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SCREENING, IDENTIFICATION AND BIOACTIVE PROPERITES OF *BACILLUS* SPP. ISOLATED FROM SHRIMP PONDS OF QUANG TRI PROVINCE

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ABSTRACT

Aquaculture is the farming of aquatic organisms by intervention in the rearing process to enhance production and private ownership of the stock being cultivated. However, the intensification of aquaculture practices requires cultivation at high densities, which has caused significant damage to the environment and, aquatic species are subjected to high-stress conditions, increasing the incidence of diseases and causing a decrease in productivity. This study was conducted to isolate bacteria from sediments of shrimp culture ponds based on antagonistic activity against shrimp pathogen, *Vibrio parahaemolyticus*, and production of exocellular enzymes, protease and amylase. Three potential strains were isolated and after analysis of 16S rRNA gene, the isolated strains were identified to *Bacillus subtilis* (isolate QTA12 with amylase activity), *Bacillus megaterium* (QTP1 with proteolytic activity) and *Bacillus subtilis* (isolate with antivibrio activity DK1). These isolated strains may be considered for future using in shrimp culture ponds as a very promising measure for sustainable aquaculture.

Keywords: antagonism, aquaculture, *Bacillus,* identification, probiotic.

1. INTRODUCTION

The half of the world’s seafood demand will be met by aquaculture in 2020, because wild capture fisheries are overexploited and are in decline. The growth demand of aquaculture products is due to several factors: (1) many fisheries have reached their maximum sustainable exploitation, (2) consumer concerns about security and safety of their food, (3) the market demand for high-quality, healthy, low-calorie, and high-protein aquatic products, and (4) aquatic breeding makes only a minimum contribution to carbon dioxide emission [1].

Nowadays, shrimp (or prawn) culture is wide spread throughout the tropical [2, 3]. The intensification of aquaculture practices requires cultivation at high densities, which has caused significant damage to the environment due to discharges of concentrated organic wastes, that deplete dissolved oxygen in ponds, giving rise to toxic metabolites (such as hydrogen sulfide, methane, ammonia, and nitrites), that often are responsible for mortality [3]. Moreover, under

these conditions of intensive production, aquatic species are subjected to high-stress conditions, increasing the incidence of diseases and outbreaks of viral, bacterial, and fungal infections leading to devastating economic losses worldwide [3, 4].

In recent decades, prevention and control of animal diseases has focused on the use of chemical additives and veterinary medicines, especially antibiotics, resulted in emergence of drug-resistant bacteria which are becoming increasingly difficult to control and eradicate [5, 6]. Controlling or preventing outbreaks in the aquatic environment is quite complicated due to the inhabited interaction between pathogens and their host in the production systems. The aquatic environment contains a redundance of opportunistic bacterial pathogens as well as beneficial and non-pathogenic bacterial strains [7]. It is recognized that microorganisms play the critical role in aquaculture systems because water quality and disease control are directly related and closely affected by microbial activity. The use of probiotics for disease prevention, improvement animal health and water quality, nutrition in aquaculture is becoming increasingly popular due to an increasing demand for environment-friendly aquaculture [7, 8].

There are several mechanisms of probiotic action, including the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, the enhancement of the immune response and improvement of water quality [9]. There have been many reports documenting activities of probiotic bacteria isolated from different habitats (intestinal aquaculture animals, sediments or water of culture ponds…) and *in vitro* selection of putative probiotic strains based on production of inhibitory compounds toward known pathogens [8, 10, 11].

The aim of this study was to isolate, identify and assess inhibition activity against *Vibrio parahaemolyticus* and ability of production extracellular enzymes of bacteria from shrimp culture ponds of Quang Tri province.

2. MATERIALS AND METHODS

2.1. Materials

Sediment samples were collected from shrimp culture ponds of Quang Tri province by special tool and put in aseptic bottles.

Starch agar medium for amylase producing bacteria (g/L): beef extract-3; soluble starch-10; agar-12.

Cazein agar for protease producing bacteria (g/L): yeast extract-0.25; KNO_3-5; KH_2PO_4-3.5; $MgSO_4.7H_2O$-0.62; cazein-2; agar-20; pH 7 ± 0.2.

TCBS medium for *Vibrio parahaemolyticus* (g/L): Sucrose-20; Dipeptone-10; Sodium Citrate-10; Sodium Thiosulfate-10; Sodium Chloride-10; Yeast Extract-5; Oxbile (Oxgall)-5; Sodium Cholate-3; Ferric Citrate-1; Bromothymol Blue-0.04; Thymol Blue-0.04; Agar-15. Final pH 8.6 ± 0.2.

LB medium (g/L): Peptone-10; Yeast Extract-5; Sodium Chloride-5; pH 7 - 7.2.

2.2. Methods

Isolation amylase and protease producing bacteria

The isolation was performed by serial dilution and spread plate method. One gram of sediment sample was serially diluted in sterilized distilled water to get a concentration range

from 10^{-1} - 10^{-7}. A volume of 0.1 ml of each dilution was transferred aseptically to starch agar or skimmed milk agar plates. The sample was spread uniformly. The plates were incubated at 37 °C for 24 hr. The bacterial isolates were further subcultured to obtain pure culture. Pure isolates on starch /skimmed milk agar slants were maintained at 4 ºC [12, 13].

Screening amylase and protease producing bacteria

The isolates were screened for amylolytic activity based on diameter of starch hydrolysis on starch agar plate. The wells were punched into starch agar plates and then filled with 100 µL of isolate culture. After 24 hour incubation at 37 °C, the plates were flooded with lugol solution for better visualization of starch hydrolysis zone. Similarly, potent protease producing bacteria were screened on skimmed milk agar for their proteolytic activity [12, 13].

In vitro antagonism tests: antagonistic activity of the isolates against *Vibrio parahaemolyticus* was determined using agar well diffusion method. 100µl *Vibrio parahaemolyticus* cultures with 10^5 CFU/ml (CFU – colony forming unit) were spread onto TCBS agar plates. Wells (8 mm) were bored into TCBS agar containing pathogenic bacterium, then 100 µl of investigated isolates were added to each well. The plates were incubated at 37 °C for 24 hours and clearing zones around the wells were observed. Sterile LB broth was used as control [14, 15].

Identification of selected isolates based on 16S rRNA profile: DNA was extracted according Sambrook & Russell (2001). Fragment of 16S rDNA gene was amplified by PCR, using universal primers for 16S rDNA. Forward primer: 5'- AGA GTT TGA TCA TGG CTC A-3' and reverse primer: 5'- AAG GAG GTG ATC CAG CC-3'. Received PCR product was electrophoresed in 1 % agarose gel. A single PCR amplicon band of 1500 bp was purified using Qiagen elute gel extraction kit and sequenced using automatic sequencer ABI 3100 Avant (Applied Biosystems). The obtained sequences were subjected to nucleotide BLAST with non-redundant database of NCBI.

3. RESULTS

3.1. Amylase producing bacteria

Figure 1. Amylolytic activity of some isolates on starch agar after lugol staining.

The separate colonies on starch agar plates were picked, subcultured on the same media for purifying and rechecked their amylolytic activity after dyeing the plates with lugol solution (Fig. 1). On the basis of the clearance area around the well, one bacterial isolate created starch hydrolytic zone of 16.4 mm was selected (QTA12).

The selected bacteria formed small colony, opaque with convex elevation. The cells were rod shaped, Gram stained positive and spore forming.

3.2. Protease producing bacteria

Based on zones of skimmed milk hydrolysis, 15 isolates exhibited different proteinase activity, with zone diameter ranges from 5 to 16.4 mm (without hole diameter).

Figure 2 showed that QTP1 has highest activity, so this isolate was chosen for identifying analysis.

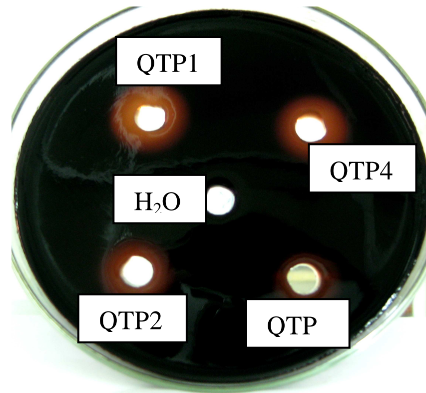

Figure 2. Proteolytic activity of some isolates on cazein agar after lugol staining.

3.3. Selection antagonistic bacteria

From sediment samples of shrimp ponds, bacteria were isolated on LB agar, purified and then assessed for their antagonictic activity by agar diffusion method. From 12 isolates, 2 isolates DK1 and DK2 were selected on the basis of inhibition zone (15.3 and 14.9 mm, respectively) developed on the TBCS plate growing *Vibrio parahaemolyticus* (Fig. 3).

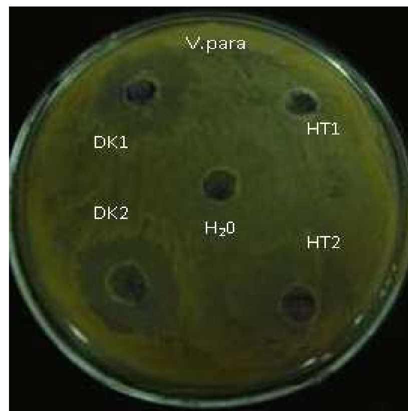

Figure 3. Antagonistic activity of DK1 and DK2 against *Vibrio parahaemolyticus.*

The isolate DK2 formed opaque colonies on LB agar with low convex (Fig. 4). The cells were also rod-shaped and Gram positive with endospore.

Beside this, isolates DK1 and DK2 were tested for their proteolytic activity. Isolate DK1 was protease producer due to the zone of cazein hydrolysis around the well (Fig. 5).

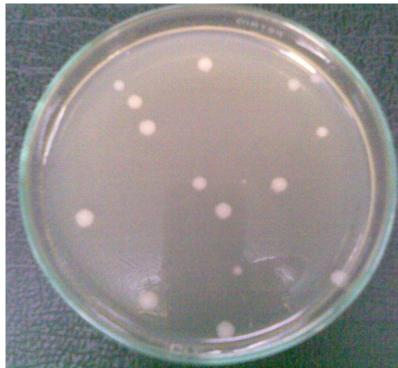

Figure 4. Colonies of DK2 on LB agar.

Figure 5. proteolytic activity of isolates DK1 and DK2.

3.4. Identification of selected isolates

DNA genome of QTA12, QTP1 and DK2 were extracted from 1.5 ml overnight culture according Sambrook & Russell (2001). The quality of the extracted DNA was analysed by electrophoresis on 1 % (w/v) agarose gel and by ratio A_{260}/A_{280} (1.8). The extracted DNA was then used as a PCR template for the amplification of 16S rRNA gene with the universal primer pair and with a thermal cycles as described above. The anticipated products of approximately 1.5 kb were analysed by agarose gel electrophoresis (Fig. 6).

The PCR products were purified and the sequence analysis of them was performed using the ABI 3100 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rRNA genes of three strains QTA12, QTP1 and DK2 with more than 1400 bp were obtained after sequencing and were aligned and compared with other 16SrRNA genes in the GenBank by using the NCBI Basic Local alignment search tools BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). The obtained results demonstrated that strains QTA12 was most similar to *Bacillus subtilis* (Fig. 7), strains QTP1 was found to be most similar to *Bacillus megaterium* (Fig 8) and strains DK2 shares closest homology with *Bacillus subtilis* (Fig. 9).

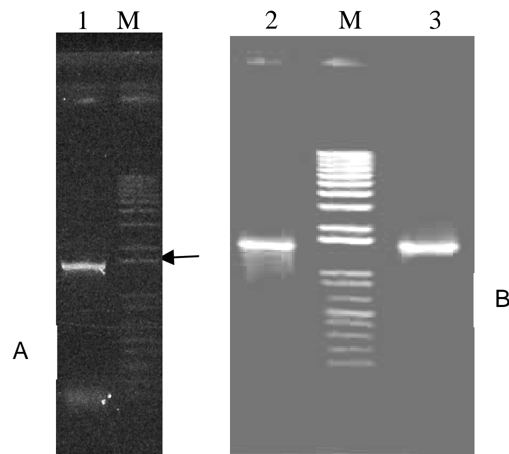

Figure 6. PCR products of 16S rRNA gene of isolates QTA12 (1A), QTP1 (2B) and DK2 (3B). M-DNA marker 1 kb (Fermentas). The arrows show the band of 1.65 kb.

Accession	Description	Query coverage	Max. identity
AB201120.1	*Bacillus subtilis* gene for 16S rRNA, partial sequence, strain: LB-01	100 %	99 %
AB188212.1	*Bacillus* sp. TUT1206 gene for 16S rRNA, partial sequence	100 %	99 %
AB110598.1	*Bacillus subtilis* gene for 16S rRNA, partial sequence	100 %	99 %
AB177641.1	*Bacillus subtilis* gene for 16S ribosomal RNA, isolate: H20	100 %	99 %
AB042061.1	*Bacillus subtilis* gene for 16S ribosomal RNA, partial sequence	100 %	99 %

Figure 7. 16S rRNA gene of strains QTA12 with others in GeneBank.

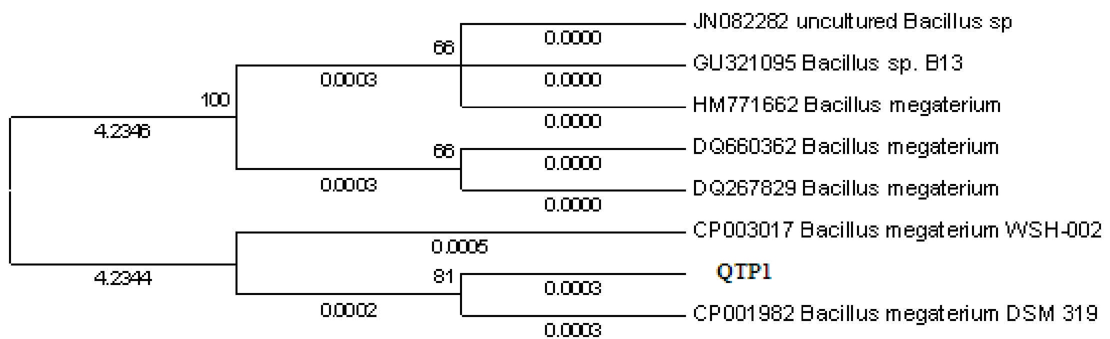

Figure 8. Phylogenetic position of the strain QTP1 based on 16S rRNA gene sequence analysis.

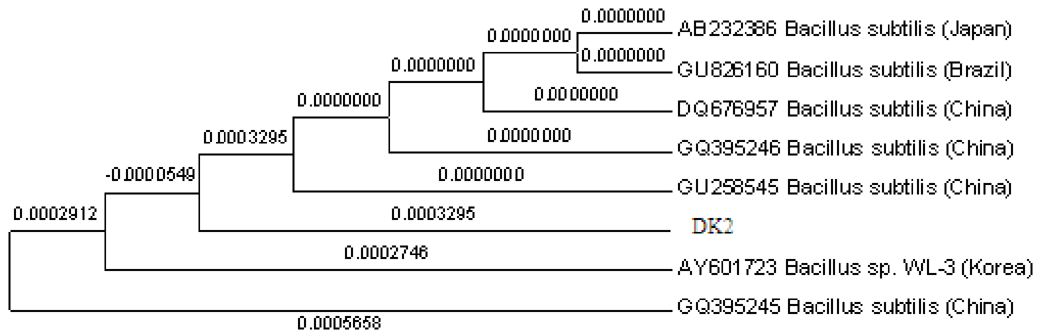

Figure 9. Phylogenetic position of the strain DK2 based on 16S rRNA gene sequence analysis.

4. DISCUSSION

Shrimp (or prawn) culture is wide spread throughout the tropical world. Aquaculture industry is besieged by disease outbreak, caused mostly by bacteria and viruses. The high density of animals in hatchery tanks and ponds let to wide spread of pathogens; In most of the shrimp farming ponds deterioration of water quality, pond bottom condition, incidence of toxic gases like NH3, NO2, H2S etc. partly due to regular applications of protein-rich feed were the

mains factors make cultured organisms stressed and thus they were more susceptible to disease outbreaks. Mostly, *Vibrio* spp. (*V.paraheamolyticus, V. harveii, V. alginolyticus*) cause major problems in aquaculture, resulting in reduced growth rate, poor feed consumption, loss of body weight and ultimately mass mortality. A number of alternative strategies for the prevention and control of diseases have been proposed and have already been applied successfully in aquaculture, such as antibiotics, vaccines and immunostimulants [2]. In view of the global antibiotic resistance crisis, there is considerable interest in developing sustainable biocontrol methods such as probiotics for disease management in aquaculture. Recently, the interest in using probiotic bacteria for improvement of aquaculture animal health and water quality has been grown rapidly. Probiotics demonstrated beneficial effects to the host by competitive exclusion potential pathogens in the digestive tract, nutritional and immunological improvement. Probiotics exhibit effective control ability various infectious diseases in aquaculture including furunculosis caused by *A.salmonicida*, lactococcosis and streptococcosis by *Lactococcus garvieae* and *Streptococcus iniea*, respectively, in rainbow trout, edwardsiellosis by *Edwardsiella tarda* in the European eel [6].

There are several mechanisms of probiotic action, including the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, the enhancement of the immune response and improvement of water quality [9]. A set of criteria has been proposed to select potential probiotic strains. However, many reports have used *in vitro* production of inhibitory compounds toward known pathogens of a particular species, or based on screening for beneficial microbial attributes such as predation, anti-virulence, competition, attachment to host surfaces, and immunostimulation as the first screen for selection of putative probiotic strains [16, 10]. The most probiotics belong to Lactic acid bacteria, genus *Vibrio, Bacillus, Pseudomonas* and *Roseobacter* [17]. Dhanasekaran et al. (2010) screened 4 Lactobacilli isolates from fresh water fishes for their antagonistic activity against *Aeromonas*, *Vibrio* sp. by agar diffusion assay. The isolate RLD3 with broad spectral activity against *Aeromonas* and *Vibrio* was evaluated for the viability of pathogen *in vitro* and then on fish. The results reveal that *Lactobacillus* is responsible for inhibition of *Aeromonas* population in cat fish (*Clarias orientalis*) [18].

Bacillus species are widely used for water remedation because they are stable for long period due to spore formation, easily prepared by fermentation and possess antagonistic effects on pathogens. They are important candidates for developing commercial biological agents for nitrogen removal and water quality enhancement [19]. Previously, strains belonging to several *Bacillus* species, such as *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus pumilus* were isolated and evaluated for their potential as biological agents for water quality enhancement [20]. Our screening result based on amylase and protease activities of the isolates, so it's obviously that the strains QTA12 and QTAP1 have potential for degradation organic matters accumulated in aquacultured ponds due to excretes of cultured animals and excessive feeds.

There have been several reports documenting the ability *Bacillus* spp. to control pathogenic bacteria in aquaculture [11]. *Bacillus* spp. were associated with improvement of water quality, reduction of pathogenic vibrios in culture environment, enhancement of survival and growth rate, and the improved health status of juvenile *Penaeus monodon* [21]. Barman et al. (2011) isolated and characterized strain PPP13 from Black Tiger Shrimp (*Penaeus monodon*), which exhibited antagonistic properties against three target pathogenic bacterial strains of *Vibrio alginolyticus, Vibrio harveyi, Vibrio vulnificus*. The strain PPP 13 was identified by morphological, physiological, biochemical characteristics and also by 16S rRNA gene sequence

data analysis as *Bacillus subtilis*. The antagonistic properties of the selected strain and the results of serum bactericidal activity of non-specific immunity proved that *Bacillus subtilis* PPP 13 was a potential probiotic for *Penaeus monodon* [22]. The probionts *Bacillus* sp. AVP03 and AVP07 were isolated from healthy adult shrimp *Penaeus monodon* inhibited the pathogenic *V. harveyi* VSH5 both *in vitro* and *in vivo* methods. Beside this, these isolates anti-vibrio probionts possessed anti-QS (quorum quenching) activity which significantly reduced the mortality when compared to the animals treated with the pathogen in post larvae and juvenile challenge study [23].

It recognized that *Bacillus* spp. not generally involved in horizontal gene transfer processes with Gram-negative organisms, so they can not acquire antibiotic resistance or virulence genes from pathogens such as *Vibrio* and *Aeromonas* spp. Another advantage of using *Bacillus* spp. that they are rapidly replicated bacteria and could tolerate a multitude of environmental conditions and exhibit a range of benefits to aquaculture organisms [24]. *Bacillus* strain IP5832 spores fed to turbot larvae resulted in a decrease in the *Vibrionaceae* population with significant improvement in weight gain and survival of the larvae [25]. *Bacillus* spp. also contribute to nitrogen removal in spite of the classical belief that this process is predominated by autotrophic bacteria [26]. The isolate DK1 was demonstrated not only anti-vibrio, but also proteolytic activity *in vitro*. So three isolates can be potential probionts for shrimp aquaculture in terms of water quality improvement and protection of cultured animals from pathogen (*V.parahaemolyticucs*). However, further investigation should be carried out to assess their effectiveness *in vivo* for improvement of water quality and ability as anti-pathogen agent in shrimp culture.

5. CONCLUSION

The paper reports on isolation and identification of three probiotic candidates consisting by two strains of *Bacillus subtilis* and *B. megaterium*, which exhibited inhibitory activity against *V. parahaemolyticus*, protease and amylase activities. The received results could be a promising measure for sustainable shrimp culture. However, the bioremedation potential and biocontrol activities of the isolated strains need to be evaluated *in vivo*.

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TÓM TẮT

TUYỂN CHỌN, ĐỊNH DANH VÀ HOẠT TÍNH SINH HỌC CỦA *BACILLUS* SPP. PHÂN LẬP TỪ HỒ NUÔI TÔM CỦA TỈNH QUẢNG TRỊ

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Nuôi trồng thủy sản là can thiệp vào quá trình nuôi thủy động vật trong các khu nuôi thủy sản nhằm tăng sản lượng và thu nhập cho chủ trang trại. Nhưng việc chuyên canh hóa nuôi trồng thủy sản đòi hỏi nuôi ở mật độ cao, điều này dẫn đến tổn hại cho môi trường và các loài thủy động vật phải chịu các điều kiện gây stress mạnh, làm tăng nguy cơ bệnh tật và làm giảm sản lượng. Trong nghiên cứu này, các chủng vi khuẩn được phân lập từ bùn của các hồ nuôi tôm dựa trên hoạt tính đối kháng của nó chống lại nguồn bệnh của tôm là *Vibrio parahaemolyticus* và sự sản sinh các enzyme ngoại bào như protease và amylase. Ba chủng vi khuẩn tiềm năng đã được phân lập và sau khi phân tích gen 16S ARN riboxom, các chủng này đã được định danh đến loài *Bacillus subtilis* (chủng QTA12 có hoạt tính amylase), *Bacillus megaterium* (QTP1 có hoạt tính protease) và *Bacillus subtilis* (chủng DK1 có hoạt tính đối kháng nguồn bệnh *Vibrio*). Các chủng phân lập này có thể xem xét để sử dụng trong tương lai cho các hồ nuôi tôm như một biện pháp đầy triển vọng cho một ngành nuôi trồng thủy sản bền vững.

Từ khóa: *Bacillus,* đối kháng, định danh, nuôi trồng thủy sản, probiotic.