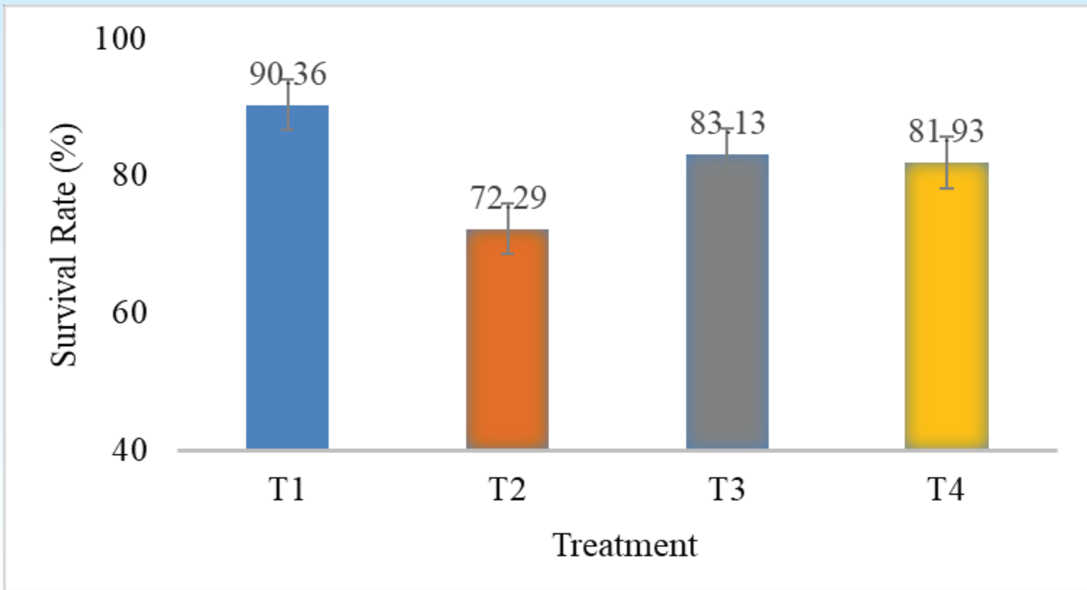


Fig 33. The survival rate (%) of *Namalycastis* sp. under four treatments.

The *Namalycastis* sp. was fed with tilapia muscles (T1) showed the highest survival rate, however, the *Namalycastis* sp. fed with T2 (tilapia fins and intestine) provided the lowest survival (Table 30; Fig 33). The relative growth rate (RGR) of *Namalycastis* sp. indicated that polychaetes growth was decreased in T2 and T3 in the first months but T1 and T2 were almost steady (Fig 34). However, the all the treatments gained weight in the 60th day and started falling thereafter (Fig 34). This data indicates that two months culturing of *Namalycastis* sp. would be optimum. However, it depends on the stocking age and size of polychaetes either juvenile or adults.

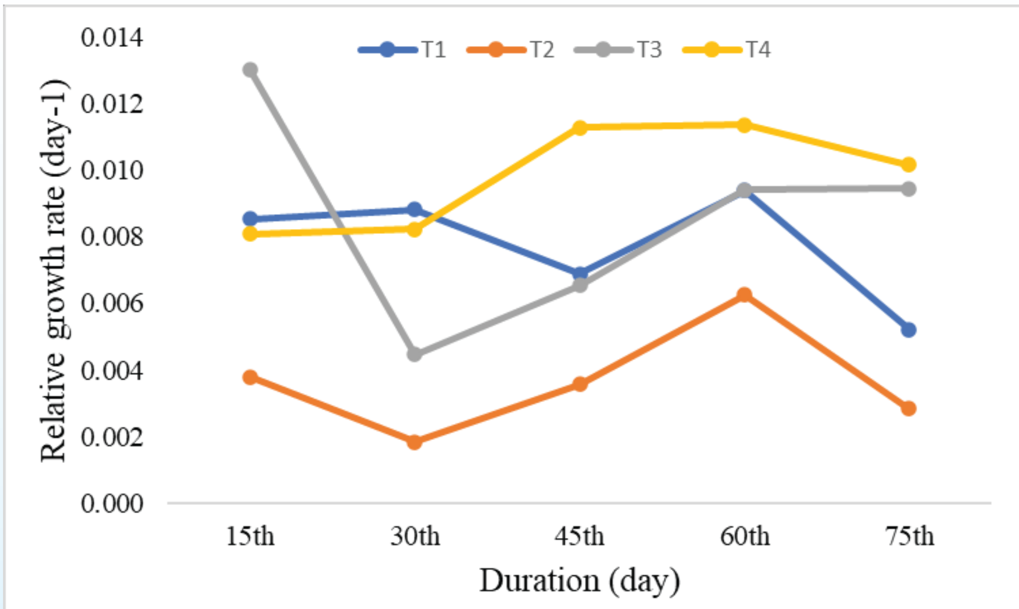


Fig 34. Relative growth rate (day^{-1}) of *Namalycastis* sp. in four diets (T1–T4).

The DGR was highest in *Nereis diversicolor* when fed PL shrimp feed code:155-FF-10 (13.9 mgDay^{-1}) followed by dry food code:155-FF-11 (9.4), code:155-FF-14 (fish dry food) (9.4), code:155-FF-12 (8.8), code:155-FF-16 (6.9) and decapsulated *Artemia* cysts (6.2) (Fidalgo E Costa et al., 2000). In addition, the SGR (% Day^{-1}) was greatest 6.8 in code:155-FF-10 and code:155-FF-14, while it was between 5.3 – 6.1 for rest of feeds. Moreover, the survival rate was 100% in code:155-FF-10, code:155-FF-11 and code:155-FF-12 feeds whereas the survival rate was 77.7-94.4% in code:155-FF-14, code:155-FF-16 and *Artemia* feeds (Fidalgo e Costa et al., 2000). High protein food (Larviva-Biomar®) showed better growth rate with commercial minimum weight (500.0 mg) after 6 weeks, recording the daily biomass production of 12,000.0 mg m^{-2} day^{-1}, while the *Hediste diversicolor* was fed with low-protein food (Classic C22-Hendrix®) reached the threshold to 9100.0 mg m^{-2} day^{-1} two weeks later (Nesto et al., 2012). The mean weight of *H. diversicolor* was significantly higher in code:155-FF-13 (920.0 ± 70.0 mg) than the code:155-FF-15 (650 ± 90 mg) and brown macroalgae (*Sargassum muticum*) (220 ± 50 mg) after culturing them eight weeks (Nesto et al., 2012).

The survival rate greatly reduced to 78%, 63% and 58% with code:155-FF-15, code:155-FF-13 and *S. muticum*, respectively from the eight weeks culture periods. Similarly, *H. diversicolor* was fed 3 different diets (two commercial diets, code:155-FF-11 and semi-wet pellets for reared sole (Moist Sole), and a non-processed diet consisting of mackerel's fillets (*Trachurus trachurus*) and the highest final weight, SGR and DGR were highest in the commercial diets (Santos et al., 2016). Likewise, the growth of our four formulated diets had significant ($p < 0.05$) variations. *Namalycastis* sp. fed with the formulation of marine wastes and potato peels (3:1) in the treatment-4 (T4) showed higher growth with maximum biomass than all other treatments. The other study with formulated feed of code:155-FF-14 feed showed 2-3-fold higher fecundity of *Dinophilia graciliana* than those fed with natural feed spinach (Prevedelli and Simonini 2000).

11.2.8. Unraveling the growth and survival of *Namalycastis* sp. (Polychaetes, Nereididae) on four variable diets in an open water culture system

Previous studies optimized the growth and survival of *Namalycastis* sp. in variable temperatures, salinities, stocking densities, diets etc. in indoor semi-circulatory culture systems. Commercial culture of Laboratory culture systems does not often justify until it is validated in the field conditions. Therefore, this study aimed to investigate the growth and survival viability of *Namalycastis* sp. in an open water culture systems with natural environmental conditions with commercial and formulated diets. A total of 15 circular sized (inner diameter: 81.28 cm and height: 30.48 cm) concrete ring was used to stock *Namalycastis* sp. Natural bottom mud was used as substrates and the rings bottom was blocked so that polychaetes cannot move out of the walls. *Namalycastis* sp. was stocked at 500 ind./m^2 by calculating the inner walls the area (πr^2). The rings were divided into five treatments (T1 to T5) triplicating of each supplied with commercial feeds (T1 and T2), formulated feeds (T3 and T4), and no feed (T5) were selected. Commercial feeds T1 (code:155-FF-03: protein 38 %, fat 5%, fiber 4 %, moisture 12%), T2 (code:155-FF-01: protein 42 %, fat 7%, fiber 3 %, moisture 9%), formulated feeds T3 (tilapia muscles: wheat flour at ratio 2:1), T4 (tilapia fins: intestine: potato peels at 1:1:1) and T5 (no feed) were selected for each treatment, respectively.

Plate 44. *Namalycastis* sp. culture in an open water system

Namalycastis sp fed with code:155-FF-01 feed (T2) (42% protein, 7% fat, 3% fiber, 9% moisture) had the highest final weight (121.250 g). On the other hand, *Namalycastis sp* fed the natural feed (T5) had the lowest (34.584 g) final average weight (Table 30).

Table 31. Proximate composition of commercial feed.

Feeds	Protein (%)	Fat (%)	Fiber (%)	Moisture (%)	References
code:155-FF-01	42	7	3	9	Present study
code:155-FF-02	42	6.5	4	11	
code:155-FF-03	38	5	4	12	

A total of four feeds were prepared as T1 (code:155-FF-04), T2 (code:155-FF-05), T3 (code:155-FF-06) and T4 (code:155-FF-07), respectively. *Namlycastis* sp. was stocked at density 500 ind./m2 with 3% feeds body weight once in a day. Specimens were sampled fortnightly and culture duration was 75 days.

Weight gain (%)

The present results indicate that *Namalycastis* sp fed with feed code:155-FF-01 (T2) (42% protein, 7% fat, 3% fiber, 9% moisture) had the highest body weight gained (45%) during experiment and followed by treatment T3, T4, and T1, which have been shown in Fig 32. In the case of T5, average weight gained can be found lowest. The statistical analysis showed that the values were significantly different ($p<0.05$) among treatments.

Namalycastis sp. was cultured for 60 days (March-April 2022) and was sampled every 10 days interval. The initial biomass (g), final biomass (g). daily growth rate (DGR) (g day^{-1}), specific growth rate (SGR) (% d^{-1}), survival rate (SR%) weight gain (WG) (%) etc. have been depicted in the Table 32. The highest SGR was measured in T2 (0.620 % d^{-1}), while a negative SGR was calculated in the T5. The SGR was decreasing as 0.399 % d^{-1}, 0.266 % d^{-1}, 0.258 % d^{-1} in T3, T4 and T1, respectively (Table 32). The highest WG (45.024 %), DGR (0.627 g d^{-1}) and feed efficiency ratio (FER) (0.426) were calculated in commercial feed T2, respectively.

Table 32. Growth and survival parameters of *Namalycastis* sp. in an open water system.

Parameters	CP feed (T1)	Frippak (T2)	Tilapia fins (T3)	Tilapia muscles (T4)	No feeds (T5)
Initial weight (g)	98.674	83.607	82.906	86.385	82.353
Final weight (g)	115.185	121.250	105.340	101.337	34.584
Weight gain (%)	16.733	45.024	27.058	17.309	-58.005
Daily growth rate (g day^{-1})	0.275	0.627	0.374	0.249	-0.796
Specific growth rate (% day^{-1})	0.258	0.620	0.399	0.266	-1.446
Survival rate (%)	73.846	72.308	80.769	75.0	30.769
Feed conversion ratio	7.172	2.665	4.435	6.933	–* Not applicable
Feed efficiency ratio	0.158	0.426	0.256	0.164	–

The highest survival was recorded in T3 (80.76 %), while, T5 (30.769 %) had the lowest survival rate (Table 32). None of the survival rates were significantly ($p > 0.05$) fluctuated in the open water systems. Survival rate ranged from 72.30 – 75.0 % both in commercial and formulated diets (Fig 35).

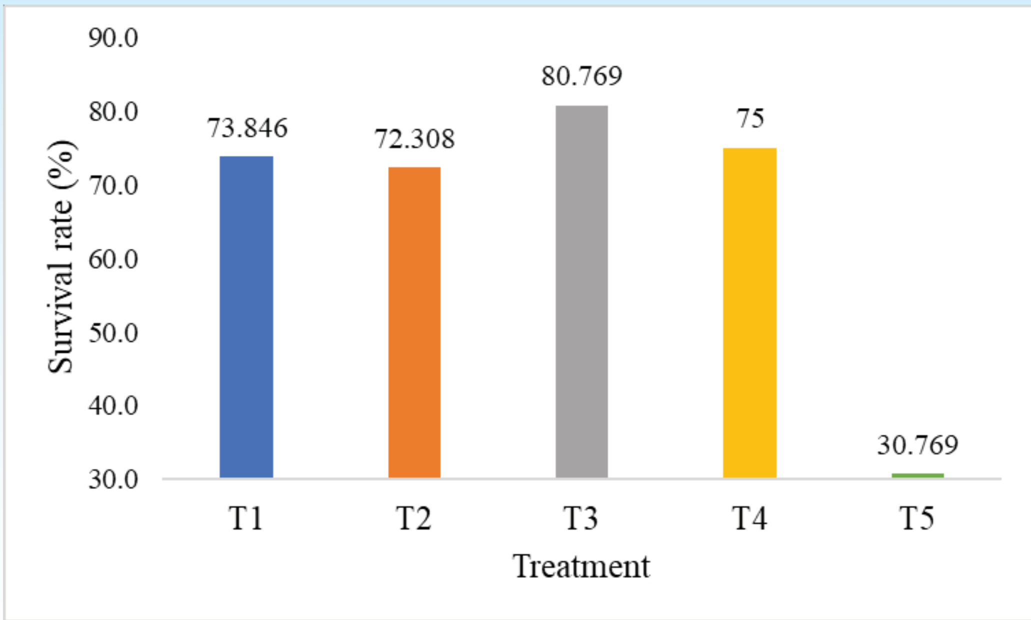


Fig 35. The survival rate (%) of *Namalycastis* sp. in an open water system.

The highest and lowest biomass was recorded in T2 (45.024 %) and T5 (-58.005 %), respectively. The biomass was significantly ($p < 0.05$) fluctuated among the treatments. The biomass of CP commercial feeds (T1) was lower than the formulated feeds (Fig 36).

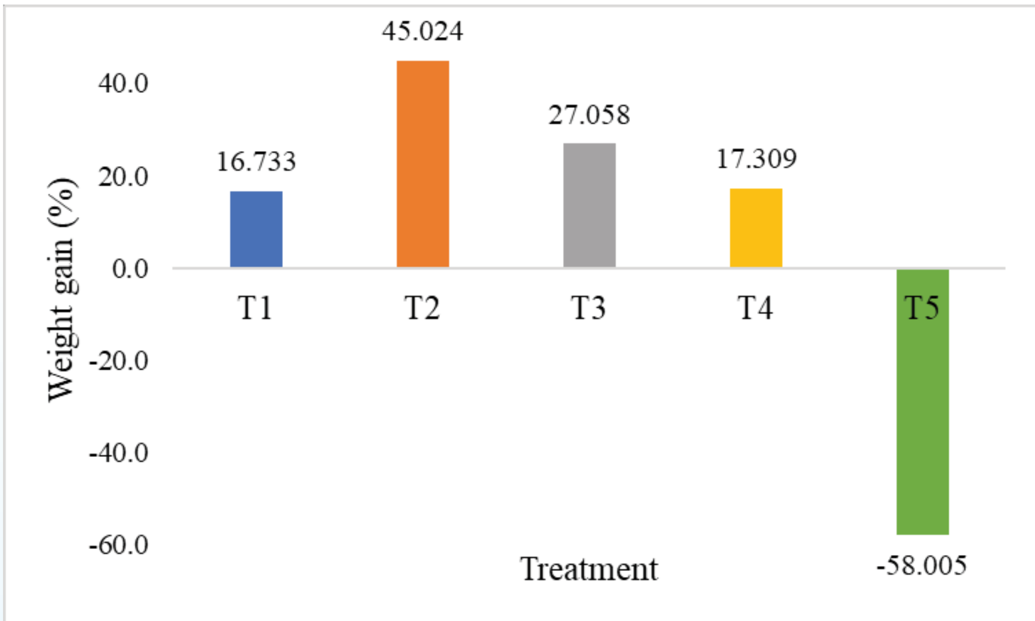


Fig 36. Biomass gain (%) of *Namalycastis* sp in an open water culture system.

During high tide, the treatments ring submerged under water, and it retained water inside when sea level was below the rings. Natural feeds in the riverbank were not enough for biomass intensification of *Namalycastis* sp.

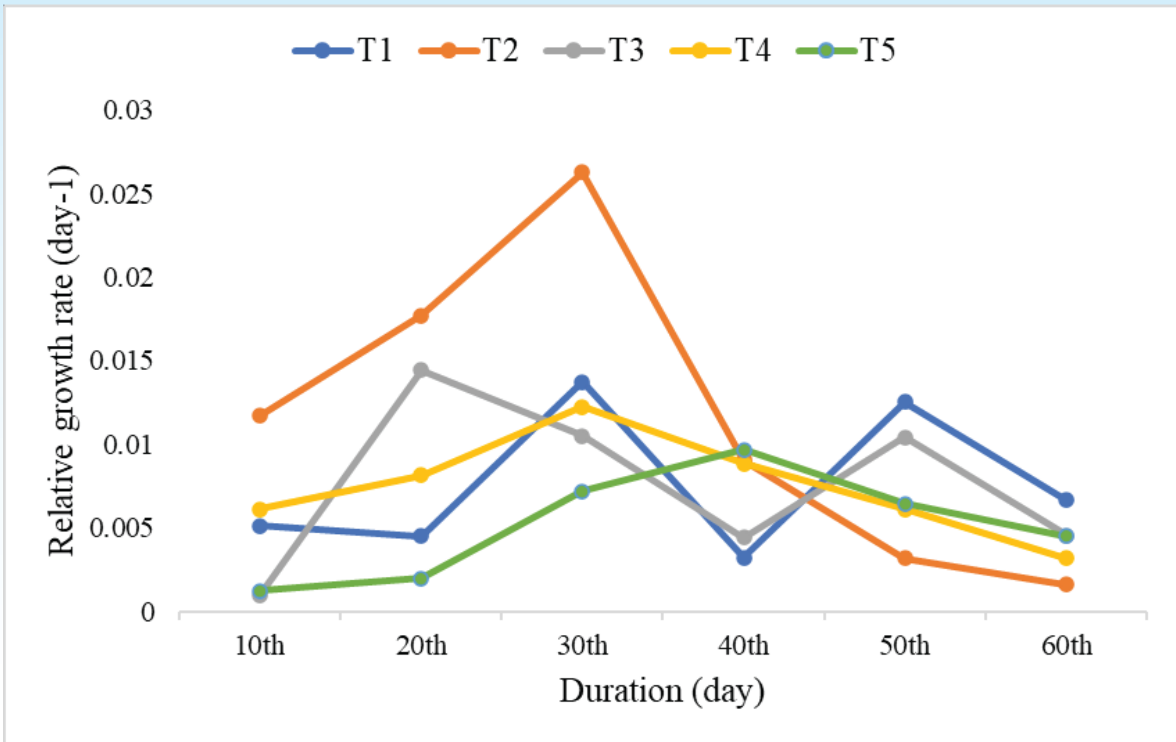


Fig 37. Relative growth rate (day $^{-1}$) of the individual mean of *Namalycastis* sp. in an open water system.

Relative growth rate (RGR) was calculated from the individuals mean during the culture intervals. The highest RGR was measured at the end of first months of the culture period (Fig 37). RGR was fluctuating in dietary treatments during the culture periods, however, RGR was almost steady in T5, where no feeds were provided (Fig 37). The RGR in formulated diets had no major variations, whereas a larger variation was noticed between the two commercial diets (Fig 37). Diet composition and quality influences the growth of survival of *Namlycastis* sp. For example, the *Hediste diversicolor* (as *Nereis diversicolor*) showed higher growth and 100.0 % in commercial PL shrimp feed code:155-FF-10 (13.9 mg day $^{-1}$) than formulated sea bream dry food (code:155-FF-11) (9.4), code:155-FF-14 (fish dry food) (9.4), soy (8.8), pollen (6.9) and decapsulated *Artemia* cysts (6.2), respectively (Fidalgo E Costa et al., 2000). Rich protein food (code:155-FF-13) showed better growth rate of *Hediste diversicolor* than low-protein food (Classic C22-Hendrix®) (Nesto et al., 2012). The mean biomass of *H. diversicolor* was higher commercial feeds than natural feed (brown macroalgae- *Sargassum muticum*) (Nesto et al., 2012). Similarly, *H. diversicolor* was fed 3 different diets (two commercial diets, dry fishfeed (code:155-FF-11 and semi-wet pellets for reared sole (Moist Sole), and a non-processed diet consisting of mackerel's fillets (*Trachurus trachurus*) and the highest final weight, SGR and DGR were highest in the commercial diets (Santos et al., 2016). Homemade formulated diets showed 2- or 3-fold higher fecundity of *Dinophilia graciliana* than those fed with natural feed spinach (Prevedelli and Simonini 2000).

11.2.9. Morphometric traits, gonadosomatic index (GSI) of mud crab (*Scylla olivacea*) under three natural diets and live polychaetes (*Namalycastis* sp.) diets

The mud crab (*Scylla olivacea* Herbst, 1796) is commonly found in the intertidal zones of mangrove forests and estuaries, where they eat and reproduce to introduce their future generations, hence driving recruitment throughout the Indo-Pacific area (Waiho et al., 2015). Crab aquaculture and fattening are quickly growing in Bangladesh due to strong demand in the worldwide market (Ali et al., 2020). Mud crabs are now used commercially and medicinally in many nations throughout the world due to their excellent nutritional quality and availability of antioxidant capabilities (Yusof et al., 2019). Furthermore, the value of *S. olivacea* as a potential export generating aquatic resource has expanded, ranking second only to shrimp (*Penaeus monodon*) in terms of earning foreign money for Bangladesh (DoF, 2016). Mud crabs mostly feeds on crustaceans, mollusks, fishes, detritus, etc. (Viswanathan and Raffi 2015). Trash fishes are typically utilized as a natural feed to mud crabs for broodstock growth or farming, whereas artificial diets are composed of prepared fish meal and differen t types of feed (Azra and Ikhwanuddin 2015). Research showed, mud crab broodstock has a high nutritional demand for lipids, fatty acids, and protein, which will be required during the maturation and spawning processes. However, as compared to the commercial diet, the natural diet produces high quality yields (Azra and Ikhwanuddin 2015). Therefore, we aimed to determine the growth and survival of *S. olivacea* in three natural diets along with its changes of morphometric traits and gonadosomatic index (GSI) in one month culture period.

A total of 125 live and healthy male and females *S. olivacea* were bought from the local farmers in the Cox's Bazar. The crabs were chilled in ice to reduce their movements and morphometric traits such as internal carapace width (ICW), propodus length (PL), chela height (CH), lower paddle width (LPW) was taken by measuring taps. Then, similarly weighted males and females were sorted out and dissected to extract gonads/testis and ovaries, respectively. The selected live crabs were acclimatized for three days. Male and female crabs were introduced into an aquarium culture system. Single specimens were introduced into each aquarium to retain the water quality clean and to avoid any competition, risks of injury etc. Three natural diets namely: Polychaetes *(Namalycastis* sp.), Tilapia muscles (*Oreochromis niloticus*) and little neck clam (*Mercenaria mercenaria*), respectively were selected for the growth performances. For each diet, five replicates were prepared separately for male and female. A total of thirty crabs were cultured dividing into three diets performances. Feeding was performed once a day at 7 % body weight and daily 100 % water was exchanged. The crabs were cultured for 25 days.

After harvesting, crabs body weights were taken and the morphometric features, gonad/testis, ovaries were taken following the previous procedure.

The culture parameters such as final body weight (BW), weight gain (WG), daily growth rate (DGR), specific growth rate (SGR) was calculated and presented in Table 33. Hundred percent (100 %) survival rate was calculated during the culture period. Crabs gained weight in three diets. The highest and lowest SGR was measured in male crabs fed with clam, tilapia, respectively (Table 33).

Table 33. Growth and culture parameters of *Scylla olivacea* in three diets. BW-body weight, WG-weight gain, DGR-daily growth rate, SGR-specific growth rate, SR- survival rate.

Treatments		Initial BW (g)	Final BW (g)	WG (%)	DGR (g /day)	SGR (% /day)	SR (%)
	Namalycastis sp.	140.50	153.00	8.897	0.500	0.341	100.0
	Tilapia	154.00	155.00	0.649	0.040	0.026	100.0
Male crab	Clam	91.50	118.00	28.962	1.060	1.017	100.0
	Namalycastis sp.	170.00	181.00	6.471	0.440	0.251	100.0
	Tilapia	134.33	145.67	8.437	0.453	0.324	100.0
Female crab	Clam	129.33	137.67	6.443	0.333	0.250	100.0
	Namalycastis sp.	155.25	167.00	7.68	0.47	0.30	100.00
	Tilapia	144.17	150.33	4.54	0.25	0.17	100.00
Average	Clam	110.42	127.83	17.70	0.70	0.63	100.00

On an average the highest weight gain (%), DGR (g/day) and SGR (%/day) were recorded in the clam feeds and followed by *Namalycastis* sp. and tilapia, respectively (Table 33). However, none of the parameters varied significantly ($p > 0.05$).

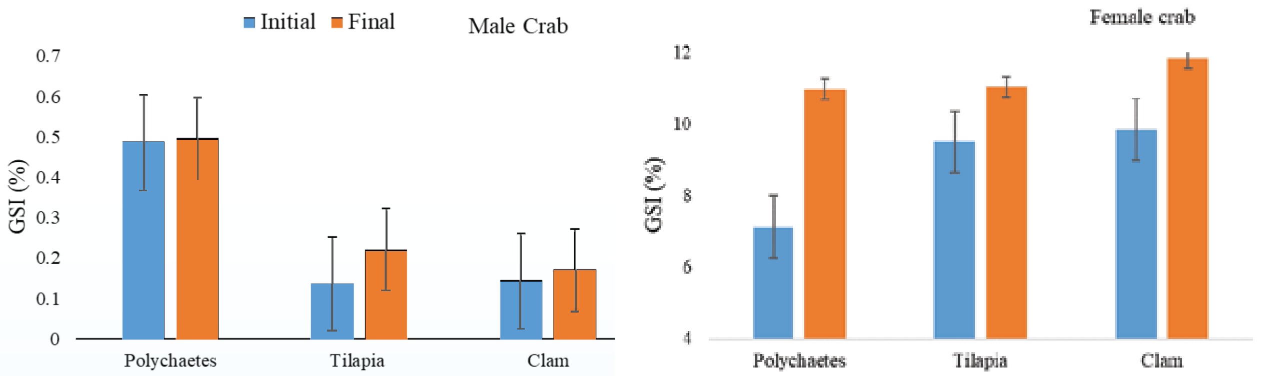


Fig 38. Gonadosomatic index (GSI) of *Scylla olivacea* in three diets before and after 25 days culture period.

The GSI of male *S. olivacea* increased slightly with the enhancement of size and weight. GSI (%) was increased in all the treatments both for male and female crabs (Fig 38.). The GSI for female in polychaetes (*Namalycastis* sp.) feeds enhanced almost two folds than tilapia and clam feeds (Fig 38.) The GSI was 0.36 ± 0.27 % in male gonads of 143.09 ± 17.23 g average body weight of *S. olivacea* collected from Setiu Wetlands, Terengganu, Malaysia (Waiho et al., 2017). This result corresponds to our finding.

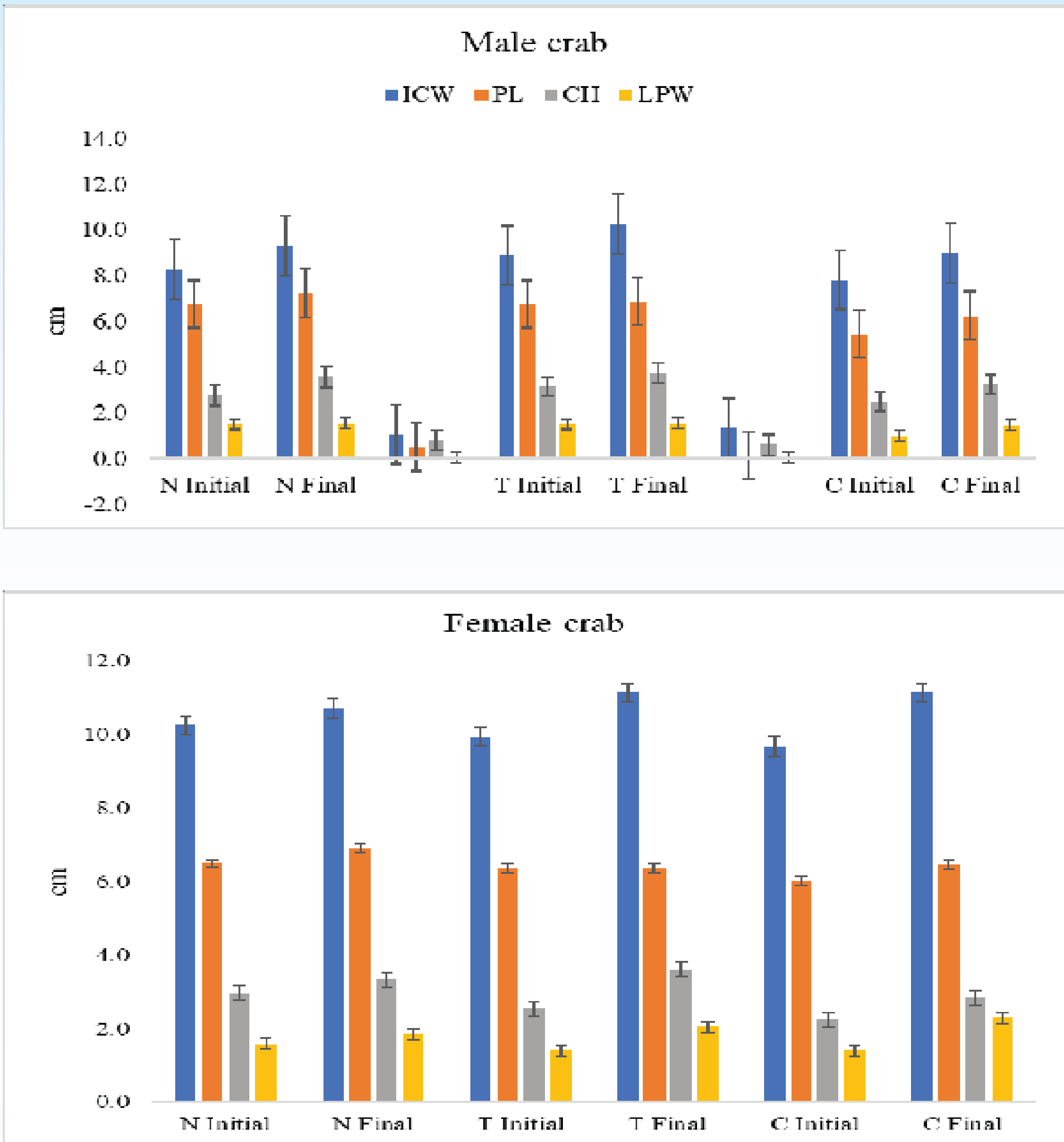


Fig 39. Variation of morphometric traits of *Scylla olivacea* in three diets. N = *Namalycastis* sp., T= Tilapia fish, C= Little neck clam, ICW = Internal carapace width, PL = Propodus length, CH = Chela height, LPW = Lower paddle width.

All the morphometric parameters were increased in size after harvesting (Fig 39). The highest increased of ICW was recorded in tilapia feeds male crabs (1.350 cm) and clam feeds for female crabs (1.467 cm), respectively (Fig 39). However, the highest PL increased in *Namalycastis* sp. feeds for female crabs (0.433 cm) and clam feeds for male crabs (0.80 cm), respectively. Overall, the highest sum of morphometric traits was recorded in clam feeds, followed by tilapia and *Namalycastis* sp., irrespective of sexes accordingly.

11.3. Component-3(BFRI)

11.3.1. *Experiment I: Determination of monovalent efficacy of Bacillus subtilis as probiotic candidate*

To conduct the experiment, initially *B. subtilis were isolated and pure culture were stored for further use.*

In-vitro challenge test for antagonistic effect of B. subtilis against V. parahaemolyticus

In-vitro challenge test were performed to assess the inhibitory effect of *B. subtilis* on the growth of *V. parahemolyticus*. In in-vitro test the growth of *V. parahaemolyticus* was inhibited by *B.* subtilis (Table 34)

Table 34. In-vitro challenge test and enumeration of *V. parahaemolyticus* load with and without *B. subtilis*

Time (hours)	*V. parahaemolyticus* load without *B.* subtilis (CFU\ml)	*V. parahaemolyticus* load with B. subtilis (CFU\ml)
0	$5.6* 10^3$	$2.8* 10^3$
4	$5.5* 10^3$	$3.3* 10^3$
8	$5.7* 10^3$	$4.2* 10^3$
12	$6.0* 10^3$	$3.9* 10^3$

11.3.2. Experiment II: Determination of efficacy of Bacillus subtilis on growth and survival of P. monodon

Growth and production of fish

The growth performances of *P. monodon* fed with different dose of *B. subtilis* as dietary supplementation are given in Table 35. The highest production was obtained at T1, where shrimp was supplied with *B. subtilis* treated diet @$2* 10^8$ cfu/g feed, whereas the control diet produced the lowest production. Highest survival was found in T1and lowest survival was found in control pond. The reason of lowest survival was disease outbreak in control ponds.

Table 35. Average body weight (ABW) of different treatments

Treatments	ABW(g) at 80 DOC	Survival rate (%)	Production (Kg/ha)
T1	16.7	64	855
T2	20.84	51	850
T3	20.2	41	662
T4 (C)	15.15	16	193

Physicochemical characteristics

Throughout 80 days of culture water quality in all ponds were observed to be normal and remained within ranges which allow high growth rate and production of *P. monodon* (Table 36). Temperature of water was 28-33° C and almost same in all ponds. Depth of water was maintained at a level of one meter in all ponds. As shown in Table 36, salinity of water was also almost same in all ponds. Salinity of water was 2-12 ppt during culture period. Initial level of morning dissolved oxygen (DO) was 5.0-8.8 mg/l which decreased to 3.2 at the later part of the culture period.

Table 36. Water quality variables of ponds under different treatments in 1st year.

Water quality Variables	Treatments			
	T_1	**T_2**	**T_3**	**T_4**
Depth (cm)	100 - 110	90- 105	95 - 108	96 -107
Transparency (cm)	35 - 45	25 - 45	25 - 40	30- 50
Temperature (°C)	28 - 33	31 - 33	31 - 33	31 - 33
Salinity (ppt)	2 - 12	2 - 12	2 - 12	2 - 12
pH	7.8- 9.4	7.8 - 9.6	7.9 - 9.6	8.0 - 9.4
Morning dissolved oxygen (mg/l)	3.2-8.0	3.8-6.6	3.6-8.2	4.3-8.8
Evening dissolved oxygen (mg/l)	6.3-10.2	6.8-11.6	6.7-10.1	6.6-10.4
Alkalinity	60 – 120	80-120	75 - 110	80 - 120

11.3.3. Experiment III: Determination of the combined effect of Bacillus sp. on growth and survival of Penaeus monodon

Under the screening process of the bacteria, different new strains of probiotic bacteria have been identified by metagenomic study

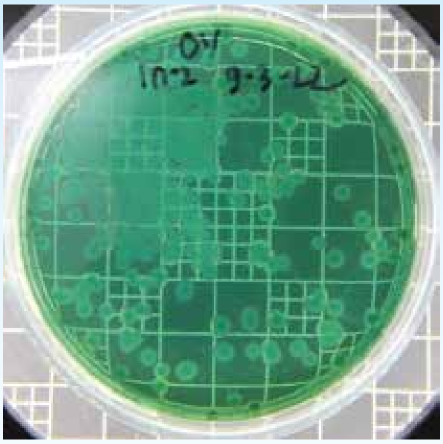
Table 37. Metagenomic Identification of probiotic bacteria

Classified upto genus	No of Unidentified	Classified Species
Lactobacillus	5	*Bacillus Bacillus_hemicentroti*
Virgibacillus	9	*Thermolongibacillus Thermolongibacillus_kozakliensis*
Bacillus	92	*Anoxybacillus Anoxybacillus_rupiensis*
Streptobacillus	6	*Alteribacillus Alteribacillus_bidgolensis*
Shewanella		*Shewanella litorisediminis*

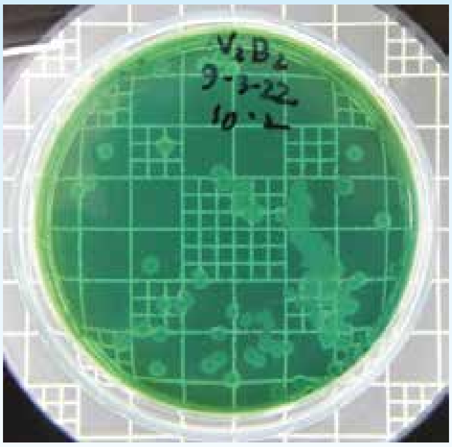
Plates 45. Field activities and monitoring of efficacy of *B. subtilis* on growth and survival of *P. monodon*

11.3.3.1. ***Inhibitory effects of*** **Bacillus** ***sp. on*** **Vibrio** ***sp.*** **in vitro.**

The effects were measured at laboratory using plate count technique and almost three times inhibitory effects of *Bacillus* sp. on *Vibrio* were observed (Plate 46 & Fig 40).



Overnight Culture plate of only *Vibrio* sp.



Overnight Culture plate of combined *Bacillus* and *Vibrio* sp.

Plate 46. Picture of Inhibitory effects of *Bacillus* sp. on *Vibrio* sp. *in vitro*

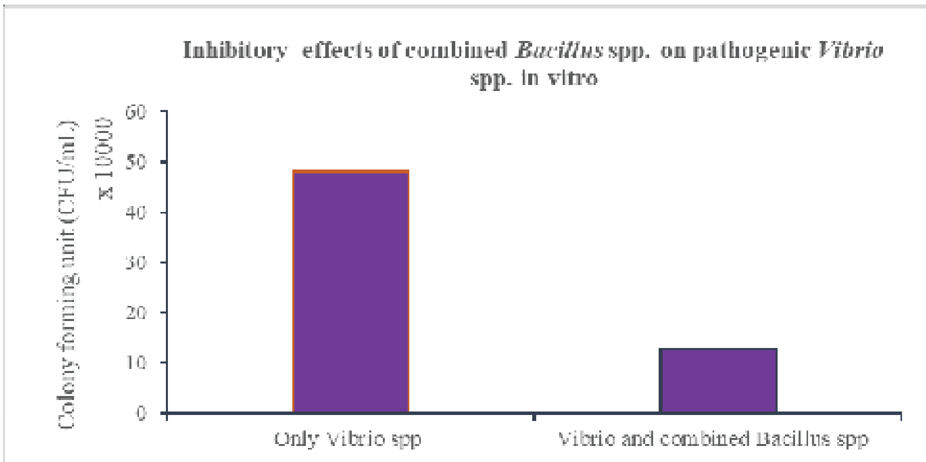
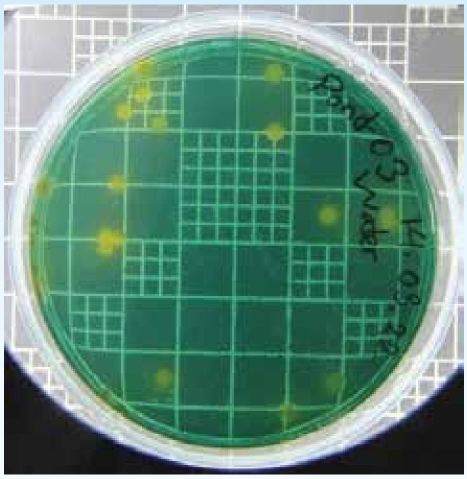


Fig 40. Inhibitory effects of *Bacillus* sp. on *Vibrio* sp. *in vitro*

11.3.3.2. *Enumeration of pathogenic (*Vibrio *sp.) in both the control and treatment pond water after probiotic application*

Pathogenic (*Vibrio* sp.) was enumerated in both the control and treatment pond water using selective agar media after probiotic application in treatment ponds and the results were observed as expected. Total *Vibrio* count were almost ten times higher in control pond than the treatments (Plate 47 & Fig 41).

Vibrio Culture plate of control pond water



Vibrio Culture plate of treatment pond water

Plate 47. Picture of Inhibitory effects of locally isolated probiotics on pathogenic *Vibrio* sp. in pond trial.

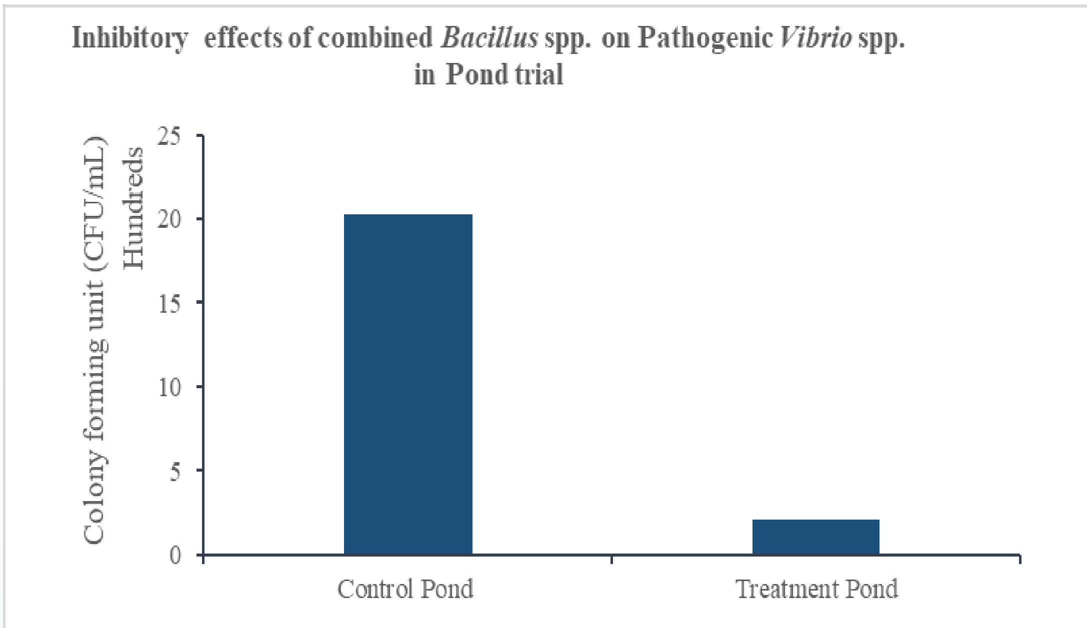


Fig 41. Inhibitory effects of locally isolated probiotics on pathogenic *Vibrio* sp. in pond trial.

Physico-chemical parameters of experimental ponds in 3rd year (April – May):

The average water quality parameters of all the treatments found suitable for shrimp culture throughout the project period (Table 38). According to Ferreira et al. (2011), the suitable range of physico-chemical parameters such as pH, DO, NH_3, temperature and salinity are 7-10, 2.5-10 ppm, >0.1 ppm, 18-33°C, 5-25 ppt, respectively for shrimp ponds.

Table 38. Average water quality parameters in experimental ponds in 3rd year (April – May)

Avarage water quality parameters					
Treatments	**pH**	**DO (ppm)**	**NH_3 (ppm)**	**Temperature (°C)**	**Salinity**
Control	8.11±0.34	6.19± 0.12	0.0± 0	32.2±0.62	8.13±1.12
Treatments	7.99± 0.11	7.36± 0.12	0.0±0	32.1± 0.91	8.33±1.20

11.3.3.3. Vibrio *load count*

Vibriosis is a bacterial disease caused by *Vibrio* which is Gram-negative, motile, facultative anaerobe bacteria of the family Vibrionaceae. It is ubiquitous throughout the world and in all marine crustaceans, including shrimp. Vibriosis is one of the major disease problems in shellfish and finfish aquaculture, especially in shrimp farming. High density of the bacterial species in culture pond increases the probability of getting diseases in the cultured stock.

In this study, *Vibrio sp* bacterial count found higher in control ponds in every sampling from the soil and water. In soil, the *vibrio* load found highest 3.7×10^3 cfu/g in the 3rd sampling from control ponds and lowest 0.3×10^3 cfu/g in the 2nd sampling from the treatment ponds.

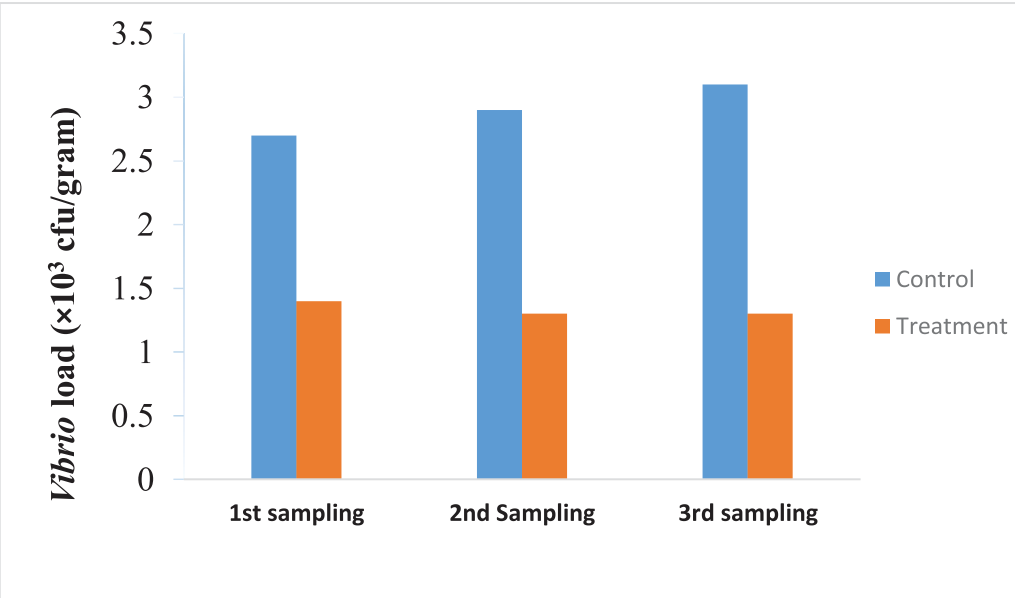


Fig 42. *Vibrio* bacterial load from the pond soil in different sampling

In case of pond water, the result was similar where the highest amount of *Vibrio* bacterial load found in the control pond and lowest in the treatment pond. It clearly indicates that application of probiotic reduced the vibrio load and also reduce the probability of disease intervention in the pond.

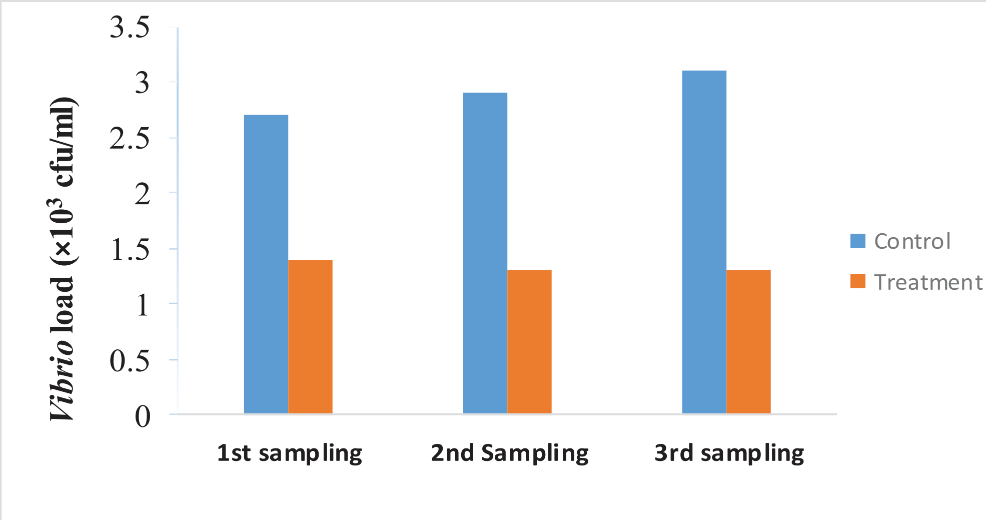


Fig 43. *Vibrio* bacterial load from the pond water in different sampling

As the *Vibrio* bacterial count in water and soil of the control pond (where probiotic was not used) was higher, in the sample of Hepatopancreas (HP) and gut content showed the similar types of result.

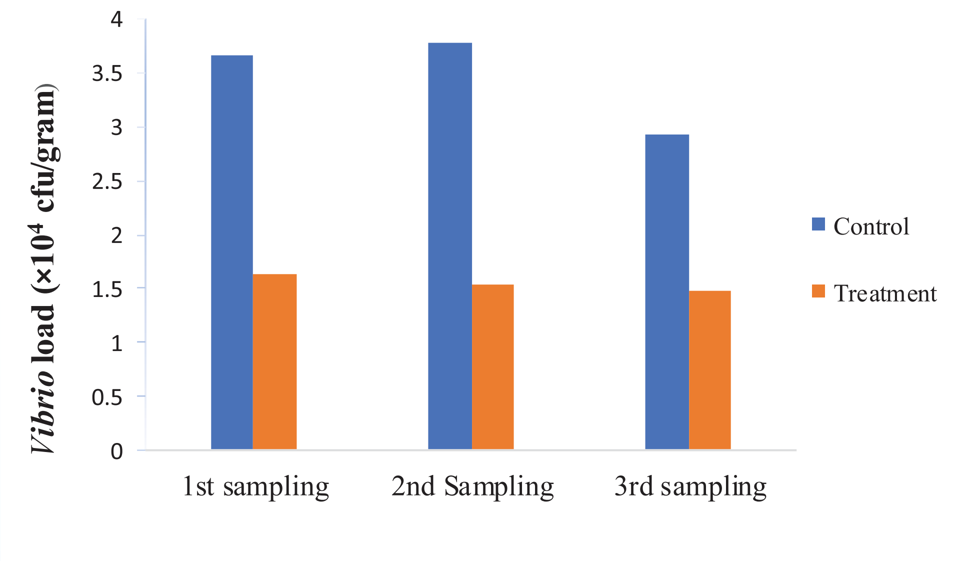


Fig 44. *Vibrio* bacterial load in the gut content from shrimp

The highest amount of *Vibrio* found in the gut and HP of shrimp from control ponds and lowest in treatment ponds (where probiotic was applied).

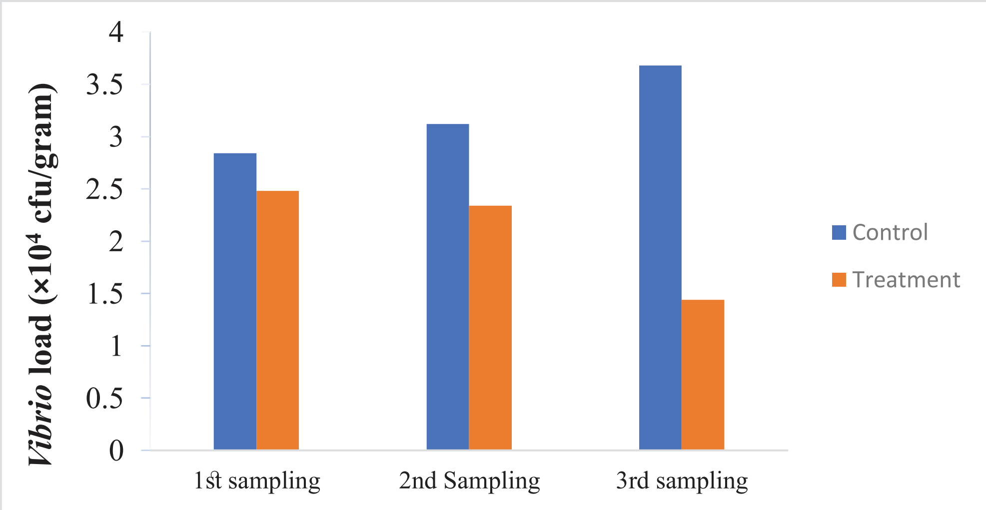


Fig 45. *Vibrio* bacterial load in the HP from shrimp

11.3.3.4. Monitoring of Growth performance

The average body weight (ABW) of the shrimp of treatment ponds were higher than the shrimp of control ponds. The ABW of the shrimp of control ponds found 12.87±1.79 g whereas the ABW from the treatment pond was 22.31±1.54 g after 60 days of stocking.

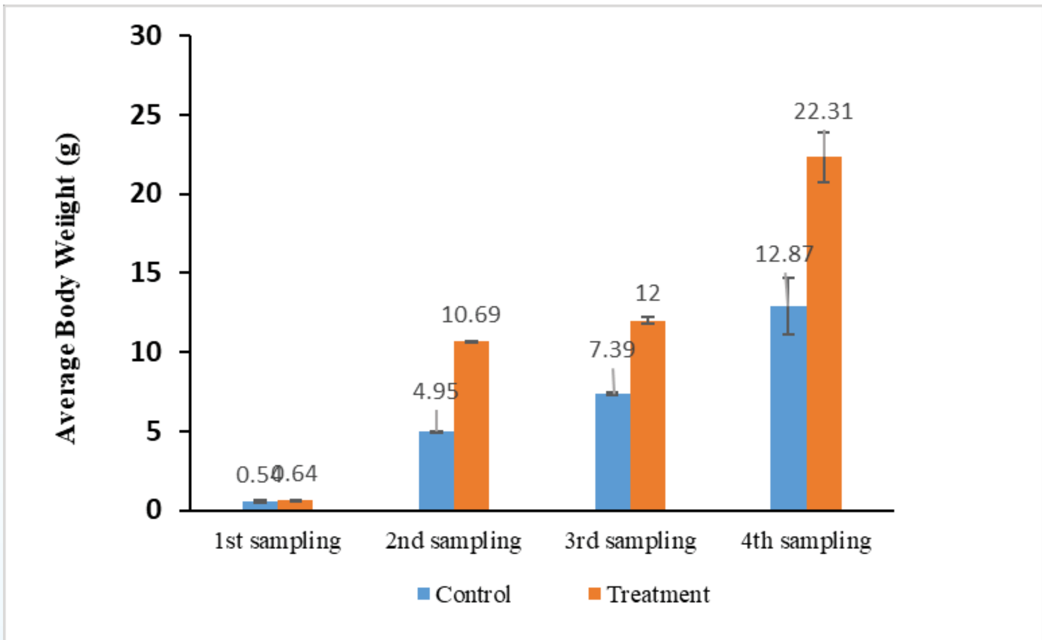


Fig 46. Growth performance of shrimp

Probiotics, as 'bio-friendly agents' helped to control and compete with pathogenic bacteria as well as to promote the growth of the cultured shrimp. In an experiment that was performed by Rengpipat (2003), the growth and resistance to *Vibrio* in black tiger shrimp (*P. monodon*) fed with a *Bacillus* probiotic were studied. It was found that the growth and survival rates of shrimps fed on the probiotic supplement were significantly greater than those of the controls. Similar results have been found in our experiments.

11.3.4. Immune Parameters

11.3.4.1. Superoxide Dismutase Activity

Superoxide dismutase (SODs) constitute a very important antioxidant defense against oxidative stress in the body. Generally, SOD activity in shrimp increases with the increasing load of pathogenic bacteria in culture environment. Higher SOD activity was found in the shrimp of control pond (542.38 U/ml), which indicates higher load of pathogenic bacteria. On the other hand, lower SOD activity in the shrimp of treatment pond (45.21 U/ml) reveals lower load of pathogenic bacteria and lower stress **(**Fig 47**).** It's might be the effect of probiotic treatment.

11.3.4.2. Total Haemocyte Count

Average THC from the shrimp of treatment showed higher amount than the shrimp of control ponds (Fig 48). Higher haemocyte count may associate with increased resistance against the pathogenic bacteria.

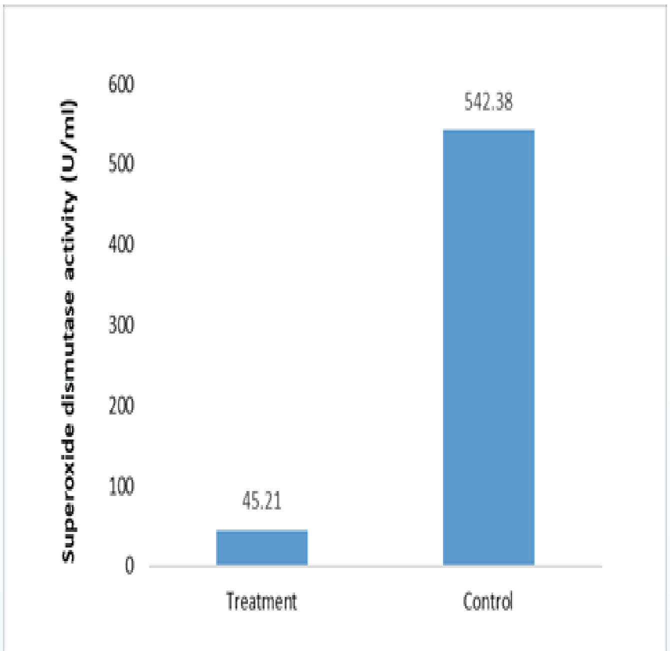


Fig 47. Superoxide dismutase activity in shrimp

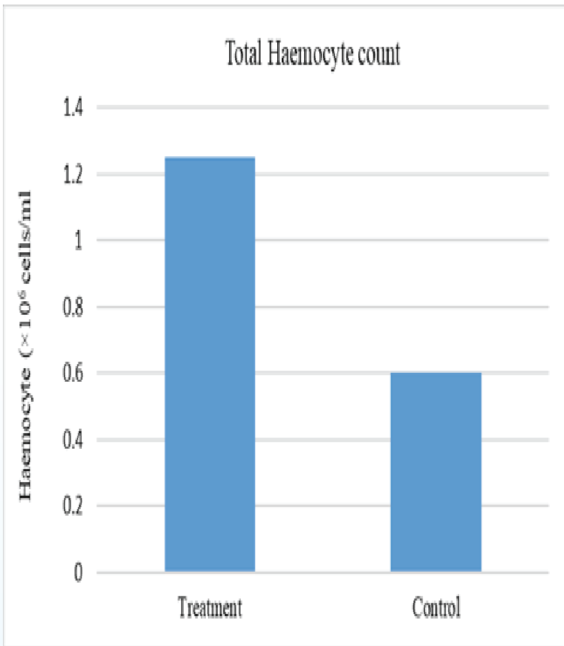


Fig 48. Total Haemocyte count from shrimp

Plates 48: Pictorial views showing sub project research and monitoring activities

12. Research highlights

Title of the sub-project

Exploration of Exogenous Enzymes, Bivalent Efficacy and Omega-3 Fatty Acid of Microbes and Small Invertebrates as Potential Feed Supplement for Enhancing Fish and Shrimp Productivity

Component 1 (BSMRAU)

Background

Today aquaculture has stand as an economically successful industry and demand for fish increases year-on-year and with increasing global population. The growth of aquaculture industry as a whole raises several issues that need to be addressed to ensure its long-term sustainability. Successful and sustainable aquaculture depends on economically viable and environmentally friendly feeds. The major feed ingredient, fishmeal, is expensive and there is increasing competition with other livestock industries for the available supply. From a nutritional standpoint, developing the use of inexpensive raw materials for feed like plant feed , increasing performance and finding way to remove or inactivate any antinutritional factors present are top priorities. This is where biological catalyst (exogenous enzymes) can play a significant role.

Objectives

i. To search and identify the suitable indigenous microorganisms for isolating bioactivity targeted exoenzymes; and
ii. To purify and characterize the isolated exoenzymes for commercial application aquaculture

Methodology

Key steps of methodology followed for the experiment are summarized as follows:

i. Collection of microbes samples from different environment and locations;
ii. Storage of samples at 4 ^{O}C at laboratory;
iii. Culture of bacteria using different culture media;
iv. Screening of bioactivity target microbes (activities included here are cellular, protolytic and phytase activity and biochemical characterization of isolated);
v. Molecular identification using PCR (molecular characterization was done through extraction of DNA from bacteriaand PCR product purification done for gene sequencing);
vi. Identification of bacteria using colony morphology;
vii. Purification of enzymes;
viii. Characterization of enzymes;
ix. Development of comprehensive database on microbes for enzyme;

Key findings

- Almost 400 samples were collected from different locations/environment of Bangladesh;
- About 520 pure isolates were separated from those collected samples;
- About 109 isolates were screened as different enzyme producer among them 44 cellulase, 58 protease and 20 phytase;
- Pure enzymes are produced having activity of cellulase, protease and phytase are 0.37, 0.28 and 0.20 U/ml, respectively;
- For commercial application, plant feed material like soybean was fermented and a significant improvement was found in term of protein content;
- A feed was formulated by replacing 30%, 40% and 50% of fish meal with FSM (Fermented Soybean Meal) Feeding trails showed that 50% replacement diet provided the best result in terms growth rate and immunity compared with control diet;

Key words

Aquaculture, Exogenous enzyme, non-conventional feed, Fermented Soybean Meal

Component 2 (NSTU)

Background

Coastal water polychaetes are considered as the best source of polyunsaturated fatty acids (PUFA) particularly of Omega-3 (n-3; alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Omega-3 rich feeds are the best quality feed for aquaculture industries globally (shrimp larvae, matured shrimp, and crab). Marine dried and powdered form of polychaetes have become a very popular supplementary diet for fish and shrimp in a number of Asian countries. Investigation on abundance, identification and qualitative assessment of polychaetes species and their performance on growth and survival of fish/shrimp and mud crab is yet to done. The present study attempted to address the gap and to commercialize the utilization of the nutrient rich animal based on the research findings for increased growth and production of aquaculture animals.

Objective

Identification and distribution of polychaete worms in the coastal/marine waters of Bangladesh, qualitative assessment of selected polychaetes with special reference Omega-3 fatty acid content, development of culture methods of selected polychaete sp. and to explore growth performance of mud crab using formulated supplementary feed from polychaetes

Methodology

i. Identification and distribution polychaetes

Based on primary information, polychaetes were collected from the sediments Sathkhira and Coxes Bazar coastal waters during March 2020 to April 2022 and identified as per taxonomical properties. Seasonal and annual abundance of polychaetes were studied and recorded. Available dominant polychaete, *Namalycastis* sp. was selected for qualitative assessment and culture trials. In addition, length-weight relationships of *Namalycastis* sp. was also done by different established equations.

Key findings

- Total 8 species of polychaetes were identified from the studied areas. Among the identified specimens, *Namalycastis* sp. was found abundant all the year round.
- The asymptotic length (L_∞) and highest recruitment were recorded in October-2021 where, temperature and salinity were 30.30 ˚C and 12.71 PSU, respectively.
- The first highest recruitment of *Namalycastis* sp. occurred in October and November, where the average temperature and salinity were 28.15 ^{0}C and 15.25 PSU, accordingly.
- *Dendronereis dayi* and *Namalycastis fauveli* are the new records for the marine polychaetes in Bangladesh.
- The evaluated exploitation level (E = 0.57) indicated that the stock of *Namalycastis* sp. was over exploited.

ii. Qualitative analysis of selected polychaete sp.

Polychaete (*Namalycastis sp.)* feeding experiment with three commercial diets with triplicate fashion were applied to a semi circulatory indoor culture system following continuous supply of *in situ* coastal waters. Water quality parameters such as temperature, DO, pH, salinity and TDS were recorded from indoor culture system during the feeding experiment daily. A 16L:8D photoperiod was maintained with an average 130 Lux light intensity. The specimens were fed treatment-wise (T1, T2 & T3) with one of the 3 types of feed namely T-1 (code:155-FF-01), T-2 (code:155-FF-02) and T-3 (code:155-FF-03). The polychaetes were cultured for 90 days from October 2020 to January 2021. Feeding was provided once a day (afternoon) at 3% BW of polychaetes. SGR%, DGR%, FCR, FER, WG and SR of *Namalycastis sp*. were recorded and analyzed as per standard procedure. Fatty acids analyses were performed both from wild and cultured species of *Namalycastis* Sp. with variable local commercial diets and salinity ranges following certified standard methods (AOAC, 2000).

Key findings

- The fatty acid composition of wild individual polychaetes measured as below the detection limits, whereas the highest recoveries were detected in cultured specimens fed with commercial feed (code:155-FF-02);
- Among three commercial diets (code:155-FF-03, code:155-FF-02, code:155-FF-01 and code:155-FF-02 feed performed better growth rate, highest weight gain and maximum feed efficiency ratio while the other commerci al feed (code:155-FF-03) showed the highest survival rate;
- The fatty acid composition among the salinity treatments were non-significant ($p > 0.05$) to each other. However, the ∑SFA, ∑MUFA and ∑PUFA were significant ($p < 0.05$).
- Most importantly, our measured omega-3 fatty acids (ALA, DHA and EPA) percentages in *Namalycastis sp* were higher than other reports of the same species observed at 30.0 PSU salinity during the controlled salinity experiment

iii. Culture trials

1. Habitat preferences and density-dependent growth of *Namalycastis* sp. (Polychaetes, Nereididae) in semi-circulatory culture systems

For density dependent growth, *Namalycastis* sp. specimens were stocked at 500, 1000 and 2000 indv./m^2 in DT1, DT2 and DT3 treatment (triplicating of each), respectively in 9 identical aquariums (46 cm length × 35.5 cm breadth × 30.5 cm height). Six types of substrates namely:

sand beds at 3.0, 7.0, 10.0 cm depth (≤ 500 µm grain size), mixer of silty sand and ≤ 7.0 mm diam. gravel (4.0 cm + 2 cm), silty sand (6.0 cm) and unmodified mangrove mud (8.0 cm) were prepared for T1, T2, T3, T4, T5 and T6, respectively in 18 identical aquariums. Commercial feed code:155-FF-08 (Protein 40 %, carbohydrate 22 %, moisture 12 %, lipid 5%, ash 16 % and fiber 5 %) was provided at the rate of 3% body weight/day for a total culture period of 90 days. Water quality parameters were maintained and measured daily. A 16L:8D photoperiod was maintained with an average 130 Lux light intensity. The survival rate and fresh weight were recorded fortnightly.

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Key findings

Among the six substrate experiments, 10.0 cm beach sand showed the highest growth and survival. In addition, the 500 indv./m^2 provided the best performance among the three density dependent growth.

2. Optimizing the growth and survival of *Namalycastis* sp. (Polychaetes, Nereididae) in variable temperature

Namalycastis sp. was stocked at density 500 indv./m^2 at five treatments namely: T26, T28, T30, T32, and T34 at 26, 28, 30, 32 and 34 ºC, respectively in 15 aquariums (22 cm length × 10 cm breadth × 30.5 cm height). Commercial shrimp nursery feeds (code:155-FF-09: 40 % protein, 10 % moisture, 9% lipid, 14 ash % and 5 % fiber) were provided at 4 % body weight/day. The stocked was cultured for 40 days and sampled at five days interval.

Key findings

The highest biomass, SR, SGR, and WG were calculated at 30 ºC and ranked as: T-30 > T-28 > T-26 > T-32 > T-34, respectively. Lower FCR indicates the higher biomass gain by these treatments. The highest survival (100 %) was recorded at T-30 treatment. A non-significant ($p > 0.05$) relative growth rate but significant ($p < 0.05$) survival were found in different treatments of *Namalycastis* sp. cultured from 26 – 34 ° C temperature.

3. Effect of growth and survival of *Namalycastis* sp. (Polychaetes, Nereididae) in different formulated diets in a semi-circulatory culture system.

A total of four feeds were prepared as T1 (code:155-FF-04), T2 (code:155-FF-05), T3 (-code:155-FF-06) and T4 (code:155-FF-07), respectively in 12 identical aquariums (46 cm length × 35.5 cm breadth × 30.5 cm height). *Namlycastis* sp. was stocked at density 500 ind./m^2 with 3% feeds body weight once in a day. Specimens were sampled fortnightly and culture duration was 75 days.

Key findings

Among the formulated diets, marine fish waste (T4) showed the highest significant ($p < 0.05$) growth and biomass gain but tilapia muscles feed (T1) had the highest survival rate. The highest SR and DGR were measured in T1, whereas, T2 had the lowest SR and minus DGR, accordingly.

4. Unraveling the growth and survival of *Namalycastis* sp. (Polychaetes, Nereididae) on four variable diets in an open water culture system

A total of 15 circular sized (inner diameter: 81.28 cm and height: 30.48 cm) concrete ring was used to stock *Namalycastis* sp. *Namalycastis* sp. was stocked at 500 ind./m^2 by calculating the inner walls the area (πr^2). The rings were divided into five treatments (T1 to T5) triplicating of each supplied with commercial feeds (T1 and T2), formulated feeds (T3 and T4), and no feed (T5) were selected. Commercial feeds T1 (code:155-FF-03: protein 38 %, fat 5%, fiber 4 %, moisture 12%), T2 (code:155-FF-01: protein 42 %, fat 7%, fiber 3 %, moisture 9%), formulated feeds T3 (code:155-FF-04), T4 (code:155-FF-06) and T5 (no feed) were selected, respectively. *Namalycastis* sp. was sampled every 10 days interval and cultured from March to April 2022 (60 days). Culture parameters (like SGR, DGR, FCR, FER, WG & SR) were calculated according to Nielsen et al., 1995; Fidalgo e Costa et al., 2000; Batista et al., 2003; Parandavar et al., 2018, Pajand et al., 2020 and Thi Thu et al., 2019

Key findings

The highest SGR (%/day), DGR (g/day) and SR (%) were measured in commercial diets (code:155-FF-01), while a negative SGR was calculated in the no feed supplied treatment (T5). The commercial diets showed higher growth but formulated diets showed the highest survival in the open water culture systems. In the open water systems, *Namalycastis* sp. with no feeds can never be substituted by supplementary diets. Comparatively, the controlled indoor culture produced higher yield than the natural open water systems.

5. Growth performance of mud crab applying formulated supplementary feed from polychaetes

A total of 125 male and female *S. olivacea* were used for this experiment. Morphometric traits namely: internal carapace width (ICW), propodus length (PL), chela height (CH), lower paddle width (LPW) was taken by measuring taps before and after the experiment. Similarly weighted male and female were sorted out and dissected to extract gonads/testis and ovaries, respectively. The gonadosomatic index (GSI) = (wet weight of gonad/wet weight of the crab) × 100 (Barber & Blake, 2006) was calculated for evert crabs. Three natural diets namely: Polychaetes (*Namalycastis* sp.), Tilapia muscles (*Oreochromis niloticus*) and little neck clam (*Mercenaria mercenaria*), respectively were selected for the growth performances.For each diet, five replicates were prepared and total of thirty crabs were cultured dividing into three diets performances. Feeding was performed once a day at 7 % body weight and daily 100 % water was exchanged. The crabs were cultured for 25 days.

Key findings

- 100 % survival rate was calculated, and crabs gained considerable weight in all diets.
- The highest and lowest SGR (specific growth rate) were measured in male crabs fed with clam, tilapia, respectively.
- The increasing of morphometric traits was comparatively higher in clam feeds than others.
- The GSI was increased in all diets, however, the GSI in female crabs were twice fed with polychaetes, *Namalycastis* sp.
- None of the growth parameters, GSI, and morphometric traits significantly ($p > 0.05$) increased in the selected diets.

Key words

Polychaetes *Namalycastis* sp. Mud crab *Scylla olivacea,* Growth and survival, Vvariable diets, density dependent growth , Omega-3 fatty acid, Distribution, Exploitation.

Component 3 (BFRI)

Background

In Bangladesh, Shrimp sector is playing a significant role in foreign exchange earnings, employment generation and poverty reduction. Since the last decades, the sector has been suffering from various issues and problems related to culture and production. In shrimp *(P. monodon*) culture, various diseases, has become a serious constraint, thereby, farmed shrimp production reduced significantly. Major suspected reasons behind the disease outbreak are poor farm management in terms of water quality, poor seed quality, less or no biosecurity, etc. To address these issues, the study aimed to produce the probiotics which will prevent diseases and develop techniques to proliferate them in farming environment.

Objective

i. To determination of efficacy of *Bacillus spp.*

ii. To evaluate growth performance

Methodology

For the screening of both pathogenic and beneficial bacteria, metagenomics approach along with conventional selective media culture were performed. Efficacy of the isolated bacteria were assessed both invitro and in-vivo with particular attention to immunological aspects.

Key findings

- In-vitro test showed growth inhibition of *V. parahaemolyticus* by *B. subtilis*.
- Almost three times inhibitory effects of *Bacillus* sp. on pathogenic *Vibrio* sp. were noticed.
- The highest production (855kg/ha) obtained with *B. subtilis* treated diet @2* 10^8 cfu/g (T1) feed whereas the control diet produced the lowest production (193kg/ha).
- Highest survival was found in T_1 (64%) and lowest survival was found control pond (16%).
- *Vibrio* were observed almost ten times lower in the treatments pond than control.
- Shrimp in the probiotic treated pond achieved almost double body weight (22.31g) than the control pond (12.87g) in only two months.

Key words

Shrimp, Diseases, Local Probiotics, *Bacillus, Vibrio,* Immunity

B. Implementation Status

1. **Procurement (component wise)**

Coordination Component (BARC): *Not applicable*

Component 1 (BSMRAU)

Description of equipment and capital items	PP Target		Achievement		Remarks
	Physical (No.)	Financial (TK.)	Physical (No.)	Financial (TK.)	
(a) Office equipment		0	0	0	
(b) Lab & field equipment (Gel Doc, -80^{0}C Freezer, Distillation Unit, On-line UPS)	04	2246000	04	2246000	100% Achieved
(c) Other capital items (Lab Table)	02	79600	02	79600	100% achieved

Component 2 (NSTU)

Description of equipment and chemical	PP target		Achievement		Remarks
a. Office equipment	**Physical No**	**Financial**	**Physical No**	**Financial**	
Executive Chair (Side Office)	2	20000	2	20000	Achieved 100%
Ceiling Fan	2	8000	2	8000	
Steel Almira (Side Office)	2	60000	2	60000	
Executive Table (Side Office)	2	40000	2	40000	
PCV chair (Side Office)	12	16000	12	16000	
b. Lab. and field equipment					
Refrigerator	2	158000	2	158000	Achieved 100%
Generator	2	80000	2	80000	
Pump	2	80000	2	80000	
multi parameter	2	397000	2	397000	
Thermostat water heater	2	199600	2	199600	
Bicycle	2	24000	2	24000	
Concrete ditch for outdoor culture of polychaetes	21	595692.2	21	595692.2	
c. Miscellaneous items					
Sampling bottles	Lot	1389000		1389000	Achieved 100%
Inputs (aerators, stones, steel, rod, rope, nipples, pipes, tanks, pipes tank	Lot	495000	Lot Lot	495000	
Apparatus & Chemicals	Lot	495000	Lot	495000	
Total		**6145192.2**		**6145192.2**	

Component 3 (BFRI)

Description of equipment and capital items	PP Target		Achievement		Remarks
	Physical (No.)	Financial (Tk.)	Physical (No.)	Financial (Tk.)	
(a) Office equipment: Digital Camera	1	**295000**	1	252000.00	All procurement completed as per plan
Desktop Computer,	1		1		
Laptop Computer,	1		1		
UPS	1		1		
Laser Printer	1		1		
High Magnification Close-up Lens	1		1		
(b) Lab &field equipment Mini Centrifuge (bench top)	1	**345000**	1	343100.00	
Handheld UV	1		1		
Heating Block)	1		1		
(c) Chemicals and reagents 100 bp DNA Ladder (ml)	LOT	**390000**	LOT	361000	
1kb DNA Ladder (ml)					
Primer (per bp)					
Gel illusion kit					
cDNA synthesis kit,	LOT	**490000**	LOT	**397000**	
DNA Extraction kit					
RNA extraction kit					
Taq PCR Premix					
Chemicals	LOT	**463500**	LOT	459500	
(d) Other capital items Computer table and chair	Table (1) Chair (1)		Table (1) Chair (1)	14000.00	

2. Establishment/renovation facilities: *Not applicable*

3.Training/study tour/ seminar/workshop/conference organized Coordination component (BARC)

Description	Number of participants			Duration (Days)	Remarks
	Male	Female	Total		
Inception Workshop (1 no)	56	7	63	1 day	All workshops held at the Conference room of BARC as per schedule of activity of the Coordination component
Half yearly Research Prog. Review Workshop (3 no.)	65+ 62+72	9+8+10	226	1+1+1 = 2 days	
Annual Research Prog. Review Workshop (2 no.)	65+63	9+8	1145	1+2 =3 days	
Project Completion Report Review Workshop (1 no)	52	6	58	1 day	
Orientation on PCR preparatory guideline	12	05	17	01 Day	All Principal Investigators of the sub project components attended the program

(*Not Applicable for BSMRAU, NSTU and BFRI components*)

C. Financial and physical progress (Combined & Component wise)

Combined progress

Fig in Taka

Items of expenditure/activities	Total approved budget	Fund received	Actual Expenditure	Balance / unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	7087007	7088207	7088207	0	100.00	No deviation noted. Expenditures made as per approved plan.
b. Field research/lab expenses and supplies	17382570	15847699	15847699	0	100.00	
c. Operating expenses	1795181	1669252	1669251.5	0	100.00	
d. Vehicle hire and fuel, oil & maintenance	1368429	1278188	1278188	0	100.00	
e. Training/workshop seminar etc.	150000	149100	149100	0	100.00	
f. Publications and printing	350000	346000	346000	0	100.00	
g. Miscellaneous	374913	306316	306316	0	100.00	
h. Capital expenses	4017300	4017300	4017300	0	100.00	
Total	**32525400**	**30702062**	**30702062**	**0**	**100.00**	

Coordination component (BARC)

Fig in Taka

Items of expenditure/activities	Total approved budget	Fund received	Actual Expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	600000	600000	600000	0	100.00	No deviation noted. Expenditures made as per approved plan.
b. Field research/lab expenses and supplies	0	0	0	0	0	
c. Operating expenses	346799	245681	245681	0	100.00	
d. Vehicle hire and fuel, oil & maintenance	255677	178074	178074	0	100.00	
e. Training/workshop seminar etc.	150000	149100	149100	0	100.00	
f. Publications and printing	350000	346000	346000	0	100.00	
g. Miscellaneous	127524	94950	94950	0	100.00	
h. Capital expenses	0	0	0	0	0	
Total	**1830000**	**1613805**	**1613805**	**0**	**100.00**	

Component 1 (BSMRAU)

Fig in Taka

Items of expenditure/activities	Total approved budget	Fund received	Actual Expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	2140400	2141600	2141600	0	100	No deviation noted. Expenditures made as per approved plan.
b. Field research/lab expenses and supplies	5498685	5347260	5347260	0	100	
c. Operating expenses	259600	235010	235010	0	100	
d. Vehicle hire and fuel, oil & maintenance	369200	356960	356960	0	100	
e. Training/workshop seminar etc.	0	0	0	0	0	
f. Publications and printing	0	0	0	0	0	
g. Miscellaneous	123915	88223	88223	0	100	
h. Capital expenses	2325600	2325600	2325600	0	100	
Total	**10717400**	**10494653**	**10494653**	**0**	100	

Component 2 (NSTU)

Fig in Taka

Items of expenditure/activities	Total Approved budget	Fund Received	Actual Expenditure	Balance/ Unspent	Physical progress (%)	Reasons for deviation
a.contractual Staff Salary	3407480	3407480	3407480	0	100	No deviation noted. Expenditure made as per approved plan.
b.Field Research / Lab expenses and supplies	7828615	7816199	7816199	0	100	
c.Operating Expenses	825651	825027	825027	0	100	
d.Vehicle Hire and Fuel, Oil & Maintenance	630180	630180	630180	0	100	
e.Training/Workshop/ Seminar etc.	0	0	0	0	0	
f.Publications and printing	0	0	0	0	0	
g.Miscellaneous	93474	93143	93143	0	100	
h.Capital Expenses	1082600	1082600	1082600	0	100	

Component 3 (BFRI)

Fig in Taka

Items of expenditure/activities	Total approved budget	Total fund received	Actual expenditure	Unspent balance	Physical Progress	Reasons for deviation
A. Contractual Staff Salary	939127	939127	939127	0	100	No deviation noted. Expenditure made as per approved plan
B. Field Research / Lab expenses and supplies	4055270	2684240	2684240	0	100	
C. Operating Expenses	363131	363534	363534	0	100	
D. Vehicle Hire and Fuel, Oil & Maintenance	113372	112974	112974	0	100	
E. Training/Workshop/ Seminar etc.	0	0	0	0	0	
F. Publications and printing	0	0	0	0	0	
G. Miscellaneous	30000	30000	30000	0	100	
H. Capital Expenses	609100	609100	609100	0	100	
Total	**6110000**	**4738975**	**4738975**	**0**	**100**	

D. Achievement of Sub-project by objectives (Tangible form): Technology generated/developed

Component 1 (BSMRAU)

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
To search and identify the suitable indigenous microorganisms for isolating bioactivity targeted exoenzymes	Sample collection	Almost 400 samples were collected from different region	A number of bacteria was identified as cellulase, protease and phytase producing agents which will be used for functional aquafeed formulation
	Isolation of microbes	About 520 pure isolates	
	Enzyme screening	44 cellulase, 58 protease and 20 phytase	Pure isolates to produce enzyme

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
To purify and characterize the isolated exoenzymes	Enzyme production, purification and pure enzyme activity assay	Activity of Cellulase, protease and phytase 0.37, 0.28 and 0.20 U/ml	Enzymes from identified pure isolates were purified and characterized which will be used for aquafeed preparation

Component 2 (NSTU)

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
Identification and distribution of polychaete worms in the coastal and/marine waters.	Polychaete sample collection, their distribution and their length weight relationship	Among 8 identified species two species were new and *Namalycastis* sp. was dominant. Index of *Namalycastis* sp. were 21.5 cm, 1.5 year^{-1} and 2.60 respectively, with total mortality (Z) of 4.56 year^{-1}. Overexploitation of polychaetes occurred.	Chechlist of polychaetes in Bangladesh. Benefit fisheries researchers for development of polychates in Bangladesh.
To analyze quality and quantity of Omega-3 fatty acid content of those identified species.	Growth studies with commercial diets were performed. Fatty acid analyses was performed from wild and cultured species of Namalytiscas Sp. with variable local commercial diets and salinity ranges.	Among three commercial diets, Uni-president performed better growth rate, highest weight gain and maximum feed efficiency ratio for *Namalycastis* sp. The fatty acid composition of wild individual polychaetes measured was below the detection limits, whereas, the highest recoveries were detected in cultured specimens fed with commercial feed. Most importantly, our measured omega-3 fatty acids (ALA, DHA and EPA) percentages were higher than other reports of the same species of *Namalycastis* sp. observed at 30.0 PSU during the	*Namalycastis* sp. could be a good omega-3 sources that could be used as feed supplement in fish and shrimp hatcheries.

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
		controlled salinity experiment.	
To develop mass culture method of Omega-3 fatty acid containing polychaetes for commercial applications in the feed industries.	Habitat preferences and density-dependent growth of *Namalycastis* sp. in semi-circulatory culture systems. Optimizing the growth and survival of *Namalycastis* sp. in variable temperatures. Effect of growth and survival of *Namalycastis* sp. in different formulated diets in semi-circulatory culture system. Unraveling the growth and survival of *Namalycastis* sp. on four variable diets in an open water culture system.	Among the six substrates 10.0 cm beach sand with 500 indv/m^2 stocking of polychaetes showed the highest growth and survival of *Namalycastis* sp. The highest biomass, SR, SGR, and WG were calculated at 30 ˚C and ranked as: T30 > T28 > T26 > T32 > T34, respectively for *Namalycastis* sp. Among the formulated diets, marine fish wastes showed the highest significant ($p < 0.05$) growth and biomass gain but tilapia muscles feed had the highest survival rate for *Namalycastis* sp. The highest SGR (%/day), DGR (g/day) and SR (%) were measured in commercial diets (code:155-FF-01), while, a negative SGR was calculated in the no feed because of higher death rate. The commercial diets showed higher growth but formulated diets showed the highest survival in the open water culture systems. In the open water systems, *Namalycastis* sp. with no feeds can never be substituted by supplementary diets	Culture of omega-3 rich *Namalycastis* sp. can be performed in dried seabeach sand substrate (10cm) following optimized stocking density. Commercial culture of *Namalycastis* sp. can be best suit of 30.0 ^{0}C. Indoor culture system followed with formulated diet of marine fish wastes could be recommended for *Namalycastis* sp. culture. Open water culture system might not be recommended for *Namalycastis* sp. culture.
To explore growth performance of mud	Morohometric traits of *Scylla olivacea*	100 % survival rate was calculated and crabs gained	Polychaete, *Namalycastis*

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
crab using formulated supplementary feed from polychaetes.	were taken by measuring taps before and after the experiment. Similarly weighted male and female were sorted out and dissected to extract gonads/testis and ovaries, respectively. The GSI was calculated for evert crabs. Three natural diets namely: Polychaetes *(Namalycastis* sp.), Tilapia muscles (*Oreochromis niloticus*) and little neck clam (*Mercenaria mercenaria*), respectively were selected for the growth performances. Feeding was performed once a day at 7 % body weight and daily 100 % water was exchanged. The crabs were cultured for 25 days.	weight in three diets. The increasing of morphometric traits were comparatively higher in clam feeds than others. The GSI was increased in all diets, however, the GSI in female crabs were twice fed with polychaetes. None of the growth parameters, GSI, and morphometric traits significantly ($p > 0.05$) increased in the selected diets.	sp. could be recommended as a supplementary feed commercially important mud crab (*Scylla olivacea)* culture in Bangladesh as this feed ensured 100% survival and manyfold GSI increment.

Component 3 (BFRI)

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e., product obtained, visible, measurable)	Outcome (short term effect of the research)
To determine the monovalent efficacy of locally isolated *Bacillus* sp.	For monovalent efficacy determination, inhibitory effects of *Bacillus* sp. on *Vibrio* were observed *in vitro* using plate count technique.	i. On in-vitro test the growth of *V. parahemolyticus* was inhibited by *B. subtilis*	Growth rate and inhibition of pathogenic bacteria in the culture system significantly increased
To determine the combined effect of *Bacillus* sp.	PL stocking, probiotics dose optimization was completed and application of probiotics and monitoring of growth and survival were being conducted fortnightly.	i. Growth performance was significantly higher in treatment ponds than control ponds ii. Highest survival was found in treatment and lowest survival was found control pond. iii. Almost three times inhibitory effects of probiotic against *Vibrio* were noticed.	This clearly indicates the positive effects of locally isolated probiotics on growth and survival of the shrimp
To assess the production feasibility of *P. monodon* using combined *Bacillus* sp.	PL was stocked and production feasibility was observed.	i. The highest production was obtained at T1, where shrimp was supplied with *B. subtilis* treated diet @2* 10^8 cfu/g feed, whereas the control diet produced the lowest production.	Shrimp production increased upto 855 kg/ha with the use of probiotic bacteria.

E: Information/knowledge generated/policy generated

Component 1 (BSMRAU)

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objective's	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
To search and identify the suitable indigenous microorganisms for isolating bioactivity targeted exoenzymes	Sample collection, Isolation of microbes, Enzyme screening, Gene and MALDI-TOF sequencing	Almost 400 samples collected 520 pure isolates separated and 44 cellulase, 58 protease and 20 phytase producing bacteria were screened	Pure isolates capable of enzyme production was identified
To purify and characterize the isolated exoenzymes	Enzyme production, purification and pure enzyme activity assay	Activity of Cellulase, protease and phytase 0.37, 0.28 and 0.20 U/ml	Pure enzymes produced for commercial application in fisheries sector

Component 2 (NSTU)

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
Identification and distribution of polychaete worms in the coastal and/marine waters.	Collection and identification of polychaetes from two study areas. *Seasonal and annual distribution of polychaetes. Length-weight relationships of dominant polychaetes. Recruitment pattern of Namalycastis sp.	Identified 8 species of polychaetes. *Dendronereis dayi* and *Namalycastis fauveli* are the new records for the marine polychaetes in Bangladesh. *Namalycastis* sp. was dominant annually. Asymptotic length, growth coefficient, and growth performance index of *Namalycastis* sp. were 21.5 cm, 1.5 $year^{-1}$ and 2.60 respectively, with total mortality (Z) of 4.56 $year^{-1}$. Recruitment mainly occurs in October and March.	Checklist of polychaetes in Bangladesh. Benefit fisheries researchers, entrepreneurs for the initiation of commercial culture technique development. Length-weight relationship determines that *Namalycastis* sp. is already overexploited in Cox's bazar coast. Policymakers should take necessary initiatives to restore polychaetes in Bangladesh.
To analyze quality and	Specific Growth Rate,	Among three commercial	Best quality feed

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
quantity of Omega-3 fatty acid content of those identified species.	Daily Growth Rate, Feed Conversion Ratio, Feed Efficiency Ratio, Weight gain (%) and survival rate of *Namalytiscas* Sp. were performed with 3 locally available commercial shrimp diets in an indoor semi circulatory culture system for 90 days. Feeding was provided once a day (afternoon) at 3% body weight of polychaetes. Fatty acid analyses was performed from wild and cultured species of Namalytiscas Sp. with variable local commercial diets and salinity ranges following certified standard methods (AOAC, 2000).	diets, Uni-president performed better growth rate, highest weight gain and maximum feed efficiency ratio for *Namalycastis* sp. Although other commercial feed (CP) showed the highest survival rate, single factor ANOVA indicated that none of the feeds significantly ($p > 0.05$) influenced the growth and survival of *Namalycastis* sp. The fatty acid composition of wild individual polychaetes measured was below the detection limits, whereas, the highest recoveries were detected in cultured specimens fed with commercial (Uni-president) feed at 26 ± 2.0 °C temperatures and 17.0 ± 2.0 PSU salinities. The fatty acid composition among the salinity treatments were non-significant ($p > 0.05$) to each other. However, the ∑SFA, ∑MUFA and ∑PUFA were significant ($p < 0.05$). Most importantly, our measured omega-3 fatty acids (ALA, DHA and EPA) percentages were higher than other reports of the same species observed at 30.0 PSU during the controlled salinity experiment. Among the salinity treatments, 30 PSU would be recommended for biomass enhancement of	for *Namalycastis* sp. is a key work to be done although commercial shrimp feed (Uni-president) showed better growth omega-3 fatty acids were below detection limit. Salinity play significant roles in the growth parameters of *Namalycastis* sp. 30 PSU salinity could be recommended for the culture of *Namalycastis* sp. as it showed the maximum percentage of omega-3 fatty acids.

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
		Namalycastis sp in aquaculture systems.	
To develop mass culture method of Omega-3 fatty acid containing polychaetes for commercial applications in the feed industries.	Habitat preferences and density-dependent growth of *Namalycastis* sp. in semi-circulatory culture systems. Optimizing the growth and survival of *Namalycastis* sp. in variable temperatures. Effect of growth and survival of *Namalycastis* sp. in different formulated diets in semi-circulatory culture system. Unraveling the growth and survival of *Namalycastis* sp. on four variable diets in an open water culture systems.	Among the six substrates 10.0 cm beach sand with 500 indv./m^2 stocking of polychaetes showed the highest growth and survival of *Namalycastis* sp. The highest biomass, SR, SGR, and WG were calculated at 30 ˚C and ranked as: T30 > T28 > T26 > T32 > T34, respectively for *Namalycastis* sp. Among the formulated diets, marine fish wastes showed the highest significant ($p < 0.05$) growth and biomass gain but tilapia muscles feed had the highest survival rate for *Namalycastis* sp. The highest SGR (%/day), DGR (g/day) and SR (%) were measured in commercial diets (FriPPaK), while, a negative SGR was calculated in the no feed because of higher death rate. The commercial diets showed higher growth but formulated diets showed the highest survival in the open water culture systems. In the open water systems, *Namalycastis* sp. with no feeds can never be substituted by supplementary diets	Culture of omega-3 rich *Namalycastis* sp. can be performed in dried seabeach sand substrate (10cm) following optimized stocking density. Commercial culture of *Namalycastis* sp. can be best suit of 30.0 ^{0}C. Indoor culture system followed with formulated diet of marine fish wastes could be recommended for *Namalycastis* sp. culture. Open water culture system might not be recommended for *Namalycastis* sp. culture.
To explore growth performance of mud crab using formulated supplementary feed from polychaetes.	Morohometric traits of *Scylla olivacea* were taken by measuring taps before and after the experiment. Similarly weighted male and female were	100 % survival rate was calculated and crabs gained weight in three diets. The increasing of morphometric traits were	Polychaete, *Namalycastis* sp. could be recommended as a supplementary feed commercially important mud

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
	sorted out and dissected to extract gonads/testis and ovaries, respectively. The GSI was calculated for evert crabs. Three natural diets namely: Polychaetes *(Namalycastis* sp.), Tilapia muscles (*Oreochromis niloticus*) and Little neck clam (*Mercenaria mercenaria*), respectively were selected for the growth performances. Feeding was performed once a day at 7 % body weight and daily 100 % water was exchanged. The crabs were cultured for 25 days.	comparatively higher in clam feeds than others. The GSI was increased in all diets, however, the GSI in female crabs were twice fed with polychaetes. None of the growth parameters, GSI, and morphometric traits significantly ($p > 0.05$) increased in the selected diets.	crab (*Scylla olivacea)* culture in Bangladesh as this feed ensured 100% survival and manyfold GSI increment.

Component 3 (BFRI)

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output	Outcome (short term effect of the research)
Development of technology for reducing fish/shrimp mortality by improving feed digestibility and disease resistance	Inhibitory effects of *Bacillus* sp. on *Vibrio* were observed *in vitro* using plate count technique. Dose optimization and application of probiotics were done. Growth and production performance were evaluated.	Locally isolated probiotics had significantly positive impact on the inhibition of pathogenic bacteria and higher growth performances of shrimp.	Application of these locally isolated probiotics will help to prevent disease and ensure sustainable production of shrimp

F. Materials development/publication made under the sub-project

Publication	Number of publications		Remarks (e.g., paper title, name of journal, conference name etc.)
	Under preparation	Completed & published	
Component 1 (BSMRAU)			
Technology bulletin/ booklet/leaflet/flyer etc.	–	–	–
Journal publication	02	–	1.Chakroborty, K., Marma, M., Alam, M. J., Alam, M. S., Rahman, M.H. and Rafiquzzaman, S. M. 2022. *Biochemical Characterization of Bacteria Isolated from Soil and Water and Their Effect on Proximate Composition of Soybean Meal after Solid-State Fermentation (Under preparation).* 2.Marma, M., Chakroborty, K., Alam, M.J., Rahman,Z.,Rahman,M.H.andRafiquzzaman, S. M. 2022. *Isolation and Characterization of Cellulase-producing Bacteria from Fermented Bamboo (Under preparation).*
Video clip/TV program	01		মাছের খাদ্যে অনুজীবের এনজাইম প্রয়োগে মৎস্য উৎপাদন বৃদ্ধির সম্ভাবনা ড. এস, এম, রফিকুজ্জামান
News Paper/Popular Article	–	–	–
Other publications, if any		03 MS thesis	1)Optimization of Replacement of Fishmeal by Fermented Soybean Meal on Growth Performance and Hematology of Nile Tilapia *(Oreochromis niloticus).* 2)Isolation and Characterization of Bacteria from Soil and Water and Their Effect on Nutrient Composition of Soybean Meal upon Solid-state Fermentation. 3)Identification and Characterization of cellulase Producing Bacteria Isolated from Fermented Bamboo Collected from Khagrachhari.
Component 2 (NSTU)			
Booklet	01		*Marine Polychaetes: A potential feed for mud crab nursing and grow-out culture (in Bangla)* By Dr. Md. Jahangir Sarker, Professor. Dept. of Fisheries and marine sciences. NSTU. Noakhali

Publication	Number of publications		Remarks (e.g., paper title, name of journal, conference name etc.)
	Under preparation	Completed & published	
Journal publication	05		*1)*Sarker, Md. Jahangir, Islam, Islam Ariful, Islam, Md. Monirul, Sarker, Pallab Kumar, Joydas Theodickal, 2022. Population dynamics of *Namalycastis* sp. (Nereididae, Polychaeta) from the Cox's Bazar coast of Bangladesh; *Journal of Frontiers in Marine Science(Submitted);* *2)*Sarker, Md. Jahangir, Islam, Islam Ariful, Islam, Md. Monirul, Sarker, Pallab Kumar, 2022. Effects of Salinity in the fatty acid profile of Namalycastis sp. in Cox's Bazar, Bangladesh. *Journal of Aquaculture (Submitted);;* *3)*Sarker, Md. Jahangir, Islam, Islam Ariful, Islam, Md. Monirul, Sarker, Pallab Kumar, 2022. Effects of various formulated diets on the growth of *Namalycastis* sp. in Cox's Bazar, Bangladesh. *Journal of Aquaculture (Submitted);* *4)*Sarker, Md. Jahangir, Islam, Islam Ariful, Islam, Md. Monirul, Sarker, Pallab Kumar, 2022. Effects of temperatures on the growth of *Namalycastis* sp. in Cox's Bazar, Bangladesh. *Journal of Environmental Science and Technology(Submitted);* **5)**Sarker, Md. Jahangir, Islam, Islam Ariful, Islam, Md. Monirul, Sarker, Pallab Kumar, 2022. Effects of substrates on the growth of *Namalycastis* sp. in Cox's Bazar, Bangladesh. International *Journal of Aquatic Ecology (Submitted);*
Video clip/TV program		01	PIU-BARC media wing
News Paper/Popular Article	01		Title: "Polycheates" a nutrient rich marine invertebrate with Omega-3 fatty acids-one of the best fish & animal feed ingredient. submitted to the Daily Prothom Alo; the Daily Star news paper
Other publications, if any	4 MS thesis awarded waiting for degree	3 MS thesis awarded	1. Effects of three diets on the growth, survival and Fatty acid contents in *Namalycastis* sp.; 2. Effect of substrate depth on the growth and survival rate of polychaetes (*Namalycastis* sp.); 3. Distribution of Coastal Water Polychaetes in Satkhira and Cox's Bazar Region;

Publication	Number of publications		Remarks (e.g., paper title, name of journal, conference name etc.)
	Under preparation	Completed & published	
			4. Study on the measurement of growth performance under various diets on mud crab (Scylla olivacea); 5. Length-weight Relationship of Polychaetes in the southern part of Bangladesh; 6. Unraveling the growth and survival of *Namalycastis sp* (Polychaetes) in four variable diets in open water culture systems; **7.** Effects of supplementary diets on the growth of marine polychaetes;
Component 3 (BFRI)			
Technology bulletin/ booklet/leaflet/flyer etc.	01		Rakib.H. 2022. *Application of locally isolated bacteria to continue pathogenic bacteria in shrimp farming. SRS, BFRI, Bagerhat.*
Journal publication	01		Rakib. H et.al. 2022. *Inhibitory effect of locally isolated probiotics on Vibrio propagation in Southwest coastal region of Bangladesh (Under preparation)*
Video clip/TV program	01		"Activities and purpose of the sub-project components and role in fish/shrimp disease control"; Bangladesh Television (BTV)
News Paper/Popular Article	–	–	–
Other publications, if Any	–	–	–

G. Description of generated technology/knowledge/policy

i. Technology fact sheet (title of the technology, introduction, description, suitable location/ecosystem, benefits, name and contact address of author)

Not applicable

ii. Effectiveness in policy support (if applicable)

Not applicable

H. Technology/knowledge generation/policy support (as applied)

i. Immediate impact on generated technology (commodity & non-commodity)

Not applicable

ii. Generation of new knowledge that help in developing more technology in future

Component 1 (BSMRAU)

1. Microbial enzymes obtained from different microorganisms will help and encourages particularly for application in industries on commercial scales;
2. Exoenzymes from microbial sources are expected to eliminate the major problems for plant-based aquafeed materials and fermentation of plant feed stuffs (soybean, maize and wheat bran) using exogenous enzyme (Protease, Cellulase and Phytase) releasing bacteria for improving nutritional properties;
3. Replacement of animal protein will be possible with low cost plant protein (generated through bacterial fermentation) will reduce feed cost and provide better growth as different anti nutrients are reduced;
4. The acquired knowledge through this researcht will help to review and revise the existing feed regulation act, 2011;

Component 2 (NSTU)

1. Eight species of marine polychaetes for the first time identified from the Cox's Bazar coastal waters;
2. Year-round dominant species, *Namalycastis* sp. in Bangladesh coastal waters is rich in omega-3 fatty acids which is even higher than the same species available in other regions;
3. Indiscriminate captured of Polychaete species containing high omega-3 fatty acids by the local fishers for shrimp hatcheries id one of the leading casual factors for stock reduction and biodiversity loss;
4. Suitability of temperatures, salinity, and habitats for the growth of *Namalycastis* sp. both in indoor and outdoor system has been explored and optimized;
5. A complete study on breeding behavior of polychaete, *Namalycastis* sp., and formulation of supplementary feed could be base for generation of a new sustainable technology for shrimp and crab production;

Component 3 (BFRI)

1. Efficacy of locally isolated probiotic bacteria was assessed for the very first time.
2. Almost three times inhibitory effects of *Bacillus* sp. on pathogenic *Vibrio* sp. were noticed.
3. In total cfu count of experimental and control pond water in TCBS selective agar media, total *Vibrio* were observed almost ten times higher in control pond than the treatments. Sample of Hepatopancreas (HP) and gut content showed the similar types of result.
4. The highest amount of *Vibrio* found in the gut and HP of shrimp from control ponds and lowest in treatment ponds (where probiotic was applied).
5. The preliminary findings immensely opt the further broad spectrum trial on the isolates followed by development of mass culture and storage condition optimization, thereby,

developing a final product for the shrimp farmers. Moreover, efficacy of this probiotic on the other aquaculture species like fish, crab, cat fish and eels can be observed.

iii. Technology transferred that help increased agricultural productivity and farmers' income

Not as such as technoogy, but the technical information generated through the research will help to increase agricultural productivity and farmers' income in following ways:

- Extraction of exo-enzymes from microbial sources are expected to eliminate the major problems for plant-based aqua feed materials and fermentation of plant feed stuffs and increase the digestibility of fish/shrimp;
- Culture and production of polychaete *Namalycastis* sp. as a rich source of omega-3 fatty acids can be a potential and quality grade fish feed ingredient;
- Higher inhibitory effects of *Bacillus* sp. on pathogenic *Vibrio* sp. in shrimp disease control could be applied as effective probiotic agent for disease control in shrimp farms.

On successful transfer of the findings of the present research, productivity of fish/shrimp will increase and income of farmers will also be increased to a significant level because of the reduced mortality of animals due to higher feed digestibility and increased disease resistance.

iv. Policy support

Bangladesh spent a significant amount of money (0.23-0.25 billion US$) for importing fish feed raw materials (fish meal, meat and bone meals) every year. However, the supply of fishmeal is insufficient and as well as in some cases degraded-underqualified and the cost is often very high. So, the profitable replacement of fishmeal with cheaper available protein sources is essential. So, it is emphasized to evaluate the actual information and nutritive value of locally available low-cost feed ingredients, which is crucial for the feed safety, increasing feed digestibility and develop disease resistant. Identification of bioactivity targeted microbes for enzyme extraction for increased feed digestibility and for most favorable growth and understanding and evaluate the most potential sources, as rich in Omega-3 fatty acid content invertebrate like marine polychaetes, *Namalycastis* sp. are the two most important areas, will satisfy the qualitative requirement of fish feed with increased return in fish and shell fish production. Therefore, the study suggesting the following amendments and proposing inclusion new opportunities under the existing regulation etc.

i. Based on the present research finding, to consider the importance of conservation of this marine potential invertebrate and to establish legal rights, it is thus recommended to amend the present definition of "Fish" by including there marine polychaetes in the Marine Fisheries Ordinance, 1983 (MFO, 1983) and to facilitate the conservation and restoration of natural *Namalycastis* sp. stocks.

ii. Besides, unrevealing extraordinary content of omega-3 fatty acids in this species, provision for alternative utilization of local resources (marine polychaetes, probiotics) for the best quality fat (with Omega -3 fatty acids) and animal protein source to supplement high quality fish and shellfish feed. Under this consideration, marine polychaetes might be included as another new potential ingredient under the Fish Feed Regulations 2011 (FFR, 2011) that will save such significant economic loss as well as ensure food security in Bangladesh.

iii. Provision for more research on identification, characterization, purify and database preparation bioactive enzyme from different sources of microbes as functional ingredient for increasing feed digestibility in fish and increased disease resistance.

I. Information regarding desk and field monitoring

i. Information on desk monitoring (combined)

Consulting meetings, workshops, seminars, coordination meetings etc	Date & Venue	Output
Component 1 (BSMRAU)		
1) Inception workshop	22/12/2019; BARC, Dhaka	Review of project document done and improved
2) 1st Co-ordination meeting	07/09/2020; BARC, Dhaka	Isolation, optimization, characterization and purification of the identified enzyme Cellulase done. Isolation and screening of all other categories of enzymes (Protease, Cellulase and Phytase) from various potential sources of bacteria also included
3) 1st Annual progress workshop	30/09/2020; BARC, Dhaka	New bacteria sources were accounted as per suggestion
4) Meeting with World bank representative	25/08/2021 BARC, Dhaka	Sub-project revision along with activity time plan
5) 2nd Annual progress workshop	23/11/2021; BARC, Dhaka	Completion of all suggested works helps to update research works
6) 2nd & 3rd Co-ordination meeting	12/12/21 & 02.01. 22 BARC, Dhaka	Molecular identification of different isolates and Characterization of different enzymes (protease, cellulase and phytase) activity done
7) 4th Coordination meeting	08/10/22 BARC, Dhaka	Finalized PCR workshop plan
8) Workshop on PCR preparation	11/05/22 BARC, Dhaka	Orientation guidelines improves the knowledge of PCR writing
9) Progress meeting on zoom platform	02.05.2021, Virtual platform	Incorporated all review suggestions of PIU-NATP

Consulting meetings, workshops, seminars, coordination meetings etc	Date & Venue	Output
10)Draft PCR review workshop	25.10.2022	Improved the PCR draft
Component 2 (NSTU)		
1)Inception report	22.12.2019; BARC, Dhaka	All the four recommendations of the meeting incorporated in the PP document and upgraded
2)1st Co-ordination meeting	07/09/2020; BARC, Dhaka	Recommendations on culture trials followed properly
3)Progress monitoring workshop	7.9.2020; BARC, Dhaka	Attended
4)1st Annual progress review workshop	30.9.2020; BARC. Dhaka	Suggested new method of culture trial improved the experiment condition
5)Meeting with World bank representative	25.8.2021; BARC, Dhaka	Sub-project revision along with activity time plan
6)2nd Annual progress review workshop	23.11.2021; BARC, Dhaka	Selection of animals and fatty acid analysis done in time
7)2nd & 3rd Co-ordination meeting	12/12/21; BARC, Dhaka	Revised activity timeframe developed; Updating and correction os bi-annual reports done.
8)Progress meeting on zoom platform	02.05.2021, Virtual platform	Incorporated all review suggestions of PIU-NATP
9)Workshop on PCR preparation	11.05.2022; BARC, Dhaka	Orientation guidelines improves the knowledge of PCR writing
10)4th Coordination meeting	08/10/22 BARC. Dhaka	PCR workshop implementation plan finalized
11)Draft PCR review workshop	25.10.2022	Improved the PCR draft
Component 3(BFRI)		
1) Inception workshop	22/12/2019; BARC, Dhaka	Expected output and outcomes and logical frame work of the PP document included
2)1st Co-ordination meeting	07/09/2020; BARC, Dhaka	Procurement delayed because of Covid-19 pandemic; However, the problem was overcoming in the post-Covid period;
3)1st Annual progress workshop	30/09/2020; BARC, Dhaka	Research condition improved
4)Meeting with World bank representative	25/08/2021; Virtual flatform,	Sub-project revision along with activity time plan

Consulting meetings, workshops, seminars, coordination meetings etc	Date & Venue	Output
5)2nd Annual progress workshop	23/11/2021; BARC. Dhaka	Revision of objectives accelerated the research works
6)2nd & 3rd Co-ordination meeting	12/12/21, 02.01.22; BARC, Dhaka	Revision of proposal prepared and submitted to BARC-EC; Discussed sub-project deviations corrections.
7)Workshop on PCR preparation	11/05/22; BARC. Dhaka	Orientation guidelines improves the knowledge of PCR writing
8)4th Coordination meeting	08/10/22 BARC. Dhaka	PCR workshop implementation plan finalized
9)Progress meeting on zoom platform	02.05.2021, Virtual platform	Incorporated all review suggestions of PIU-NATP
10)Draft PCR review workshop	25.10.2022	Adaption of recommendations of the workshop helped to improved the PCR draft

ii. Information on field monitoring (combined)

Date	No. of visit	Name/s of visitors	Addresses	Output
Component 1 (BSMRAU)				
23.02.2020	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	As per advice new sources of microbes were taken as potential source
21.09.2021	01	1. Dr Md Sirazul Islam 2. Md Asaduzzaman 3. Munshi Mamunur Rahman 4. Md Abdur Rahman	Team members from Department of Fisheries Biology and Aquatic Environment, BSMRAU	Monitoring of the field work done

Date	No. of visit	Name/s of visitors	Addresses	Output
27.12.2021	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	Procedural system of lab operation for bioactive enzyme research enhanced
Component 2 (NSTU)				
10. 02. 2019	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	Suggested experimental design accelerated research work
19. 12. 2020	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	Suggested Dark:Light condition was maintained
11.08. 2021	01	Dr. Saleh Uddin Ahmed	Consultant PBRG sub project (Fisheries),BARC	Suggested feeding experiment was followed
20.01. 2021	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	Salinity experiment was developed
30 January 2022	01	Dr. Harunur Rashid Dr. Mohammad Abdullah Al Faroque Md. Ashequr Rahman	Director PIU, NATP-II, BARC	Financial and technical review was corrected upon comments.
26-27.02. 2022	01	Dr. Harunur Rashid Dr. Mohammad Abdullah Al Faroque	Director PIU, NATP-II, BARC	Overall experimental procedures were monitored.
05/03.2022	01	Dr. Saleh Uddin Ahmed	Consultant PBRG sub project (Fisheries),BARC	Rearrangement of indoor culture system of polychaetes and field culture of shellfish/mud crab started
25.03.2022	01	Dipok Qumar Md. Hasan Mahmud	Monitoring Expert PIU, NATP-II,	Video clip was prepared as per suggestion

Date	No. of visit	Name/s of visitors	Addresses	Output
			BARC	
Component 3 (BFRI)				
20.04. 2021	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	Financial and technical review was corrected upon comments
17.05.2021	01	Dipok Qumar Md. Hasan Mahmud	Monitoring Expert PIU, NATP-II, BARC	Video clip preparation initiated
10.07.2021	01	Dr. Harunur Rashid Dr. Mohammad Abdullah Al Faroque	Director.PIU, NATP-II, BARC	Overall experimental procedures were monitored
27.10.2021	01	Dr. Saleh Uddin Ahmed and Sayed Lutfur Rahman	Consultant PBRG sub project (Fish), BARC & CSO, BS-BFRI	Lab activities and procurement process reviewed and suggestion complied
13.12.2021	01	Dr. Yahia Mahmud	Director General BFRI, Mymensing	Overall suggestion on activities under the sub project help to speed up research work
20.01. 2022	01	Dr. Md. Monirul Islam	Coordinator PBRG sub project And MD(Fisheries) BARC	Suggested guidelines followed and revision of objectives
28.01.2022	01	Dr. Saleh Uddin Ahmed	Consultant PBRG sub project (Fisheries), BARC	Coordination of activities improved

iii. Weather data, flood/salinity/drought level (if applicable) and natural calamities:

Component 1 (BSMRAU): *Not applicable*

Component 2 (NSTU)

Average three years weather information of Cox'sBazar region (2019 -2021)

Parameters	Seasons						Remarks
	Pre-Monson (January – April)		Monson (May – August)		Post Monson (Sept – December)		
	Max	Min	Max	Min	Max	Min	
Av. Rainfall(mm)	146	6	370	106	145	7	
Av. Temperature (OC)	32.32	14.60	36.44	28.63	31.67	16.20	
Av. Humidity (%)	80.66	60.65	84.23	86.89	89.29	77.30	
Flood (year & category)							Not recorded
Av. Salinity (ppt)	31.6	16.3	27.4	15.7	23.0	16.5	
Natural calamity (Frequency & category)							Not recorded

Ref. Regional meteorological Department, Khulna zone

Average three years weather information of Satkhira region (2019 -2021)

Parameters	Seasons						Remarks
	Pre-Monson (January – April)		Monson (May – August)		Post Monson (Sept – December)		
	Max	Min	Max	Min	Max	Min	
Av. Rainfall(mm)	130	6	310	156	200	9	
Av. Temperature (OC)	33.41	13.13	37.22	27.65	32.63	16.3	
Av. Humidity (%)	76.66	70.66	85.44	76.73	82.73	75.80	
Flood (year & category)			01		01		Mild flood due to river overflow
Av. Salinity (ppt)	22.6	4.5	24.8	0.0	21.7	0	
Natural calamity (Frequency & category)							Occasional costal storms

Ref. Regional meteorological Department, Khulna zone

Component 3 (BFRI)

Average three years weather information of Bagertat region (2019 -2021)

Parameters	Seasons						Remarks
	Pre-Monson (January – April)		Monson (May – August)		Post Monson (Sept – December)		
	Max	Min	Max	Min	Max	Min	
Av. Rainfall(mm)	136	1	300	146	187	5	
Av. Temperature (OC)	34.43	12.63	35.04	25.63	33.67	15.1	
Av. Humidity (%)	77.67	70.67	86.00	78.00	84.33	78.33	
Flood (year & category)			01				
Av. Salinity (ppt)	23.67	4.00	25.00	0.00	23.00	0.00	
Natural calamity (Frequency & category)							Occasional costal storms

Source: *Statistical Year Book of Bangladesh. Ministry of Planning (2019 -2022)*

J. Sub-project auditing (covers all types of audits performed):

Coordination component (BARC)

Types of audit	Major observation/ issues/ objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial audit by FAPAD for the year 2019- 20 on 09-12-2020	No objection raised, found all relevant documents updated as per guideline	30917.50	Financial management of the component found running smoothly till the end of the project No query or objection raised at any stage of operation by the audit teams	Financial management & project performance found satisfactory in all the audit cases
Financial audit by FAPAD for the year 2020- 21 on 11-10-2021		135258.00		
Financial audit by FAPAD for the year 2021- 22 on 17-10-22		604312.00		

Component 1 (BSMRAU)

Types of audits	Major observation/ issues/objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial audit by FAPAD for the year 2019-2020	No objections raised	1741426.00	No query or objection raised at any stage of operation by the audit teams	Financial management & project performance found satisfactory
Financial audit by FAPAD for the year 2020-2021		5000000.00		
Financial audit by FAPAD for the July'21– June '22		3515556.00		

Component 2 (NSTU)

Types of audits	Major observation/ issues/ objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial audit by FAPAD for the year 2019-20	No objection raised	4209731.00	No query or objection raised at any stage of operation by the audit teams	Financial management & project performance found satisfactory
Financial audit by FAPAD for the year 2020-21		4448368.00		
Audit Farm: M.I Chowdhury & Co. for the year 2020-21		4448368.00		
Financial audit by FAPAD from July'21– Jun'22		4568239.00		

Component 3 (BFRI)

Types of audits	Major observation/ issues/ objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial audit by FAPAD for the year 2019-20	No objection raised, found all relevant documents updated as per guideline	783584.00	Financial management of the component found satisfactory. No query or objection raised at any stage of operation by the audit teams.	Financial management & project performance found satisfactory
Financial audit by FAPAD for the year 2020-21		1533829.00		
Financial audit by FAPAD from July'2021- Jun'22		2106957.00		

K. Lessons learned

i. Screening of exogenous enzyme producing microbes, production and fermentation of plant feed materials;

ii. Commercial farming of polychaetes particularly of highly omega-3 rich fatty acid species, *Namalycastis* sp. could be one of the best alternative feed supplements for high production of mud crab;

iii. Detailed studies on the breeding behavior of *Namalycastis* sp. will lead a new horizon in polychaete farming in Bangladesh;

iv. Noticeable natural decrease of marine polychaetes often hampered our experiment;

v. Indiscriminate harvest of wild polychaetes regularly applied to shrimp hatcheries in the Cox's bazar areas should be stopped for future ecological restoration;

vi. Specific primer base detection process may not be appropriate for isolating bacteria;

vii. Preservation and storage of different bacteria may be required special attention and steps to take down;

L. Challenges (if any)

i. Fishing bans disrupted research data collection from the nature;

ii. Intensive field work even procurement process delayed the project work due to COVID-19;

iii. It was difficult tasks to send samples to foreign laboratories for analysis and also there was no available credible operational laboratory in Bangladesh for omega-3 fatty acid analysis during Corona pandamic;

iv. Dealing with local people, fishers and other stakeholders was very challenging work.

v. Daily mobilization to the very remote muddy areas by speed boat for polychaete collection was very expensive and risky job;

vi. Reagents are costly and sometimes rarely available;

M. Suggestions for future planning (if any)

i. Exogenous enzyme production from other sources like fungi, yeast etc to be explored.

ii. Further research is needed for carrying out molecular cloning and over expression of gene of interest (enzyme producing gene) from bacteria for maximizing enzyme production.

iii. Optimization of fermentation procedure of different plant based feed-stuffs by enzyme releasing bacteria and inclusion level during the feed preparation need to be accomplished.

iv. Present research on polychaete, is pioneer in Bangladesh and its outputs determines that polychaete, *Namalycastis* sp. is very rich in omega-3 fatty acid (even higher than the same species in other regions) and its causal factors behind its growth have been optimized already. Therefore, further long-term studies particularly with optimization of their feeds and breeding behavior will help honestly for commercial polychaete farming first time in Bangladesh.

v. This work under the component aimed with the alternative feed development was limited with mud crab due to time limitation and budget. Shrimp hatcheries are facing severe problems for quality feeds for which it is crying need to redo this research with brood shrimp and shrimp larvae. Therefore, this work needs to be continued on priority basis.

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Signature of the Coordinator	**Counter signature of the Head of the organization/authorized representative**
	
(Dr. Mohammad Rafiqul Islam) Date: 20.12.2022 ***Director (Nutrition)*** ***Bangladesh Agricultural Research Council***	***(Dr. Shaikh Mohammad Bokhtiar)*** Date: 20.12.2022 ***Executive Chairman*** ***Bangladesh Agricultural Research Council***

Annexures

1. Coordination component (BARC)

Annexure – BARC: A

Recommendation of the inception workshop and status of action taken

Recommendations	Action Taken
Component 1 (BSMRAU)	
No specific comment made;	
Component 2 (NSTU)	
• Detail evaluation of nutritional status of fatty acid profile in the available polychaetes should be done particularly while selecting the species;	Complied as per suggestion
• Mostly two factors like bio-conversion mechanism and PUFA preparation ability of polychaetes should be given priority;	Recommendation followed
• Qualitative analysis of Omega-3 fatty acid should be explained properly;	Done as per requirement
• Methodology for objective no-3 should clearly mention the feeds to be provided in the respective culture system;	Necessary modification done
Component 3 (BFRI)	
• Expected output and outcomes and logical frame work not included in the presentation. The component PP should include all those properly;	Followed in the next presentations

Annexure – BARC:B

Recommendation of the half yearly workshops

Recommendations of the first half yearly workshop	Action taken
General comment	
• Baseline survey informatin are yet completed by any of the component. Initially emphasize should be given for a completed baseline report development.	Baseline survey report presented in the next progress revieqw meeting
• Preparation and submission of a procurement plan of the respective year by all components are urgently needed	Complied accordingly
Recommendations of the second half yearly workshop	
General comment	
• Data on available fish species in the baor water, their bioloy, breeding, growth survival and management practices should be properly reviewed and compare with the available literature and secondary data.	Recommendation followed accordingly

Annexure – BARC: C

Recommendation of the Annual workshops

Recommendations of the First Annual Workshop	**Action taken**
Component 1 (BSMRAU)	
• Sidal sutki as fermented products may be taken for trials as potential source for indigenous microorganisms for isolating bioactivity targeted exozymes;	Necessary attempt taken
Component 2 (NSTU)	
• Suggested more trials on effect of different photoperiods on the growth and breeding performance of the animal;	Observation recorded under suggested trial condition
• Observation should be made on properties of sands and depth of sand layer in aquarium culture of the animals;	Observation recorded
• Sand samples from the naturally available areas of animals (e.g. Satkhira coastal area) may be tested for culture performance trials;	Complied
• A qualitative assessment of the collected animals from two different coastal regions is important for selecting the best quality animal. But it is still not done. As the result will determine the particular species to be selected for culture and breeding, therefore, more attention should be given on qualitative assessment of the collected animals;	Done later in time as per revised plan
Component 3 (BFRI)	
• Progress of the sub project component is still in initial stage. Monovalent efficacy of *B. subtilis* as probiotic candidate test is still under process. Completion of the above experiment along with the experiment for bivalent efficacy and in-vivo challenge test for antagonistic effect of *B. subtilis* against *V. parahaemolyticus* and immune response of shrimp should be started immediately as per prescribed design;	Necessary attempts taken
Recommendations of the Second Annual Workshop	**Action taken**
Component 1 (BSMRAU)	
• As per revised work plan of the sub-project, it is suggested to give more emphasize to complete the following activities within the project extension period. Activities are: • Completion of molecular identification of different isolates;	Completed all suggested

• Completion of optimization and purification of enzymes produced from the target isolates which has already been screened for protease, cellulose and phytase. • Completion of characterization of different enzymes (protease, cellulose and phytase)	works timely as per revised plan of activity
Component 2 (NSTU)	
• Though activities on collection of live polychaets and their identification (under NSTU component) completed so far. Partial activities on qualitative assessment of the animal also completed locally. It is re-emphasizing to complete the fatty acid profile analysis reports from the accredited labs immediately and compare the respective data for the species and finally select the appropriate animal to be put under culture program.	Fatty acid analysis done properly and animal selection completed in time.
• It is also suggested to complete all sorts of facilities and arrangements for indoor and field culture trails of the animal, meanwhile.	Timely completion was not possible due to Covid-19. But complete later.
Component 3 (BFRI)	
• Under the activities of component 3 (BFRI), construction of recombinant plasmid to assess the bivalent efficacy of Bacillus sp. hampered due to prolong lockdown situation worldwide and unavailability of the required plasmid vector. Therefore, the house suggested to evaluate the efficacy of the locally isolated probiotics as initial experiment showed better result of locally isolated bacillus against disease outbreak. A proposal should be initiated by the respective PI through proper channel seeking approval of the revised sub-project component specific objective.	Proposal placed as per recommendation and was finally approved by the EC BARC

Annexure – BARC:D

Recommendation of the Coordination meetings

Centrally organized two (1st & 2nd) virtual Coordination meeting at BARC	
Recommendations	**Action taken**
Component 1 (BSMRAU)	
• So far, 20 bacteria has been screened and colony developed and studied. Isolation, optimization, characterization and purification of the identified enzyme Cellulase is under process. Isolation and screening of all other categories of enzymes (Protease, Cellulase and Phytase) from various potential sources of bacteria will continue for next 3-4 months which should be followed by experiments on	Complied as per recommendation

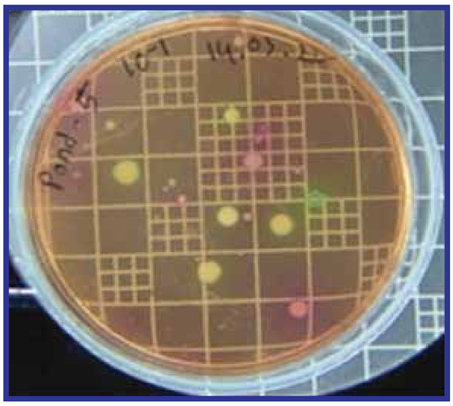
commercial application of the isolated exoenzymes within the next 2-3 months	
Component 2 (NSTU)	
• Culture trials with the polycheate species collected from Munsigonj, Satkhira region suggested to continue with special emphasize to identify the suitability of media property, stocking and feeding so that the findings will guide to apply proper method for final attempt of culture of quality grade polycheates after identification of amino acids properties;	Recommendations followed properly
• In case of denaturation of amino acid properties due to long time preservation after isolating from polycheate body, due to present unavailability of laboratory facilities (due to Covid- 19), new samples are to be provided from the same sources when analysis done next (if required);	Analysis was done on new samples collected
• Continuous liaison with the potential recognized laboratories of the country or abroad for analyzing of Omega-3 fatty acids suggested;	Maintained liaison
• Immediate attempt for identify the available polycheates species in the marine waters of south east region (Coz'sBazar) should be taken by this month;	Completed timely as per plan
• Remaining work of the project on growth performance of shrimp/fish using formulated supplementary feed from polychaetes is not possible at this stage before identification of Omega-3 fatty acid property. Therefore, it is important to complete all the previous works under objective 1-3 within October'20. Formulated feed development and growth performance of fish/shrimp will have to be completed within the next 3 months of that period;	New revised plan was developed and the activities were completed as per plan
Component 3 (BFRI)	
• Delay floating of procurement process due to late approval of revised procurement plan hangs the laboratory activities due to shortage of chemicals and other supporting materials which is also related with the experiments animals under test (*P. monodon*) and its development stage. If not procurement of goods completed by July'20' than it is advised to take another attempt for animal culture and growth before coming winter (August-November,20) and complete the due research next as per plan. No work should be left for next year;	Attempt was taken accordingly

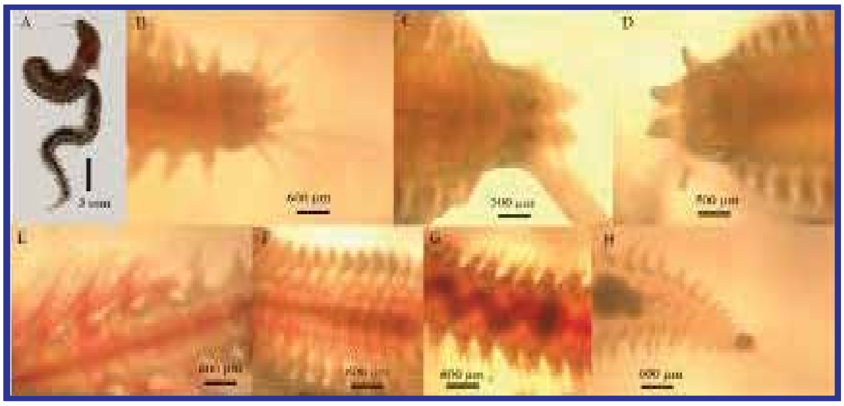
3rd Coordination meeting	
Component 1 (BSMRAU)	
• Activities on molecular identification of different isolates; • Optimization and purification of enzyme produced from target isolates which has already been screened for protease, cellulase and phytase; • Characterization of different enzymes (protease, cellulase and phytase) activity;	Completed all suggested works intime
Component 2 (NSTU)	
• Emphasize to complete the qualitative study of the selected worms followed by culture attempts under different conditions and designs (such as, density dependent growth and survival of *Namalycastis* sp.; growth and survival of *Namalycastis* sp. in variable salinity; survival and growth of *Namalycastis* sp. in three formulated feeds and setting up polychaetes culture in natural systems by circulating open water and perform culture for the supplementary diets of shrimp/fish.	Done under revised activity plan
Component 3 (BFRI)	
• Due to some technical reasons, progress of the Component 3 (BFRI) is still far behind the activity plan of the component. The component will get about seven months working period for the rest of the activities, but, unfortunately, the procurement of desired plasmid vector pHT43 will not be possible due to its unavailability in the international market which will hamper the total activity of the component. Therefore, further continuation of the research activity in the proposed form or in other form may require consent/approval of the respective authority.	Proposal done and work continued as per new plan
4th Coordination meeting on organizing of PCR review workshop	
• Decision was taken to organize the two days workshop by the Coordination component within the first half of Oct'22; • The sub-project will be presented by Prof. Dr. S. M. Rafiquzzaman of BSMRAU, Principal Investigator on behalf of the other sub-project components; • Coordination component will prepare a list of expert members, general participants, session Chairman, Chief guest/Special guest along with a draft copy of workshop program; • The two days workshop will be held in the auditorium of the Training building of BARC. Coordination component will ensure the proposed venue;	PCR workshop held smoothly on 24-25 Oct'22 under the organizership of the Coordination component following all the instruction of the respective meeting

Annexure – BARC:E

Recommendation of the draft PCR review workshop

General comment	**Action taken**
• Updating of financial, procurement and audit reports of all the sub-project components including the Co-ordination component	Complied
Component 1 (BSMRAU)	
• Under key findings of research highlight, indicate some beneficiary enzymes from the study	Included
• Indicate the short-term outcomes of objective 1 & 2	Shown in respective place
Component 2 (NSTU)	
• Culture and trials are to be shown in details in case of indoor culture of polychaetes	Details are included in the main text
• Similarly, crab culture through applying of commercial feed and polycheate mixed feed trials and findings should be focused more clearly in the report	
• Avoid showing of segmentwise summary box in the report	Follwed
Component 3(BFRI)	
• Present the key findings of BFRI under research highlight as bullet points.	Necessary correction done





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