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Full Length Research Paper

Biochemical and molecular characterization of hemolytic *Bacillus licheniformis* strains isolated from shrimp and clam aquacultures

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A polyphasic approach was used to discriminate among the significantly hemolytic and closely related *Bacillus licheniformis* strains, NE-1, NE-2 and NE-3. Utilization of the API 20E kit revealed that *B. licheniformis* NE-1 is unique in its ability to utilize sorbitol as a sole carbon source, whereas *B. licheniformis* NE-2 is an exceptional in L-arabinose utilization. The phylogenetic analysis using the 16S rDNA 5´end revealed a strong relatedness to *B. licheniformis* strains found in the GenBank with a similarity percentage ranging from 97 to 99. Moreover, the partial sequences was successfully differentiated among the novel strains. The plasmid pattern is almost the same for the strains and plasmid curing using acridine orange showed no effect on the hemolytic activity of them.

Key words: *Bacillus licheniformis*, aquacultures, hemolytic activity, plasmids, 16S rDNA.

INTRODUCTION

Microbes play a critical role in aquaculture systems for shrimp, fish and mollusks at both the hatchery and the grow-out stages since water and soil quality and their effects on aquatic animal health are related to, and closely affected by, microbial activity. Recently, there has been increasing interest in understanding the relevance of microbial communities in aquaculture systems and their importance (Rengpipat, 2007) . Global aquaculture is challenged by poor water quality and the outbreak of diseases which lead to reduced growth (Lallo et al., 2009). Microorganisms, particularly bacteria, play a vital role in aquaculture systems. A part from indigenous bacteria in estuarine water, application of artificial feed and fertilizers, high stocking density, induced breeding and shallow nature of water in intensive and semi-intensive farms. These factors lead to high bacterial population and thus cause diseases to the aquaculture life. Further, the dominant roles in the spoilage of marine products are mainly due to bacteria (Palaniappan, 1982; Ganesh et al., 2010). In addition, *Bacillus* spp. has been successfully isolated from shrimp (Balcazar and Rojas-Luna, 2007) and clam aquacultures (Bourne et al., 2004; Romanenko et al., 2008). Some of these species such as *B. cereus* and *B. licheniformis* have the ability to produce exotoxins of strong hemolytic activity and this may lead to reduction in the aquaculture products (Romanenko et al., 2008; Kashid and Gosh, 2010).

Bacillus species are ubiquitous and diverse both in the terrestrial and marine ecosystems (Oguntoyinbo, 2007). The genus comprised phylogenetically and phenotypically heterogenous members (Oguntoyinbo, 2007). The taxonomic position of the species belonging to *Bacillus* can be precisely identified by molecular techniques including sequencing of the 16S rDNA (Goto et al., 2002; Ki et al., 2009; Mohamed and ElSersy, 2009). In addition, the characterization of *Bacillus* group members can be evaluated using polyphasic approaches. To our knowledge, few publications are devoted to the study of the *Bacillus* species isolated from marine aquacultures.

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Abbreviations: TVC, Total viable count; **Mg,** magnesium; **KCl,** potassium chloride; **HCl,** hydrochloric acid; **PCR,** polymerase chain reaction; **EDTA,** ethylene diamine tetraacetic acid; **DDBJ,** DNA data base Japan; **H_2S,** hydrogen sulphide.

However, *B. licheniformis* is one of the commonly found *Bacillus* species in the marine habitats (Ivanova et al., 1999).

The aim of the present paper is to study the taxonomic affiliation and to characterize some hemolytic Bacilli isolated from *Penaeus japonicas* and *Paphia undulate* aquacultures using a polyphasic approach. This will lead to study the possibility of using them as probiotic tool in aquaculture future researches.

MATERIALS AND METHODS

Sample collection

Water samples were collected from different invertebrate aquaculture tanks, shrimp (*P. japonicas*) and clam (*P. undulate*). However, water samples were collected from different shrimp growth phase tanks, such as, eggs, Zoea and larvae.

Bacterial isolation and enumeration

Water samples were diluted up to 10^{-6} in sterile sea water. To estimate total viable count (TVC), 1 ml of each dilution were cultivated on sea water agar using poor plate method and the plates were incubated at 28°C for 24-48 h.

For detecting spore forming *Bacillus*, the samples were subjected to spore formation test (Elsersy, 2001). All resulted colonies were examined microscopically for Gram stain and spore formation. *Bacillus* strains were kept on nutrient agar slants for further investigations. Resulted data were the mean of triplicate tests.

Hemolytic activity test

All isolated spore forming *Bacillus* strains were tested for their hemolytic activity. This activity was determined as a clear zone of hemolysis around colonies on human blood agar plates with 5% (v/v) (Brender and Janda, 1987; Abou-Elela et al., 2009). *Bacillus* spore former isolates with the highest hemolytic activity (largest hemolytic zone diameter) were selected for further investigations.

Culture media

All culture media which were used for isolation were of pure grade and purchased from Difco, Detroit, USA, and prepared according to the manufacturer's instructions.

Phenotyping

Pure cultures were examined for colony morphology using a simple stain, safranine, Gram reaction and sporulation ability, after incubation in 2XSG plates at 35°C for 24 h. Biochemical characterization was performed using API 20E kit (Biomereux, France) . 0.1 ml of overnight *Bacillus* cultures were used to inoculate each of the 22 wells, containing freeze-dried test substrates. The inoculated strips were incubated at 37°C for 24 h.

Molecular techniques

Plasmid isolation

Plasmids, which may have genes that code for hemolysins, were isolated as described by Ombui et al. (1996) with some modifications.

Plasmid curing

In order to use *Bacillus* isolated strains as probiotic bacteria in aquacultures, a trial to remove the hemolysin gene(s) was applied. Plasmid curing was done using acridine orange as described by Trevors (1985).

DNA extraction, amplification and purification

DNA was extracted from *Bacillus* cultures and purified using EZNA bacterial DNA extraction kit (Omega Biotek, USA). The 16S rDNA region was amplified (approx 1500 bp) by polymerase chain reaction (PCR) using the forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse one 5'-TACGGYTACCTTGTTACGACTT -3'. The PCR mixture consists of 30 pmol of each primer, 100 ng of DNA, 200 µM dNTPs, 1.5 mM magnesium (Mg) Cl2, 20 mM potassium chloride (KCl), 10 mM tris-hydrochloric acid (HCl), pH 8.3, and 2.5 U of Taq polymerase (Pharmacia Biotech, USA). The 50 µl PCR mixture containing tube was placed in the DNA thermocycler, Gene cycler ^{TM} BIO- RAD, USA. The PCR conditions were as follows: initial denaturation of DNA at 95°C for 3 min and then 30 cycles of three-step PCR amplifications consisting of denaturation at 94°C for 1 min, primer reannealing at 55°C for 1 min and extension at 72°C for 2 min. Samples were subjected to an additional extension at 72°C for 10 min at the end of the amplification cycles (Ausubel et al., 1999). The amplicons were finally purified using QIA quick PCR purification kit (QIAgen, USA).

Gel electrophoresis

For plasmid running, 15 µl of the plasmid DNA, mixed with the loading buffer, were loaded on a 1.5% (w/v) agarose gel. 10 µl of PCR products, mixed with the loading dye, were loaded on a 1X agarose gel. Electrophoresis was carried out with 1X TEA (Tris EDTA Acetate) buffer. DNA was visualized by UV transillumination after ethidium bromide staining. The molecular sizes of the isolated plasmids and the amplified fragments were estimated using Lambda DNA/Ecor1 marker and 100 bp of DNA ladder, respectively.

Sequencing, data analysis and phylogeny

Sequencing was performed using 3130 X Sequencer (Genetic analyzer, Applied Biosystems, Hitatchi, Japan), BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and the same primers used for PCR. Homology search was done against DNA Data Base Japan (DDBJ), using Blast program to find the sequences producing significant alignment with the obtained ones. Similarity percentages among the sequences were obtained using Biology WorkBench software version 3.2. Multisequence alignment and molecular phylogeny were performed using ClustalW (a distance-based analysis program at DDBJ) program. The tree topology was evaluated using the neighbor -joining method (Saitou and Nei, 1987) based on 1000 resamplings.

RESULTS

Bacillus isolates were Gram positive, spore forming and

Table 1. Viable bacterial count of *P. Japonicas* and *P. undulate* aquaculture.

Sample origin (tank name)	TVC (CFU/ml)	Spore former *Bacillus* count (CFU/ml)	*Bacillus* sp. with hemolytic activity count (CFU/ml)
P. japonicas eggs	120± 0.07	50±0.006	12±1.02
P. japonicas zoea	200±1.2	10±0.05	2±0.06
P. japonicas larvae	100±0.02	40±0.034	15±0.003
P. undulate	80±0.02	33±0.05	30±0.07

± Standered error.

Table 2. Codes of some bacterial isolates and their hemolytic zones diameters (mm).

Bacterial code no. (Shrimp)*	Hemolytic zone (mm)	Bacterial code no. (Clam)**	Hemolytic zone (mm)
SH 1	10	CL 1	12
SH 2	14	CL 2	15
SH 4	11	CL 3	13
SH 5	13	CL 4	14
SH 12	8	CL 15	7
SH 10	9	CL 22	9
SH 20	11	CL 30	7

*SH, Shrimp; **CL, clam.

rod-shaped bacteria. They comprise 5 to 42% from the TVC of the different shrimp and clam aquacultures (Table 1). However, among the *Bacillus* isolates, hemolytic spore formers have been detected at high concentration, especially in *P. undulate* aquacultures (99%) (Table 1). Seven hemolytic isolates, four from clam and three from shrimp aquacultures, have been subjected to further investigation due to their significant hemolytic activity (Table 2), especially CL2 and CL4 (Figure 1) which have been isolated from clam aquacultures. Biochemical characterization using API 20E kit has been performed and the results are shown in Table 3. All of the tested *Bacillus* isolates can utilize D- glucose, D -mannitol and D-sucrose. On the other hand, neither of them can utilize inositol, D-rhamnose nor D-melibiose. Only SH2, a shrimp aquaculture isolate, can utilize D-sorbitol. Besides, SH5 and CL4, a clam aquaculture isolate, are distinguished due to their ability to utilize L-arabinose.

All isolates can produce enzymes such as catalase, oxidase, urease, gelatinase, arginine dihydrolase and others. SH4 can be differentiated by its inability to produce -galactosidase. SH5 is exceptional in production of hydrogen sulphide (H_2S) and only SH4 and CL1 cannot utilize amygdaline.

For more characterization, three isolates, CL2, CL4 and SH2, were subjected to some molecular investigations. Plasmid isolation, which may carry genes coding for the hemolysins, was carried out and showed no differentiation among the tested organisms. Each of them has two different plasmids. One of them is approximately 21.226 kbp and the other one has slightly more weight (Figure 2). Plasmid curing using acridine orange showed no change in the hemolytic activity for all of the tested strains (data not shown).

The precise differentiation among the three tested isolates was successfully performed upon isolation of the DNA, amplification of the 16S rDNA, approx 1500 bp, (Figure 3 (only CL2 DNA was shown)) and sequencing of its nucleotides. The sequences were deposited in the GenBank and the accession numbers are illustrated in Table 4.

Phylogentic analysis of the target sequences and other homologous ones from the data base revealed that the new isolates are strongly belonging to three different *B. licheniformis* strains, divided within three separate clusters (Figure 4). Therefore, SH2, CL4 and CL2 were given the names *B. licheniformis* NE-1, *B. licheniformis* NE-2 and *B. licheniformis* NE-3, respectively. Biology WorkBench 3.2 program showed that the three tested strains have similarity percentages ranging from 97 to 99 with some *B. licheniformis* strains collected from the GenBank (Table 5).

DISCUSSION

Ganesh et al. (2010) have demonstrated that the generic composition of the total heterotrophic bacterial isolates from an Indian aquaculture bond is basically formed of *Vibrio, Pseudomonas, Bacillus* and *Micrococcus* spp. In

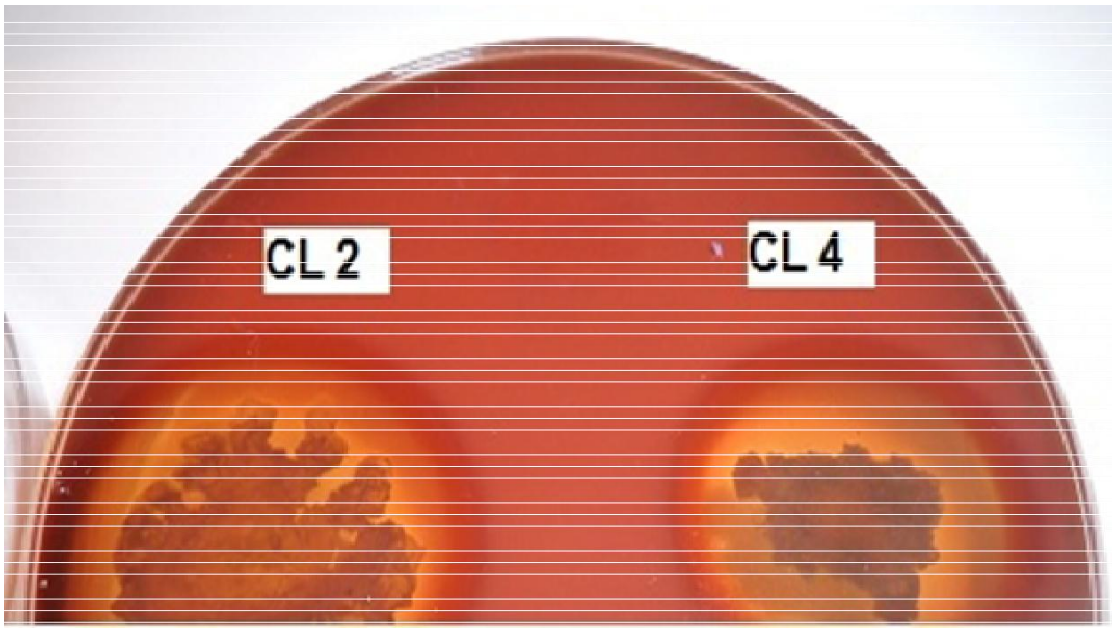

Figure 1. The hemolytic activity of two *Bacillus* isolates coded as CL2 and CL4 isolated from clam samples.

Table 3. Characterization of the isolated *Bacillus spp* using API 20E.

SH5	SH4	SH2	CL4	CL3	CL2	CL1	Characteristic
+	-	+	+	+	+	+	-Galactosidase
+	+	+	+	+	+	+	Arginine dihydrolase
+	+	+	+	+	+	+	Lysine decarboxylase
+	+	+	+	+	+	+	Omithine decarboxylase
+	+	+	+	+	+	+	Citrate utilization
+	-	-	-	-	-	-	H_2S production
+	+	+	+	+	+	+	Urease production
+	+	+	+	+	+	+	Tryptophan deaminase
-	-	-	-	-	-	-	Indole production
+	+	+	+	+	+	+	Acetoin production
+	+	+	+	+	+	+	Gelatinase production
+	+	+	+	+	+	+	D-Glucose utilization
+	+	+	+	+	+	+	D-Mannitol utilization
-	-	-	-	-	-	-	Inositol utilization
-	-	+	-	-	-	-	D-Sorbitol utilization
-	-	-	-	-	-	-	D-Rhamnose utilization
+	+	+	+	+	+	+	D-Sucrose utilization
-	-	-	-	-	-	-	D-Melibiose utilization
+	-	+	+	+	+	-	Amygdalin utilization
+	-	-	+	-	-	-	L-Arabinose utilization
+	+	+	+	+	+	+	Oxidase production
+	+	+	+	+	+	+	Catalase production

addition, many *Bacillus* spp have been successfully isolated from shrimp and clam aquacultures (Bourne et al., 2004; Balcazar and Rojas-Luna, 2007; Romanenko et al., 2008). The culturing technique used in this study has focused on marine *Bacillus* as spore forming-bacteria which can be easily isolated and abundantly found in shrimp and clam aquacultures. Due to their significant hemolytic activity in comparison with the other isolated

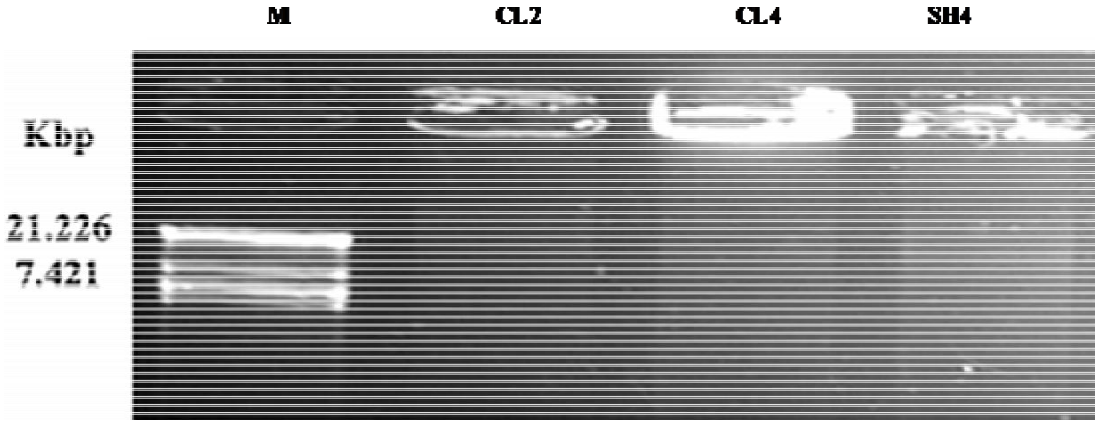

Figure 2. Plasmid isolation from three *Bacillus* spp. isolated from clam, CL2 and CL4, and shrimp, SH4, aquacultures.

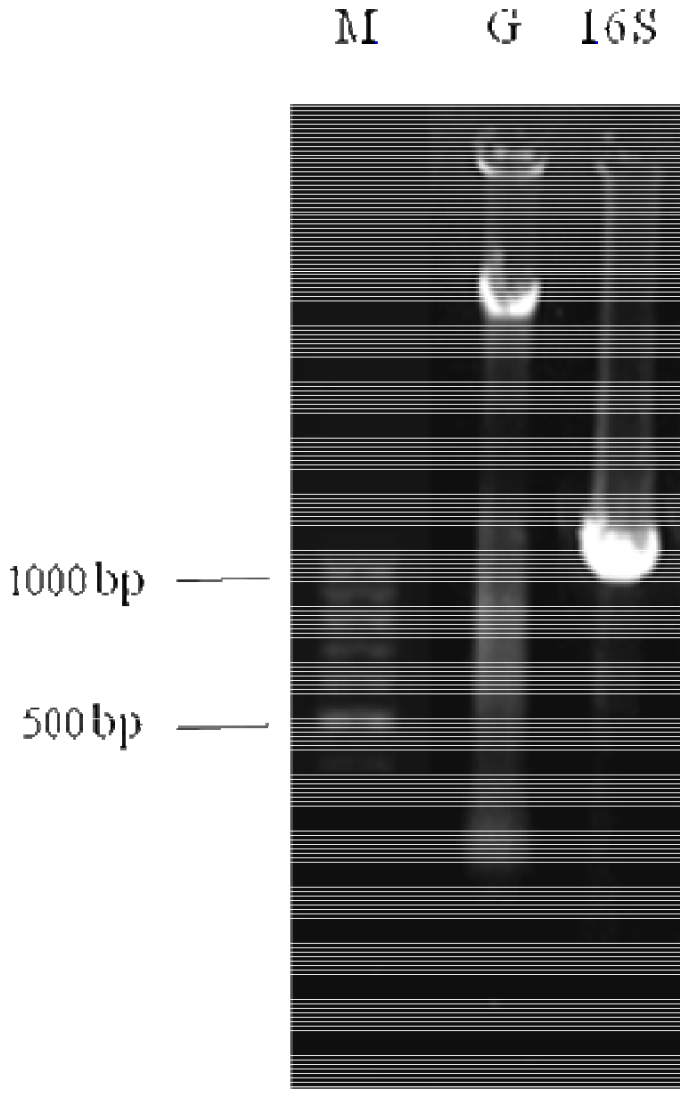

Figure 3. Gel electrophoresis of the isolated genomic DNA, G, and the amplified 16S rDNA region (approx 1500 bp), 16S, from the clam aquaculture isolate, CL2. M, 100 bp genetic marker.

Table 4. Accession numbers of *Bacillus* strains.

Bacillus	Accession number
B. licheniformis NE-1	HM776580
B. licheniformis NE-2	HM776581
B. licheniformis NE-3	HM776582

spore forming *Bacillus*, seven hemolytic bacteria were selected for further investigation using API 20E kit. Although the API 20E system showed, to some extent, a variation among the seven hemolytic isolates, the accurate characterization was achieved upon partial sequencing of the 16S rRNA gene. The phylogenetic analysis of the target sequences revealed a strong relatedness of the isolates to *B*. *licheniformis.* Using the 5' end of the 16S rDNA has been successfully used in many studies to identify *Bacillus* spp. (Goto et al., 2002; Mohamed and ElSersy, 2009). In this study, the 16S rDNA hyper variant region (Goto et al., 2002) not only located the strains in its precise taxonomic position, but also exhibited a discriminating tool among the target sequences (1-3% sequence differences) . Although *B*. *cereus* is a well known hemolytic strain in the genus *Bacillus* (Liu et al., 2009), there are little reported on hemolytic activity of *B*. *licheniformis*. Hemolysis is often associated with pathogenicity of strains because hemolysins have been considered to contribute to the virulence (Wekh et al., 1981; Waalwlk et al., 1982; Hughes et al., 1983). For example, Staphylococcal -hemolysin (Phillips et al., 2006), Streptolysin-O (Kehoe et al., 1987), and *E. coli* hemolysin (Beutin, 1991) are all major pathogenecity factors that can cause a variety of diseases in humans (Bhakdi et al., 1996). Many toxins have been reported to be produced by *Bacillus* as well. For instance, *B*. *cereus* produces a number of cytotoxins and enzymes that could contribute to the rapid course and severity of endophthalmitis including hemolysins,

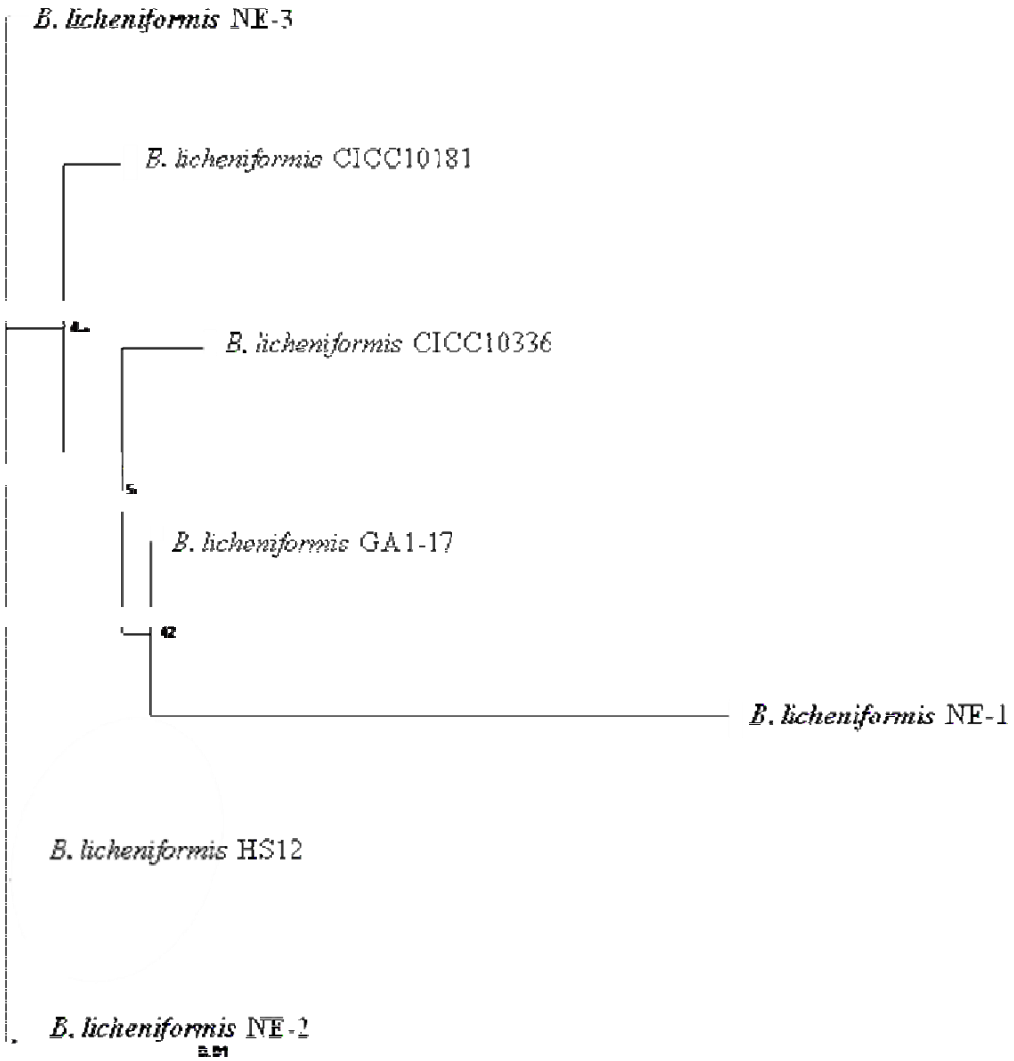

Figure 4. Phylogenetic position of *B. licheniformis* NE-1 (SH2), *B. licheniformis* NE-2 (CL4) and *B. licheniformis* NE-3 (CL2), based on partial sequences (approx 400 bp) of the 16S rRNA gene. The tree constructed by neighbor-joining method using ClustalW software. The scale indicates substitutions per site.

Table 5. Similarity percentages among *B. licheniformis* NE-1, *B. licheniformis* NE-2, *B. licheniformis* NE-3, and other homologous *B. licheniformis* strains from the data base.

***B. licheniformis* strain**	**NE-1**	**NE-2**	**NE-3**	**CICC10181**	**HS12**	**CICC10336**	**GA1-17**
NE-1		97	97	98	98	98	98
NE-2			99	99	99	98	99
NE-3				98	99	98	98
CICC1018					99	99	99
HS12					99	99	99
CICC10336							99
GA1-17							

lipases,enterotoxins, and proteases (Turnbull, 1981; Davey and Tauber, 1987; Drobniewski, 1993). Moreover, hemolysin BL which is produced by *B. cereus* has a severe toxicity (Callegan et al., 1999).

Despite being hemolytic, the three isolates *B. licheniformis* strains, NE-1, NE-2 and NE-3, have the ability to produce some highly benefit enzymes for aquacultures. Therefore, they can be probiotic if hemolysin gene(s) are removed. Plasmids, which may harbor these hemolysin genes, have been isolated from r

the three strains under test and the gel electrophoresis revealed that there are only two plasmids for each strain. One of them is approx 21.226 kbp and the other is slightly larger. However, plasmid curing using acridine orange did not affect the hemolytic activity. Although this finding may indicates that the genes coding fohemolysins are mostly chromosomal, different methods for plasmid curing must be used to confirm this finding. In a previous study (Kamat and Nair, 1992), a strain of *B. cereus*, isolated from shrimps, harbored a 7.6 mDa plasmids. When cells were cured, they still produce hemolytic toxins. Besides, some secondary metabolites of *Bacillus* could cause hemolysis too (Liu et al., 2009).

Conclusion

A differentiation among some *B. licheniformis* strains recently isolated from shrimp and clam aquacultures has been achieved using polyphasic approach including biochemical and molecular characterizations. The 16S rRNA gene partial sequencing revealed a strong tool for both identification and discrimination. Although plasmid profiling showed no significant differences among them, an interesting finding was gained. The hemolytic activity in marine *B*. *licheniformis* is not necessary to be harbored in plasmids and may be chromosomal. Very few reports had studied the hemolytic activity of marine *B*. *licheniformis* and more study is needed to be conducted to characterize the hemolysin genes in *B. licheniformis.*

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