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Colonization of enzymatic bacterial flora in biofloc grown shrimp *Penaeus vannamei* and evaluation of their beneficial effect

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Abstract

Experiments were conducted to explore the colonization of beneficial bacteria in shrimp *Penaeus vannamei* grown in different sources of biofloc and clear water. Beneficial effect in terms of extracellular enzyme production and antibiofilm activity of the isolated strains was determined. Heterotrophic bacterial population were isolated by using different agar plates and resulted in isolation of 94 isolates in total. Extracellular enzyme production such as amylase, protease, lipase, cellulase, xylanase, and pectinase were screened. Antibiofilm activity of culture supernatants of enzymatic strains against pathogenic *Vibrio* was also determined. Out of 94 strains screened, 36 strains were found to produce amylase enzyme, 20 strains protease, 27 strains lipase, 6 strains cellulase, and 8 strains xylanase. Totally, 21 isolates selected for further identification and different species of *Cobetia*, *Exiguobacterium*, *Bacillus*, *Marinilactibacillus*, *Staphyllococcus*, and *Novosphingobium* genera from biofloc treatments were identified. In control group animals, strains of *Bacillus* and *Exiguobacterium* were isolated and identified. The genus *Exiguobacterium* was found common in all the different treatments and control. The result showed that shrimp grown on biofloc system allows colonizing more beneficial bacteria in gut than control. Few promising strains under *Bacillus* genus were found to produce all the extracellular enzymes along with antibiofilm activity.

Keywords Biofloc · Shrimp · Enzymatic bacteria · Protease · Amylase · Lipase

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Introduction

Aquaculture is a rapidly growing food-producing sector increasing fast to meet the global fish demand and it was estimated reaching the 140 Mt. tons in 2050 from 67 Mt. tons in 2012.This development brings to fulfill the animal protein consumption and enhance the employment opportunities and income generation to the world especially developing countries. (Waite et al. 2014). However, this rapid growth faces a lot of environmental issues mainly generating waste due to unconsumed feed, excretory materials by candidate animals, antibiotic residues leading to the deposition of nitrogenous pollutants, and other toxic materials to the water bodies. Photoautotrophic culture system and regular water exchange will be useful to avoid such pollution commonly (Crab et al. 2010). But water exchange is one of the major concerns for biosecurity and limiting its usage because of water scarcity and conservation issues (Hargreaves 2006; Emerenciano et al. 2012).

Biofloc technology, a new approach popularly adopted in aquaculture, enables to overcome above said problem because it works in minimal or zero water exchange. This is a microbial-based nutrient utilization technology requiring the addition of carbon source to the aquaculture medium to raise the C/N ratio which is subsequently utilized by the microbial consortium (Avnimelech 1999; Crab et al. 2010; Suryakumar and Avnimelech 2017; Panigrahi et al. 2019a, b). The carbon sources normally used are rice bran, molasses, tapioca, and millets. The heterotrophic bacteria forms aggregates with this nutrients and serves as additional nutrient sources for candidate species; moreover, this carbon sources are cost-effective and this technology significantly reduced the feed cost to the farmers (Crab et al. 2009, 2010; Panigrahi et al. 2019c). These microbial aggregates with the carbon sources improve the water quality of the production system.

Ammonia generated from the unutilized feed, excretions, and other inclusions from aquaculture system leads to poor water quality and creates toxic environment for the shrimp. The microbial consortium along with nitrifying bacteria enables the waste nutrient recycling by utilizing the ammonia for cellular protein production (Ebeling et al. 2006; Ray et al. 2011; Panigrahi et al. 2018). Improving water quality and nutrient recycling enables the cultured shrimp free from stress and diseases and allows intensification. This technology widely applied now in shrimp culture system and observed improved results than the clear water culture system. For example, the culture of *Farfantepenaeus brasiliensis* in biofloc system improved the growth performance and water quality than shrimp cultured in clear water system. In addition, the growth was almost same in shrimp cultured with or without the commercial feed drawing great attention to this technology (Emerenciano et al. 2012). Different carbon sources used in this technology proved to improve the immune status and survival of the shrimp (Panigrahi et al. 2019c) and elevated phenol oxidase activity, respiratory burst activity, and other immune responses (Ekasari et al. 2014; Panigrahi et al. 2018). Studies related to the growth enhancement and microbial colonization in the host will improve the concept of the mechanism of biofloc study for better understanding. It was observed that bacterial community from water and intestine of shrimp *Litopenaeus stylirostris* greatly varied from clear water to biofloc medium and found abundance in biofloc system. This is because the organic nutrient in rich biofloc system enables the colonization (Cardona et al. 2016). The intestinal colonization of beneficial microbes is very important, as it plays an important role in nutrient digestion, prevention of pathogenic invasion, and upregulation of immune system by the host (Nicholson et al. 2012; Panigrahi et al. 2005). Beneficial effects by microbes are categorized mostly by the production of extracellular enzymes (protease, amylase, lipase, cellulase, etc.) and production

of antimicrobial molecules against invasive pathogens. This enzyme producing potential and probiotic effect of these isolates could be useful for the optimized production of healthy shrimps in the future (Tzuc et al. 2014). Considering the importance of the above said points, the present study was conducted to explore the characteristics of beneficial bacteria and their colonization in shrimp *Penaeus vannamei* cultured in the biofloc system developed with different nutrient sources.

Materials and methods

Chemicals

All analytical chemicals, culture media DNA isolation, and PCR product purification kits were purchased from Himedia (India). Primers were purchased from Sigma (India) and plasticwares were purchased from Tarson (India).

Experiment design

A 30-day experiment was conducted to study their beneficial effects of colonization bacteria on shrimp *Penaeus vannamei* larval culture on Biofloc and clear water system. The experiment was carried out in 1-ton fiber reinforced plastic tanks (FRP) and 3500.nos *P. vannamei* post larvae III stage was stocked in triplicate. The experimental variations as follows, treatment 1 (Rice bran, molasses, ragi, wheat, branless wheat, and rice flour.), treatment 2 (probiotics only), treatment 3 (sugar), treatment 4 (Rice bran, molasses, ragi, wheat, branless wheat and rice flour.), and control (no biofloc), were conducted. Carbon sources of treatments 1 and 3 were respectively mixed with autoclaved sea water (1 l) and fermented overnight with microbial consortium (mixture of certain *Bacillus* bacterial strains; isolated from the previous biofloc culture (*Bacillus subtilis, Bacillus licheniformis, Oceanobacillus,* and *Bacillus halosaccharomyces)*, in the concentration of 1×10^6 CFU/ml). In treatment 2, only microbial consortium was mixed in sterile water and in treatment 4, carbon sources mixed in seawater and kept overnight without microbial consortium. After overnight incubation the contents filtered in algal mesh and mixed in respective tanks. Before starting the experiment, seawater (35 ppt) was filled in all treatment and control tanks initially and average water temperature recorded was 30 ± 3 °C. Tanks were bio-fertilized in all the treatment and control tanks to develop bloom or primary producers.

Experimental diet

The experimental diet was procured from Inve feed pvt Ltd., Thailand. The experimental animals were fed five times daily at 06:00, 10:00, 14:00, 18:00, and 22:00 h initially for 2 weeks @ 20% of body weight which declined gradually to 5% towards the end of the experiment. Experimental diet with 38–40% crude protein was used and manipulation of C/N ratio was followed by our previous study (Panigrahi et al. 2018).

Isolation of heterotrophic bacteria

Sampling was done at 10-day interval and 10 larvae from each treatment were collected and abdominal part of the larvae was macerated aseptically with saline water then serially diluted.

Heterotrophic bacteria were isolated by using Zobell marine agar (32 °C; 24 h). Lactic acid bacteria, *Bacillus* strains, and presence of *Vibrio* were enumerated respectively using MRS agar (32 °C; 48 h), Hichrome *Bacillus* agar and TCBS agar (32 °C; 24 h). After enumeration, colonies with different morphology were picked and stored in 4 °C for further study.

Beneficial effect of isolated strains

The beneficial effect of isolated strains was analyzed in terms of extracellular enzyme production and antibiofilm activity against pathogenic *Vibrio* sp.

Extracellular enzyme production activity

Extracellular enzyme production by isolated strains was analyzed by using specific agar plates (Banerjee and Ghosh 2014). Amylase production by isolated strains was screened by using starch agar (meat extract, 0.3%; peptone, 0.5%; starch soluble, 0.2; Agar, 1.5%). The isolated strains were streaked into the starch agar plates and incubated for 24 h and after that, the plates were flooded with iodine solution for observing zone formation. For extracellular protease activity, the colonies were streaked into skim milk agar plates (skim milk powder, 2.8%; tryptone, 0.5%; yeast extract, 0.25%; dextrose, 0.1%; agar, 1.5%) and incubated for 24 h and the results were observed with regard to zone formation after incubation. Lipase activity was observed by streaking the colonies in spirit blue agar plates (casein enzymatic hydrolysate, 1%; yeast extract, 0.5%; spirit blue, 0.015;agar, 1.7%) supplied with tributyrin (1%).The respective colonies were streaked on this plates and zone of clearance was observed after 24–48 h of incubation. Cellulase activity and xylanase activity was screened by using the following media (%); yeast extract-3.0, sodium nitrate-0.2, KH_2PO_4-0.1, $MgSO_4{\cdot}7H_2O$-0.003 Agar-2. In this media, 1% carboxymethyl cellulose and 0.1% beechwood xylan were respectively added for cellulase and xylanase. The isolated colonies were streaked on the plates and incubated for 24–48 h. After incubation, the plates are flooded with Congo red solution (100 mg/100 ml) and kept for 15 min. The solution drained off, and plates washed with distilled water. Cellulase and xylanase positive colonies produce clear zone around it. For pectinase activity, the colonies were streaked into following agar medium (%); apple pomace pectin - 0.5, peptone - 0.2, KH_2PO_4- 0.05, $MgSO_4$ - 0.025, sodium nitrate - 0.1, and agar - 1.5. The isolated colonies were streaked on the plates and incubated for 24–48 h. After incubation, the plates were flooded with iodine solution and kept for 15 min. After that the iodine drained off and pectinase positive colonies produce clear zone around it (Supplementary figure 1a-1d).

Antibiofilm activity

Microtiter-plate assay was used for the estimation the antibiofilm activity of culture supernatants of isolated strains (Yuvaraj and Arul 2014). A loopful of different isolates were inoculated in Zobell marine broth and kept in shaker (150 rpm) for 3 days at 32 °C. Then, it was centrifuged at 10,000 rpm and the supernatant was used to check the antibiofilm activity. The pathogenic strain selected was *Vibrio* strain LB4 previously isolated from diseased shrimp culture ponds. In 96 well plates, individual wells were filled with sterile nutrient broth and were inoculated with 1 μl of overnight grown pathogenic *Vibrio* LB4. Then, the wells were added with crude supernatant of isolates with the concentration of 25 μl, 50 μl, and 75 μl and the total volume made to 200 μl in each well with sterile broth. Wells with only *Vibrio* LB4

culture were used as a control. After incubation (32 °C for 24 h), the contents were removed and washed with phosphate buffer saline (PBS pH 7.2). The adherence of the sessile bacteria was fixed (99% methanol; 15 min). Further stained with crystal violet (0.1% *w*/*v*) and washed with deionized water then ethanol (99%). After that, glacial acetic acid (100 μl) was added and plates were read at 595 nm (Xie et al. 2019)

Identification

The potential enzymatic and antibiofilm activity producing strains were identified further using 16S rRNA gene amplification and sequencing (Panigrahi et al. 2005). Genomic DNA was isolated using DNA isolation kit and amplified by using forward and reverse primer (95 °C, 3 min; 95 °C, 30 s; 56 °C, 45 s; 72 °C, 60 s; 72 °C, 10 min; 30 cycles). The amplified product was purified using PCR product purification kit and sequencing was performed at Apical Scientific SdnBhd, Malaysia. Homology search and similarity findings were done by using NCBI-BLAST. Phylogeny was constructed by using MEGA 5.0 software.

Statistical analysis

One-way ANOVA was assessed to determine the significance of antibiofilm activity between different selected isolates from the treatments using SPSS version 20 (SPSS Inc., Chicago, IL, USA).

Results

The total viable count of heterotrophic bacteria on Zobell marine agar resulted that high TVC values observed in treatment 1 other treatments. TVC of 4.2×10^4, 2.0×10^4, and 1.93×10^4 respectively observed in 10, 20, and 30 days of sampling. Low TVC of 1.0×10^4, 1.75×10^4, and 1.05×10^4 were observed in treatment 2 in different days of sampling. Moderate values of TVC was observed treatment 3 (1.1×10^4, 2×10^4, and 1.93), treatment 4 (1.25×10^4, 1.75×10^4, and 1.0×10^4), and control (1.1×10^4, 2.5×10^4, and 1.5×10^4). In case of *Vibrio* control (0.9×10^2, 1.1×10^2, and 1.4×10). The Vibrio count also found in treatments but it is too low to count. There are no colonies found in MRS and Hichrome *Bacillus* agar.

After enumeration in each sampling, colonies with different morphology were picked, purified, and stored. Totally, 94 different morphologically different heterotrophic bacteria were isolated at different days of intervals and screened for extracellular enzyme activity. Highest number morphologically different colonies isolated from treatment 1 (36 isolates) followed by treatment 2 (22 isolates), treatment 4 (15 isolates). Comparatively, lower isolates found in control (11 isolates) and treatment 2 (10 isolates).

Extracellular enzyme production

Among the extracellular enzymes screened, amylase producers are predominantly found in all the treatments. Out of 94 strains screened, 36 strains were found to produce amylase enzyme. Totally, 18 amylase-producing strains are isolated from shrimp that have been grown in treatment 1. Next, highest amylase producers found in treatment 2 (11 isolates). A comparatively lower amount of amylase producers found in treatment 3 (3 isolates), 4 (1 isolate), and

control (3 isolates). Not like amylase, protease-producing strains found less in the animals grown on different treatments. Out of 94 strains screened 20 strains were found to produce protease enzyme. The highest number of 6 isolates were found to produced protease in treatment 1 followed by treatment 2 (4 isolates), treatment 3 (4 isolates) treatment 4 (2 isolates), and control (2 isolates).

Lipase activities of isolated strains from different treatments were screened and 27 isolates were found to produce lipase. Ten isolates from treatment 2 were found to produce lipase enzyme followed by treatment 1 (9 isolates). Four isolates were found in treatment 3 and each 2 isolates were found in treatment 4 and control.

Interestingly, the cellulase activity was also found in the bacterial strains isolated from shrimp grown on different treatments. Among the 94 strains, 6 were found to produce cellulase enzymes. Among the 6, 3 isolates are from treatment 1. Respectively, one isolate was found to produce cellulase from treatments 3, 4, and control. There is no cellulase producer found in treatment 2. Among the strains tested, 8 were found to produce xylanase enzyme. Five isolates were found in treatment 1 followed by treatment 2 (1 isolate), treatment 3 (1), and treatment 4 (1 isolate). Different strains isolated from animal samples of respective treatments were screened for pectinase activity and none of the strain produced pectinase.

Antibiofilm activity

The antibiofilm activity of culture extracts of selected isolates was tested against pathogenic biofilm of *Vibrio* LB4. Most of the selected isolates from different days of sampling showed very good results in inhibition of biofilm of *Vibrio*. These isolates selected for screening because of its potential enzymatic profile screened earlier. The culture extracts of bacterial isolates from treatment 1 potentially reduced the biofilm of *Vibrio* LB4 followed by treatments 2 and 3. Treatment 4 and control (Fig. 1; Supplementary Fig. 2a-c) one-way ANOVA for the antibiofilm activity of isolates in restive treatments were statistically significant ($P < 0.001$).

Molecular identification

Further, the identification of potential isolates was done by using biochemical characteristics and 16S rRNA gene amplification and sequencing. Totally, 94 strains were isolated from 5 treatments and control from three intervals of sampling and 21 strains were selected for identification based on the morphological, biochemical characteristics, enzyme screening, and antibiofilm activity, because of repeated isolates from each sampling and the sequences of the identified strains were submitted to GenBank (Accession no: MK934549-MK934569).

Among this, shrimp cultured on treatment 1 allows to colonize the different variety of bacteria in terms of beneficial effects. Totally, six different bacterial strains were isolated and their beneficial effects already mentioned in Table 1. The strains identified were phylogenetically closely related with the following strains *Cobetia marina*, *Bacillus aquamaris*, *Bacillus tequilensis*, *Exiguobacterium* sp., *Exiguobacterium aurantiacum*, and *Novosphingobium* sp. (Fig. 2). In treatment 2, there are five different strains selected and further identified and it was phylogenetically closely related to *Exiguobacterium aurantiacum*, *Marinilactibacillus piezotolerans*, *Bacillus* sp., *Exiguobacterium profundum*, and *Exiguobacterium indicum* (Supplementary Fig. 3a). There were five different strains identified from shrimp grown on treatment 3 and the blast analysis of its sequences phylogenetically closely related to *Bacillus subtilis*, *Staphylococcus haemolyticus*, *Bacillus thuringiensis*, *Exiguobacterium aurantiacum*,

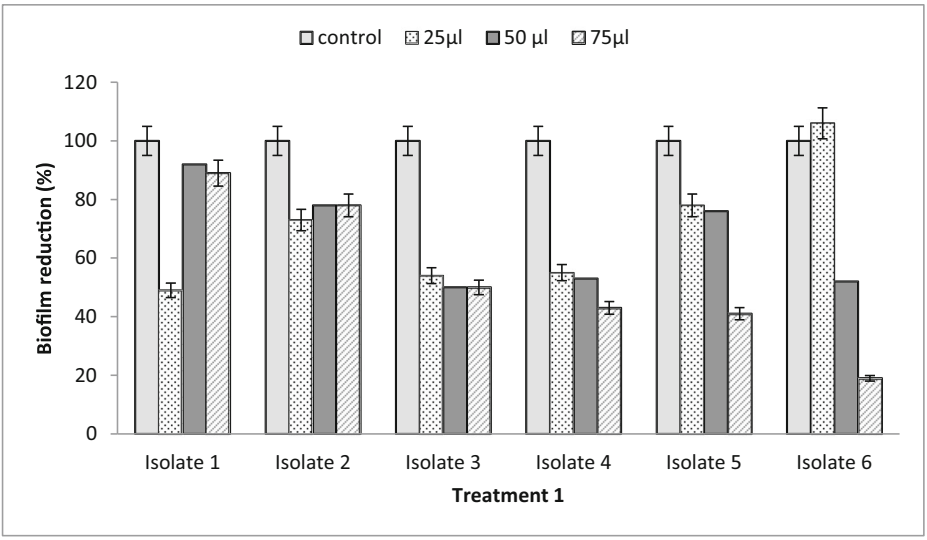

Fig. 1 Antibiofilm activity of strains isolated from treatment 1

and *Exiguobacterium* sp. (Supplementary Fig. 3b). In treatment 4, three different strains were identified and phylogenetically closely related with *Bacillus niabensis*, *Novosphingobium panipatense*, and *Exiguobacterium profundum*.(Supplementary Fig. 3c). In control, two different strains were identified and phylogenetically closely related to *Bacillus cereus* and *Exiguobacterium aurantiacum* (Supplementary Fig. 3d).

Discussion

Biofloc system is more advantageous medium made up of particulate-rich organic matter which allows the microbial utilization and subsequent nutrient source for candidate aquaculture species. This system activated mainly by the colonization of bacteria, in addition to phyto and zoo planktons (Aguilera-Rivera et al. 2014). Biofloc is generated in the culture system based on C/N ratio (Panigrahi et al. 2019a, b) and heterotrophic bacteria plays the main role in this nutrient digestion in BFT system and for this digestion, they will use extracellular enzymes. In this study, there were 94 isolates enumerated from shrimp gut grown on different sources of biofloc (83) and control (11). The strains isolated from the biofloc sources were found to produce extracellular amylase, proteases, and lipases. Extracellular cellulase activity also found mostly in bacteria isolated from biofloc system than control. Xylanase activity only found in bacteria isolated from shrimp reared in biofloc system not in control. This proves the important role of bacteria in the hydrolysis of carbohydrates, proteins, and lipids by generating essential nutrients for subsequent food for shrimp grown on biofloc based system. Microbial populations colonized in the animal intestine with extracellular enzyme activity will give potential benefits to host in terms of growth and metabolism (Tzuc et al. 2014). Fishes are popularly studied for enzymatic microflora (Saha et al. 2006; Esakkiraj et al. 2009; Banerjee et al. 2015; Liu et al. 2016), but there are not much studies in shrimp in this area (Esakkiraj et al. 2010). Bacteria colonized in animal generally related with digestion of host species and

Table 1 Enzymatic profile of selected isolates from shrimp grown on different treatments

Isolates	Amylase	Protease	Lipase	Cellulase	Xylanase
Treatment 1					
T I isolate 1	+	−	−	−	−
T I isolate 2	−	+	−	−	−
T I isolate 3	+	+	+	+	+
T I isolate 4	+	−	−	−	−
T I isolate 5	+	+	+	−	−
T I isolate 6	−	+	+	−	−
Treatment 2					
T II isolate 1	+	−	+	−	+
T II isolate 2	+	−	+	−	−
T II isolate 3	+	+	+	−	−
T II isolate 4	+	+	−	−	−
T II isolate 5	+	+	+	−	−
Treatment 3					
T III isolate 1	+	+	+	+	+
T III isolate 2	+	−	+	−	+
T III isolate 3	+	+	−	−	−
T III isolate 4	−	+	+	−	−
T III isolate 5	+	+	+	−	−
Treatment 4					
T IV isolate 1	+	−	+	+	−
T IV isolate 2	−	+	−	−	+
T IV isolate 3	−	+	+	−	+
Control					
C1	+	−	−	+	−
C2	+	+	+	−	−

the colonization of enzymatic flora greatly influenced by trophic level the of host species (Liu et al. 2016). In the same way, the present study proves biofloc system is an aggregate of rich nutrients and allows colonizing the variety of enzymatic bacterial flora in host species than the shrimp grown on clear water.

In addition to enzyme production, inhibitory activity against *Vibrio* in terms biofilm reduction was also studied and the results observed best to give a supplementary effect to shrimp health. Normally, shrimp culture systems are prone to pathogenic bacteria and subsequently have reduced survival rate (Otta et al. 2018). Biofloc system guaranteed the shrimp health and many studies proved by means of a higher survival rate (Emerenciano et al. 2012; Xu et al. 2013; Ekasari et al. 2014; Panigrahi et al. 2018, 2019b). In the present study, the isolates mostly produced extracellular enzymes which can not only act as a digestive aid but may also serve as an antibiofilm agent. Because biofilm formation is an important primary step for pathogenic attack and which is mainly composed of exopolysaccharides, proteins, lipids, and other macromolecules. Enzymes especially amylase and protease were previously reported to hydrolyse the pathogenic bacterial biofilm (Kalpana et al. 2012; Esakkiraj et al. 2016). This study also explores the possible mechanistic role of enzymatic bacteria in biofloc system for shrimp health.

In the present study, there are 21 strains selected and identified from shrimp gut grown on biofloc and clear water system. Among all, the genus *Exiguobacterium* predominantly present in all the treatments and control. In treatment 2, three different strains were isolated. *Exiguobacterium* is previously studied mainly for extracellular protease activity (Kumar and Suresh 2014; Lei et al. 2014). In the present study, in addition to protease, other enzyme-

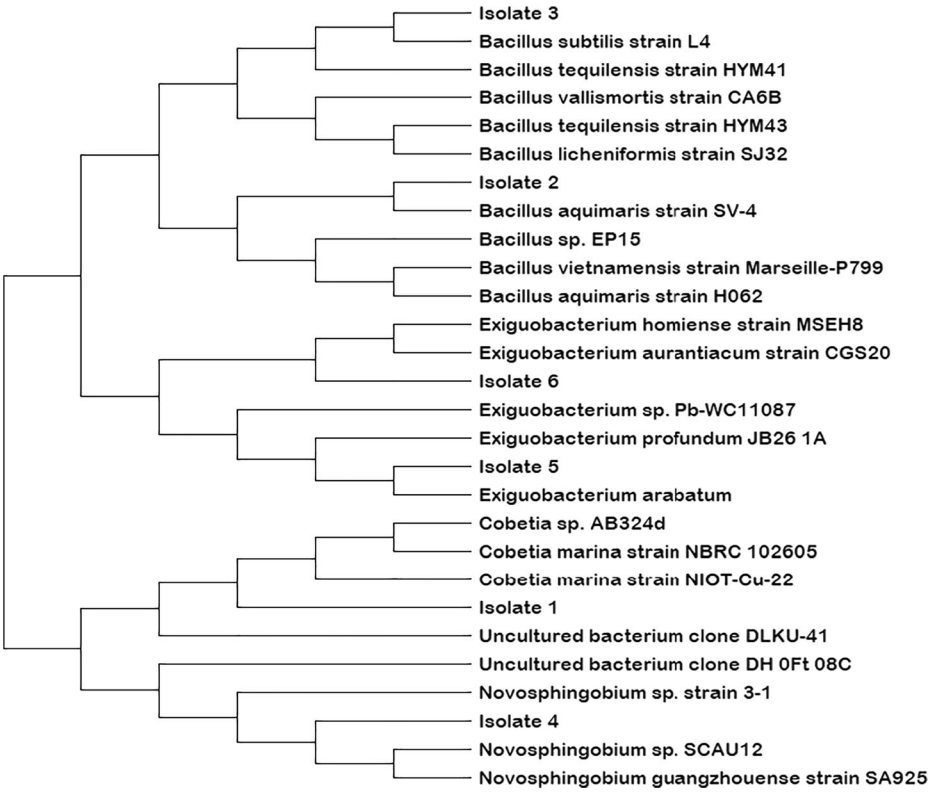

Fig. 2 Phylogenetic relationship of strains isolated from treatment 1

producing strains of *Exiguobacterium* were also isolated. Interestingly, the genus *Novosphingobium* also isolated and identified from treatments 1 and 4. These strains popularly studied for bioremediation of hydrocarbons (Yuan et al. 2009; Lyu et al. 2014). Enzyme system from *Novosphingobium* was reported to degrade lignin, a major discharged as terrigenous organic carbon into the marine environment (Ohta et al. 2015). In the present study, also *Novosphingobium* strain isolated from treatment 4 was found to produce xylanase which adds value to the biofloc system for bioremediation, because this system mainly adopted to reduce the organic discharge. These enzymatic strains have important roles in BFT system so as to utilize the input carbohydrates which in turn give better performance and immunity to the reared animals (Panigrahi et al. 2018, 2019c)

The genus *Bacillus* is common and different species of *Bacillus* were isolated from different treatments (*Bacillus aquamaris*, *Bacillus tequilensis*, *Bacillus* sp., *Bacillus niabensis* and *Bacillus subtilis*) and control (*Bacillus cereus*). Many strains of *Bacillus* are reported to produce extracellular enzymes and antagonistic compounds. In the present study, two potential *Bacillus* strains from treatment 1 and treatment 3 were isolated to produce all the enzymes except pectinase. Also, the strain *Bacillus niabensis* which is a close neighbor to *Bacillus cellulasensis* (Mawlankar et al. 2016) was isolated from treatment 4 and was found to produce cellulase.

Lactic acid-producing marine bacterium phylogenetically close with *Marinilactibacillus piezotolerans* was isolated from treatment 2. Lactic acid bacteria were widespread in the food

production industry for their probiotic effect (Nikoskelainen et al. 2003; Panigrahi et al. 2005; Balcázar et al. 2008). *Cobetia merina* is Halomonadaceae bacterium found in treatment 1. This strain was previously reported to produce bioflocculant (Ugbenyen et al. 2012; Ugbenyen and Okoh 2014). In this study, this may take a part in promoting the floc formation by yoking the nutrients. *Staphylococcus haemolyticus* was also observed in treatment 3; early reported *Staphylococcus epidermidis* from shrimp was found to produce lipase (Esakkiraj et al. 2010).

In conclusion, culturing shrimp in biofloc system has many benefits in terms of growth and health. This study proves that biofloc system allows colonizing shrimp with more of enzyme-producing and biofilm-inhibiting bacteria than the control. Also this system encourages colonizing the bioremediating bacteria such as *Novosphingobium* which will be helpful to reduce the organic discharge. Few of the strains which are isolated and identified to produce different types of enzymes and also have active antibiofilm activities could be promising as probiotic bacteria, even constituting biofloc consortium for commercial shrimp aquaculture.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The research undertaken complies with the current animal welfare laws in India. Care and treatment of the experimental animal used in this study were in accordance with the guidelines of the CPCSEA [Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment & Forests (Animal Welfare Division), Govt. of India] on care and use of animals in scientific research. The study was undertaken with approval of statutory authorities of the Central Institute of Brackishwater Aquaculture, Chennai, India. The experimental animal *Penaeus vannamei* is not an endangered shrimp, the provisions of the Govt. of India's Wildlife Protection Act of 1972 are not applicable for experiments on this fish.

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