Journal of Animal Prod.., *35*(3), https://rpa.reduc.edu.cu/index.php/rpa/article/view/e4569

aquaculture

Review article

Effects of *Bacillus* sp. as Biofloculant Bacteria in Shrimp Culture

Yudelquis Aldana Calderón *, Amílcar Arenal Cruz **, Golnaz Naderkhani **

* Shrimp Production Facility Laboratory, south coast of Granma Province (Calisur) ECCAM, Cuba.
**School of Veterinary Medicine, Saint Nicholas University, Morne Daniel, Roseau 00152, Dominica.

Correspondence: yudelquis.aldana1982@gmail.com

Received: October 2023; Accepted: November 2023; Published: November 2023; Published.

ABSTRACT

Background: Genus *Bacillus* is among the most widely studied bacteria used as probiotics in aquaculture. Besides its bioflocculant activity, it can offer other benefits, such as nitrogen recycling in the culture pond. **Aim.** To review the application of *Bacillus* in shrimp culture, emphasizing its bioflocculant effects in Biofloc systems. **Development:** *Bacillus* sp. produces a broad range of extracellular polymeric substances (EPS) and antimicrobial peptides against a variety of microorganisms, in addition to improving the immune response and growth of culture animals and disease control. The bioflocculant potential of *Bacillus* species makes these bacteria good candidates for use in *Penaeus vannamei* cultures in Bioflock systems. **Conclusions:** However, new biofloculant *Bacillus* strains must be isolated and characterized, along with the evaluation of the biofloculant effect on *Penaeus vannamei*' s metabolism and immune response.
Keywords: bacterium, bioflocculants, shrimp, culture, polymers (*Source: MESH*)

INTRODUCTION

The accelerated growth of the population brought about the development of aquaculture worldwide (Kasan *et al*., 2017), and the Pacific white shrimp *Penaeus vannamei* is the most widely cultivated crustacean species, native to the Pacific coast of Latin America, and introduced in China and other Asian countries in 1996 (Wyban, 2019). This species is a good candidate for culturing, among other factors, due to its high tolerance to salinity. However, high levels of concentrations of nitrogenated compounds may affect the health of cultivated animals and harm growth and survival (Valencia-Castañeda *et al*., 2019).

Various researchers have reported ammonium toxicity in shrimp (Valencia-Castañeda *et al*., 2018; Valencia-Castañeda *et al*., 2019; Thi *et al*., 2022), so the conversion of toxic ammonium into the non-toxic form of nitrogen is one of the most important issues in handling water quality

Citation (APA)
Aldana Calderón, Y., Arenal Cruz, A., & Naderkhani, G. (2023). Effects of Bacillus sp. as Biofloculant Bacteria in Shrimp Culture *Journal of Animal Prod*, *35*(3). https://rpa.reduc.edu.cu/index.php/rpa/article/view/e4569

in intensive culture systems (Ren *et al*., 2019). The increase in the microbial community may improve water quality by removing the toxic nitrogen compounds, whereas the microbial proteins could be used as feeds (Reis *et al.*, 2019). Bioflock is based on previous knowledge, it is environmentally friendly and relies on *in situ* microorganism production, where nutrients can be recycled continuously, and benefit from minimum or no water changes (Gustavo *et al*., 2017).

Fortunately, among the bacteria used as probiotics in aquaculture, Genus *Bacillus* is among the most widely studied bacteria; besides its biofloculant activity, it can offer other benefits, such as nitrogen recycling in the culture pond (Harun *et al*., 2018). However, new bioflocculant *Bacillus* strains must be isolated and characterized, then used as inoculants in Bioflock systems for *Penaeus vannamei* production. Additionally, the effect of bioflocculants on *Penaeus vannamei*’ s metabolism and immune response could be evaluated. Accordingly, the aim of this paper is to review the application of *Bacillus* in shrimp culture, emphasizing its bioflocculant effects in Biofloc systems.

DEVELOPMENT

The influence of bacterial communities on the health of aquatic animals is critical. The shrimp’ s gut and its aquatic setting are complex ecosystems where several bacterial communities live together, and where some microorganisms are probiotic, whereas others are pathogenic (Zheng *et al*., 2017).

The microorganisms found in biofloc play three main roles in the Bioflock technology, water maintenance by using nitrogenated compounds, as a feed source for cultivated animals, since they offer probiotic properties and compete against pathogens. Heterotroph bacteria, such as *Bacillus* sp. have a critical role in biofloc systems (Hossein and Mohammadi, 2022).

Genus *Bacillus*, its effects on shrimp culture

Among the bacteria used as probiotics in aquaculture, the Genus *Bacillus* is among the most widely studied microorganisms used in aquaculture to enhance cultivated animal growth, and to control diseases (Kewcharoen and Srisapoome, 2019; Nimrat *et al.*, 2020). Members of the genus *Bacillus* belong to phylum Firmicutes, qualifying as Gram-positive and walking stick-like spore-forming bacteria (Abu Tawila *et al*., 2018). They may be aerobic or facultative anaerobic. *Bacillus* sp. produces a broad range of extracellular substances and antimicrobial peptides against a variety of microorganisms, in addition to improving the immune response culture in animals (Niu *et al*., 2014).

Species of *Bacillus* grow efficiently in carbon and low-cost nitrogen sources. They have gained commercial relevance as producers of secondary metabolites, such as antibiotics, bioinsecticides, and enzymes. *Bacillus* enzymes are highly efficient in decomposing a great variety of carbohydrates, lipids, and proteins in small amounts. Considering these characteristics, *Bacillus* could be considered good probiotic candidates in the shrimp diet to improve the digestibility of different ingredients in the diet (Omont *et al*., 2021).

In their study, Ochoa-Solano and Olmos-Soto (2006) reported the protease, α-glucosidase, and lipase activity of *Bacillus subtilis* and *Bacillus megaterium.* strains. Meanwhile, Alsalman *et al*. (2022) reported the production of enzyme chitinase by *Bacillus salmalaya*. The results of this study showed the capacity of these bacteria to produce large amounts of chitinase in a short time, so it could degrade complex polysaccharides present in organic residues and contribute to the conservation of the environment (Alsalman *et al*., 2022)

Several researchers describe the effect of *Bacillus* strains on the immune response of culture animals. The immune system of crustaceans depends on the immune innate response, consisting of cell and humoral immune responses. The cellular response involves hemocytes that participate in phagocytosis, apoptosis, nodulation, and encapsulation. Meanwhile, humoral response depends on immune factors such as prophenoloxidase, lectins, and antimicrobial peptides (Huang *et al*., 2020).

Niu *et al*. (2014) reported the probiotic potential of the *Bacillus* sp strain LT3 in sea shrimp larvae infected with *Vibrio campbellii*, which improved their survival due to a reduction of *in vivo* activity of the pathogen and stimulation of the immune response with the activation of the prophenoloxidase system. *Bacillus* strains have been isolated and identified as having the ability to degrade N-acyl-homoserine lactones (AHLs), on+e of the *quorum-sensing* molecules involved in the regulation of virulence factor production in many pathogenic bacteria.

Ramírez *et al*. (2022) evaluated the probiotic potential of the microbial consortium holding three strains *Bacillus cereus* sensu stricto (P64), *Vibrio diabolicus* (Ili), and *Vibrio hepatarius* (P62), used to control vibriosis. The probiotic improved *P. vannamei* larval quality, previously exposed to *Vibrio parahaemolyticus.* The results indicate that the previous treatment of the larva using the probiotic limited the colonization of pathogenic *Vibrio*.

Moreover, Luna-González *et al.* (2017) found that the addition of *Bacillus licheniformis*, molasses, and the reduction of feeding rates did not affect shrimp growth and survival. The nitrogenated residues were kept within the optimum range for shrimps. *Bacillus* strains may play a relevant role in nitrification-denitrification. The reduction of the feeding rate could help maintain water quality and contribute to a reduction of costs in shrimp culture (Luna-González *et al*., 2017).

Classification of flocculants

There are three types of flocculants: inorganic, like aluminum sulfate and aluminum chloride; synthetic inorganic flocculants such as polyacrylamide derivatives and polyethylamines; and natural flocculants, like the ones produced by microbes. Inorganic and organic flocculants are used frequently in industrial processes due to their high activity and low cost. However, studies have demonstrated that organic and inorganic flocculant substances can harm health and the environment. In that sense, the biopolymers produced by microorganisms are being studied today, since they are biodegradable and safe for humans and the environment (Wandong *et al*., 2021).

Bacteria are the main component of Biofloc, reported as bioflocculant microorganisms that produce biopolymers, substances that can flocculate suspended solids. These biopolymers are known as extracellular polymeric substances (EPS) produced by microorganisms when growing, which have a critical role in flocculation (Kasan *et al*., 2017). Each bacterium produces different EPS, which differ in their ability for flocculation. Hence, bacteria with high flocculation activity are potentially used as inoculum to raise bioflocculation in biofloc systems for *P. vannamei* production (Harun *et al*., 2018).

Among the bacteria reported as bioflocculant producers, are *Bacillus* sp., *Pseudomonas alcaligenes, Citrobactor* sp., *Enterobacter cloacae, Halomonas* sp.*, and Klebsiella pneumoniae*. Microbial bioflocculants comprise polymers like cellulose, protein, glycoproteins, polysaccharides, and nucleic acids (Gosai and Narolkar, 2022). The molecular weight and functional groups of molecular chains are determinants in flocculation performed by bioflocculants. The functional groups determine the type of load, its distribution, and consequently, the type of interaction (Kushwaha *et al*., 2020).

Bacillus sp. as bioflocculant bacteria

Bacillus species produce different EPS, *B. subtilis produces* polysaccharides; *B. consortium* produces glycoproteins; *B. safensis* produces functional proteins, and some other species produce several EPS. Fortunately, among the bacteria used as probiotics in aquaculture, the Genus *Bacillus* is among the most widely studied bacteria used as probiotics in aquaculture; it can offer other benefits, such as nitrogen recycling in the culture pond (Harun *et al*., 2018).

Several authors have reported the bioflocculant potential of the genus *Bacillus* bacteria. Abu Tawila *et al*. (2018) reported the production, optimization, and characterization of bioflocculant QZ-7 synthesized by *Bacillus salmalaya strain* 139SI isolated from the soil of a farm in Malaysia. The maximum flocculant activity was 92.6%; the optimum conditions for the production of the bioflocculant were temperature above 35.5 ºC, pH 7±0.2, using sucrose and yeast extract as sources of carbon and nitrogen, respectively. The chemical analyses revealed that the bioflocculant consisted of 79.08% carbohydrates and 15.4% proteins. Bioflocculant QZ-7 was heat stable and maintained over 80% activity after being heated at 80 °C for 30 min. Besides, the effect of *Bacillus salmalaya* strain 139SI on the removal of organic matter was demonstrated, so bioflocculant QZ-7 can be a promising factor in treating residual waters.

Other results showed the optimal bioflocculation parameters in a study done by Gosai and Narolkar (2022), who used glucose and ammonium chloride as sources of carbon and nitrogen in the presence of ferric chloride cation and pH = 8. The chemical analysis of the bioflocculant showed a protein content of 2.81 mg/ml and a carbohydrate content at a concentration of 1.86 mg/ml. In the same study, *Bacillus* flocculants were isolated from the water of shrimp culture ponds and other sources. Three isolates showed bioflocculant activity; the isolate BF9 showed higher bioflocculant activity.

Harun *et al*. (2018) evaluated the flocculant potential of different bacteria using the Clay suspension method and found that six species had flocculant activity. They were identified as *Bacillus infantis*, *B. cereus*, *B. safensis*, *Halomonas venusta*, *Nitratireductor aquamarinus*, and *Pseudoalteromonas*. The greatest bioflocculant activity was observed in *Bacillus infantis* (93%), which indicated the highest production of EPS, favoring flocculation. In this paper, they reported low bioflocculant activity in *Pseudoalteromonas*, whereas Wandong *et al.* (2021) reported the high bioflocculant ability of *Pseudoalteromonas* sp. NUM8. The optimum production of bioflocculant by the bacterium was by using sucrose and sodium nitrate as a source of carbon and nitrogen, respectively, along with the presence of a divalent cation (Ca) and pH = 5.

Fakriah *et al.* (2019) reported the protein content of EPS in biofloculant bacteria isolated from *Penaeus vannamei* culturing ponds. Each species showed different protein concentrations of EPS, from 1.377 µg/mL to 1.455 µg/mL. In the study, *Bacillus cereus* and *Bacillus pumilus* showed the highest bioflocculant activity, among other bacteria of genera *Nitratireductor*, *Pseudoalteromonas*, and *Halomonas*. Coincidentally, Kasan *et al.* (2017) isolated and identified bioflocculant-producing bacteria, particularly genera *Bacillus* and *Halomonas*, which could be used as inoculum for Biofloc. Other genera were identified, such as *Providencia* sp., *Nitratireductor* sp., and *Pseudoalteromonas* sp.

Although microbial bioflocculants comprise polymers like cellulose, protein, glycoproteins, polysaccharides, and nucleic acids (Gosai and Narolkar, 2022), the chemical composition reported for *Bacillus* produced bioflocculants consisted of polysaccharides and proteins. Budi *et al*. (2023) noted that the bioflocculant produced by *Serratia marcescens* included several carboxylic acids and intermediate enzymes in their chemical composition, indicating the presence of polysaccharides and proteins, and suggesting that they are the main ingredients of bacterial bioflocculants. At pH = 7, its protein content was 1.3 µg/mL, and the overall carbohydrate content was 0.53 mg/L.

The preparation of the ponds is a critical stage in aquaculture, it influences the survival and growth of cultured animals. The post-larva from crustaceans are susceptible to sudden changes in water quality. In a study conducted by Fakriah *et al*. (2022), they added the bacterium *Bacillus infantis* reported for its high bioflocculant potential, as inoculum for the preparation of a pond under the Biofloc system. *B. infantis* stain *BF3* isolated from Biofloc pond samples containing shrimp cultures was used to evaluate its effect on water quality and bacterial communities. The application of the bioflocculant showed a strong effect on Biofloc development, since in the 10-day study, the Biofloc volume was significantly higher in the treatment than in the control group. The treatment with *Bacillus infantis* in the Biofloc system resulted in a higher number of viable heterotrophic bacteria, and the maintenance of inorganic nutrients (ammonium, nitrite, and dissolved oxygen) at sub-lethal levels (Fakriah *et al*., 2022).

Other microorganisms with bioflocculant potential

In addition to bacteria, other microorganisms like fungi and yeast are reported as bioflocculant producers (Gosai and Narolkar, 2022). Fungi are heterotrophs and compete against bacteria over sources of hexose sugars (fructose and glucose) in biofloc systems. The presence of fungal filaments could lead to the formation of large Biofloc particles. Some species like *Aspergillus* sp. and *Penicillium* sp. have proven high bioflocculant activity. The interaction and association of fungi with other microorganisms in the Biofloc system could stimulate shrimp growth and survival, and enhance disease resistance (Hossein and Mohammadi, 2022).

Filamentous fungi such as *Aspergillus, Penicillium, Trichoderma, Spicaria,* and *Hyaloflorea* have been reported to have the ability to catch suspended solids and ensure bioflocculation thanks to their filamentous properties. Fungal filaments made up of hypha favor bioaggregation and absorption of microalga cells that can be removed from the surrounding water (Mohd *et al*., 2019). Hypha and mycelia contain polysaccharides with active sites responsible for the absorption; these polysaccharides also offer load to fungal cells (Kushwaha *et al*., 2020)

Mohd *et al*. (2019) used *Aspergillus niger* as a bioflocculant of microalgae in a water system. The bioflocculant demonstrated the ability to adapt to a broad range of pH, between 3.0 and 9.0, and 100-150 rpm, with a greater flocculation efficiency above 90%. The treated water showed a low concentration of chlorophyll, and low cell density, indicating that *Aspergillus niger* is a promising biofloculant for micro-alga flocculation to treat residual water in aquaculture (Mohd *et al*., 2019).

Micro-algae have an important role in biofloc systems, using nitrogenated compounds for proteins and sugars, and supplying oxygen in the presence of light. Micro-alga like diatom and *Chlorella* sp. are good sources of essential amino acids and unsaturated fatty acids for shrimp. In biofloc systems, the addition of diatom in the water improves yields and fatty acid content of *P. vannamei* larva. The relation between alga and bacterial communities in biofloc systems may be mutuality or antagonistic. For instance, some bacteria can contribute to alga growth, and the extracellular substances secreted by diatoms could be used by bacteria as a source of energy. These interactions can help control pathogenic bacteria in biofloc systems (Hossein and Mohammadi, 2022).

Micro-alga bind bacterial EPS and form large flocculates that stimulate bioflocculation. In that sense, glutamic acid from *Bacillus subtilis* is used for collecting micro-alga biomass, such as *Nanochlropsis occulata* LICME 002, *Phaeodactylum tricornutum*, *C. vulgaris* LICME 001 and *Botryococcus braunii* LICME 003. *Bacillus licheniformis* CGMCC 2876 is used for collecting micro-alga *Desmodesmus* sp. F51 has a flocculation efficiency of 92% (Kushwaha et al., 2020).

Moreover, the implementation of artificial substrates to confer sites for microbial community development has demonstrated benefits. Viau *et al*. (2014) evaluated the effect of biofilm on *Farfantepenaeus brasiliensis* shrimp post-larva using polyethylene sheets as artificial substrates.

To create a heterotrophic medium, the tanks were inoculated with diatoms *Thalassiosira weissflogii* an artificial feed, molasses, and bran. The carbon: Nitrogen ratio (C/N) was 20:1. Post-larval survival was higher and the nitrite levels were lower in the treatments with the presence of biofilm. These results indicate that the utilization of biofilm could be a reasonable alternative to improve postlarval survival by maintaining water quality.

Likewise, Thompson and Abreu (2002) demonstrated the effectiveness of biofilm, mainly made up of diatoms (*Amphora, Campylopyxis, Navícula, Sinedra, Hantschia,* and *Cilindrotheca*) in maintaining water quality by reducing the ammonium and phosphorous levels, and as a source of feed for shrimps. The biofilm associated with the submerged substrate was a significant complementary source of shrimp feed that promoted growth.

Carbon: Nitrogen ratio (C/N)

In biofloc technology, microorganisms have different functional characteristics and have three main roles: (i) helping improve water quality by removing inorganic compounds from nitrogen (bioaccumulation, bioassimilation, nitrification, and denitrification); (ii) acting as a source of complementary feed; and (iii) creating probiotic properties, which are critical to any aquatic culture system.(Khanjani *et al.*, 2022).

The addition of carbon sources to aquaculture systems promotes the growth of heterotrophic microorganisms, in which nitrogen is used to generate microbial proteins. These sources of carbon include molasses, bran, tapioca, and others (Azhar *et al*., 2016). The C./N ratio is another factor that influences the density of heterotrophic bacteria and other microorganisms in biofloc systems. This ratio is used for ammonia nitrogen control, so it influences the growth and well-being of aquaculture organisms (Hossein and Mohammadi, 2022).

The utilization of the C/N ratio in a culture system can promote a bacterial community dominated by heterotrophic bacteria, which use organic carbohydrates to generate energy and grow, for protein synthesis and new cells. An optimal C/N ratio is key to controlling inorganic nitrogen; it may improve the production and growth, and nutrient recycling (Avnimelech, 1999). The meta-genomics analysis revealed that Vibrio was 90% of the population in the biofloc, whereas *Pseualteromonas, Photobacterium, Shewanella, Alteromonas, Bacillus, Lactobacillus, Acinetobacter, Clostridium, Marinifilum and Pseudomonas* were detected as well (Tepaamorndech *et al.*, 2020). The density of Vibrio, opportunistic pathogens, drops when C/N ratios increase, thus confirming the density of heterotroph bacteria in treatments where the C/N ratio is high. it explains that the survival rates improve when C/N is higher (Panigrahi *et al.*, 2019).

Recently, in a biofloc culture containing *P. vannamei*, six species of sea bacteria known as *Halomonas venusta, Bacillus cereus, Bacillus subtilis, Bacillus pumilus, Nitratireductor aquimarinus* and *Pseudoalteromonas* sp., were isolated. They were bioflocculant producers. The highest flocculant activity was found in *Bacillus cereus* (93%) (Minaz and Kubilay, 2021). There is a tendency to use *Bacillus* spp., among them *Bacillus infantis* and *Bacillus eclensis* as bioflocculants to improve biofloc quality (Che Hashim *et al*., 2022).

CONCLUSIONS

Genus *Bacillus* bacteria are used for their beneficial effects on shrimp culture. Although the bioflocculant potential of several species of *Bacillus* has been demonstrated, a new strain should be isolated for further research that characterizes its bioflocculants, and to evaluate the effect on the metabolism and immune system of *Penaeus vannamei.* Other optimization studies are recommended for bioflocculant production using new sources of carbon and nitrogen.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: YAC, AAC, GN; data analysis and interpretation: YAC, AAC, GN; redaction of the manuscript: YAC, AAC, GN.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.