P-FNN-28

Screening of potential probiotic Bacillus for aquaculture industry

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ABSTRACT

The objective of this study was to isolate probiotic Bacillus from intestinal contents of fishes and shrimps. 154 Bacillus- like bacteria were isolated and studied further based on probiotic characteristics. A total of 5 isolated bacteria was selected as they were not potential pathogenic bacteria**.** Besides, they exhibited antimicrobial activity against specific pathogens such as *Vibrio alginolyticus*, *Vibrio parahaemolyticus, Pseudomonas aeroginosa and Bacillus cereus* and tolerance to some antimicrobial substances**.** These isolates were identified by using the API 50 CHB identification kit and confirmed by using the 16S rRNA sequence**.** Results showed that the potential probiotic bacteria were *Bacillus safensis, Bacillus subtilis and Brevibacillus agri.*

Keywords: Bacillus, probiotic, aquaculture

INTRODUCTION

Fisheries and aquaculture industries remain important sources of food, nutrition, income, and livelihood for hundreds of millions of people around the world. In recent decades, disease prevention and control have led to sustainable increase in the use of antimicrobial drugs, pesticides and disinfectants (Done et al, 2015). The continuous applications of antibiotics brings important changes in the microbiota of the aquaculture systems, causing the development

of bacterial resistance to frequently used antimicrobials and which even affect the natural beneficial bacteria (Kavitha et al, 2018). Therefore, the use of probiotics is now considered a viable prophylactic alternative.

Probiotics are defined by Food and Agriculture Organization/World Health Organization as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO/FAO, 2002). Many microorganisms have been used as probiotics such as lactic acid bacteria *Bifidobacterium* spp. and *Bacillus* sp. Most Bacillus microbes are rarely found in the intestine. Because Bacillus needs oxygen to grow although it is a group of bacteria with some species that can cause disease and is harmful to humans and certain animals. From various study data, it was found that some species were useful. *Bacillus* sp. can produce many types of enzymes and also inhibit some pathogens which can developed into probiotics for use in fish farming industry instead of antibiotics. The administration of a mixture of bacteria positively influenced on survival and had protective effect against *Vibrio harveyi* and the white spot syndrome virus (WSSV) (Balcázar et al., 2006). Moreover probiotic strains of *Bacillus* sp. increased the quality and viability of pond-raised shrimp. (Moriarty, 1998). The aim of this study was to screening of *Bacillus* sp. isolated from aquatic animal such as fish and shrimp. The selected strains were investigated further based on probiotic characteristics.

MATERIAL AND METHODS

Isolation of Bacillus

Twenty one samples consisting of fishes and shrimps were purchased from fresh markets in Pathum Thani province, Thailand. Their intestinal contents weighing about 1 g was diluted 10 times with 0.85% NaCl solution and destroyed spores by heat shock method. Then, each diluted samples was serially diluted with 0.85% NaCl solution at dilution level of 10^{-1} to 10^{-10}. The 0.1 ml of appropriate dilutions was placed on the surface of prepared Nutrient agar plate. After aerobic cultivation at 37ºC for 24 – 48 h, the well-isolated colonies like Bacillus were picked up and re-streak twice on Nutrient agar plate to obtain pure isolates. All selected isolates were tested for gram staining and spore formation

Haemolysis

Blood haemolysis of the isolates was determined on Brain heart infusion agar supplemented with 5% human blood after incubation at 37°C for

24 h. The plates were examined for α- haemolysis, β- haemolysis and γ- haemolytic properties (Vesterlund et al., 2007).

Detection of antimicrobial activity

Antimicrobial activity was analyzed using the agar well diffusion method described by (lin et al. , 2007) . The pathogenic bacteria used as indicators included Gram negative bacteria and Gram positive bacteria such as *Escherichia coli TISTR 887, Salmonella typhimurium ATCC 11331, Salmonella enteritidis TISTR 2202, Staphylococcus aureus TISTR 1466, Bacillus cereus ATCC 687, Pseudomonas aruginosa TISTR 1468, Vibrio alginolyticus TISTR 1572, Vibrio harveyi TISTR 2088, Vibrio parahaemolyticus TISTR 1596, Aeromonas hydrophila TISTR 1321*. Indicator strains were cultured in Nutrient broth overnight at 37ºC. Aliquots of 100 µl of cell culture were spread on nutrient agar plates. Spent culture supernatant obtained from the 48 h of NB cultures of Bacillus- like isolates were filtrated through a 0. 45 µm pore- size sterile filter. The 100 µl of spent culture supernatant was dropped into the wells on nutrient agar drilled with sterile cork borer no. 3. The agar plates were incubated at 37ºC overnight and the diameters of the inhibition zones on the agar plates were measured. Each essay were performed in triplicates.

Quantitative enzyme activity of the selected bacterial strains

Extracellular amylase, protease, lipase and cellulase activities of the selected bacterial strains were quantitatively estimated in starch agar, skim milk agar, Tween 80 agar and carboxymethyl cellulose agar media plates, respectively according to the method of Kavitha et al (2018).

Antibiotic susceptibility assay

The susceptibility of LAB isolates was determined according to the method described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) using antibiotic discs (Oxoid, England). *Escherichia coli TISTR 887* and *Staphylococcus aureus TISTR 1466* were used as the control bacterial strains.

Bacterial identification

The selected strains were firstly identified by the API 50 CHB identification kit (Biomerieux, Marcy I'Etoile, France) and further confirmed by partial sequencing 16S rRNA analysis. Chromasomal DNA of unknown strain was extracted from cells grown in MRS agar at 37 ºC for 24-72 h (or

suitable cultured condition) using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACC TTGTTACGACTT-3'), purified by the DNeasy Tissue Kit (Qiagen, Germany), sequenced and analyzed as described by Tanasupawat et al. (2004). PCR condition was as follows; 94 °C for 3 min, 30 cycles of 94 °C for 60 s, 55 °C for 60 s, 72 °C for 2 min with a final extension at 72 °C for 3 min. Multiple sequence alignment was done with the program CLUSTAL X (version 1.83; Thompson et al, 1997). Alignment gaps and unidentified bases were eliminated. Distance matrices for the aligned sequences were calculated using the two-parameter method of Kimura (1980) and find regions of similarity between query nucleotide sequence and sequence database in Ezbio Cloud (Yoon et al., 2017).

RESULTS

Selection of microorganisms

154 Bacillus-like isolates were selected by colony characteristics on Nutrient agar. All isolates were also monitored with gram positive and spore formation. The selected colony were steaked twice on Nutrient agar and incubated at 37 °C for 24–48 hours for purification.

Haemolysis

Haemolysis of red blood cells was used to indicate the pathogenic potential of the isolated bacteria. The haemolytic reactions were recorded through the observations of a clear zone around the colonies (β-haemolysis), a partial hydrolysis and greening zone (α-haemolysis) or no reaction (γ-haemolysis). The results showed that among 154 isolates, 13 isolates showed γ-haemolysis and appeared as simple growth with no change to the medium. The others were shown α-haemolysis and β-haemolysis. Thus, these 13 isolates were selected and used for further studies.

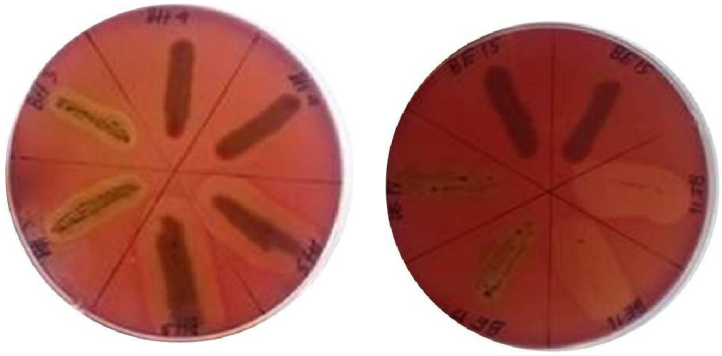

Figure 1. Growth of the isolates on blood-agar plate.

Antimicrobial activity

The selected isolates were tested for the inhibitory properties against pathogenic bacteria. Out of 13 isolates, only 5 isolates were able to inhibit specific pathogens such as *Vibrio alginolyticus, Vibrio parahaemolyticus, Pseudomonas aeroginosa* and *Bacillus cereus.* The results were showed in Figure 2. As reported by Kuebutornye et al (2019), many Bacillus species were able to produce antibiotics/metabolites which have antagonistic effects against pathogenic microorganisms.

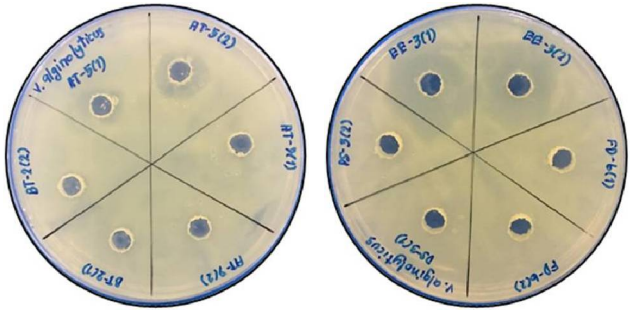

Figure 2. Antimicrobial activity of the selected isolates

The ability of enzyme production

The ability as enzyme producer of the selected isolates was shown in Figure 3. Three isolates (AT-5, EE-3 and NG-5) were able to create various enzymes such as cellulose, amylase, protease and lipase while the isolates of BE8 and BE10 could produce only amylase and protease. The results were shown in Table 1.

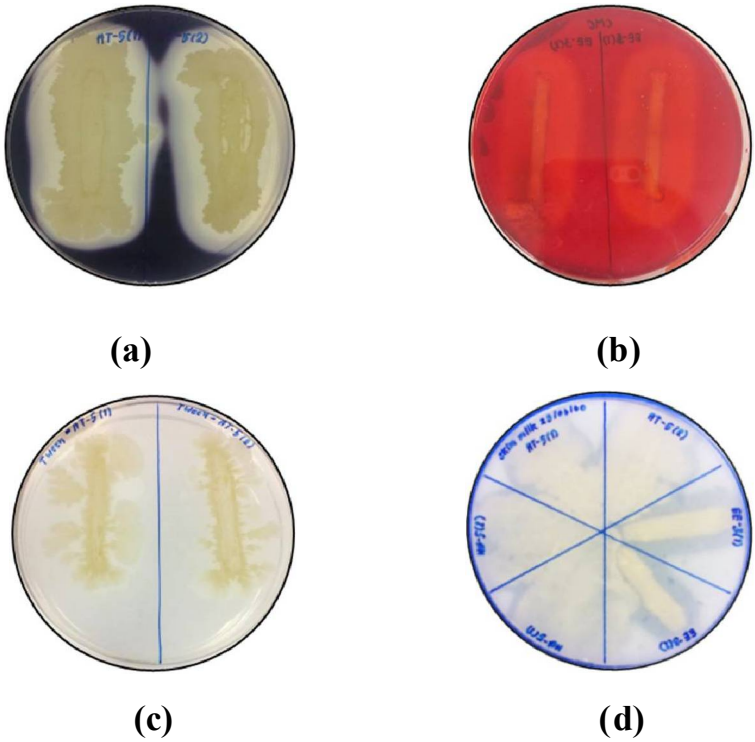

Figure 3. Enzyme activity of the selected isolates testing on (a) starch agar plate (b) CMC agar plate (c) tween 80 agar plate and (d) skim milk agar plate

Table 1. The ability as enzyme producer of the selected isolates

Isolates	Enzyme production			
	Cellulase	Amylase	Lipase	Protease
AT-5	+	+	+	+
EE-3	+	+	+	+
NG-5	+	+	+	+
BE8	-	+	-	+
BE10	-	+	-	+

+ = Positive
- = negative

Antibiotic susceptibility assay

The results of antibiotic susceptibility of 5 isolates were shown in Table 2. All isolates showed resistance to azteonam while sensitivity to cinoxacin, imipenem, streptomysin, ampicillin, cephalothin, erythromycin polymycin-B, vancomycin, bacitracin and norfloxacin. The other isolates showed differently in antibiotic susceptibility. A beneficial effect of antibiotic resistant strains is that they can be co-administered with therapeutic antibiotics for disease treatment. However, the emergence of antibiotic resistant organisms was a potentially serious threat to public health (Del Piano et al., 2006). Ideally, probiotic bacteria should exhibit tolerance to antimicrobial substances used in clinical practice but should not be able to transmit such resistance to other bacteria. As reported by Courvalin (2006), there was the possibility of resistance gene transfer between probiotics and pathogenic bacteria in the gastrointestinal tract if the gene was located on the plasmid. In contrast, if the gene was localized on the chromosome, it was not transferable.

Table 2. Antibiotic susceptibility test of Bacillus isolates.

Isolates	antibiotic																					
	Amoxicillin 30 µg	Cefoperazone 75 µg	Cinoxacin 100 µg	Imipenem 10 µg	Nystatin 100 unit	Streptomysin 10 µg	Ampicillin 10 µg	Ceftazidime 30 µg	Clindamycin 2 µg	Kanamycin 30 µg	Penicillin-G 10 unit	Tetracycline 30 µg	Azteonam 30 µg	Cephalothin 30 µg	Erythromycin 15 µg	Metronidazole 5 µg	Polymycin-B 300 unit	Vancomycin 30 µg	Bacitracin 10 unit	Chloramphenicol 30 µg	Gentamicin 10 µg	Rifampicin 5 µg	Norfloxacin 10 µg
AT-5	S	S	S	S	S	S	S	I	S	I	S	S	R	S	S	I	S	S	S	S	I	S	S
EE-3	S	S	S	S	R	S	S	R	R	S	S	I	R	S	S	R	S	S	S	I	S	R	S
NG-5	S	S	S	S	R	S	S	S	S	S	S	I	R	S	S	R	S	S	S	S	I	S	S
BE8	S	S	S	S	R	S	S	S	R	S	R	S	R	S	S	R	S	S	S	S	S	S	S
BE10	R	I	S	S	R	S	S	S	R	S	R	S	R	S	S	R	S	S	S	S	S	I	S

I: intermediately susceptible; R: resistant; S: sensitive;

Identification by API 50 CHB Kit test and 16s rDNA

Carbohydrate fermentation patterns of the selected 5 strains which displayed probiotic properties were tested using an API50 CHB kit. The results showed that AT-5 and NG5 were identified as *Bacillus subtilis* (99% identity).

EE3 was identified as *Bacillus pumilus* (99% identity). BE8 and BE10 were identified as *Bacillus megaterium* (98% identity) and *Bacillus licheniformis* (99% identity) respectively.

Identification was confirmed by 16S rRNA sequence analysis. Based on sequence analysis, The isolates of AT-5 and NG5 were correctly identified as *Bacillus subtilis*, but BE8 and BE 20 were identified as *Brevibacillus agri* (99% homology). EE3 was identified as *Bacillus safensis* (99% homology).

CONCLUSION

In this study, potential probiotic Bacillus were isolated from intestinal contents of fishes and shrimps. Five isolated were selected base on their probiotic properties. They were not potential pathogenic bacteria as they showed γ-hemolysis. Besides, they exhibited antimicrobial activity against specific pathogens and tolerance to some specific antimicrobial substances. These isolates were identified by using the carbohydrate fermentation method and confirmed by using the 16S rRNA sequence. Results showed that the potential probiotic were identified as *Bacillus subtilis, Bacillus safensis, and Brevibacillus agri.* All identified strains should be studied further in beneficial characteristics and industrial application.

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