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Isolation, identification and characterization of *Lactobacillus* on black tiger shrimp

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Abstract
Shrimp farming has been a significant agro based economic activity. Shrimp farming shows a booming expansion. But there are also many risks and challenges, especially in the culture techniques, the degradation of environmental quality, biodiversity and natural brood stocks. Pathogenic microorganisms implicated in these outbreaks are viruses, bacteria, algae, fungi and protozoan parasites. The most prevalent bacterial disease is Vibriosis which causes a mass mortality both in larval cultures and shrimp production. In this research we have successfully isolated 5 species of *Lactobacillus* having ability to compete with 7 species of *Vibrio* spp. Among 5 species of *Lactobacillus* having capability to resist with *Vibrio* spp., we define two *Lactobacillus* species L1.2 and L1.3 having the strongest resistant capability after 24 hours of cultivation. We have also noticed optimal growing conditions for these *Lactobacillus* species: L1.2: temperature 37 ^{o}C, pH= 6.5 – 7.0, cultivation time to get biomass 24 – 28 h, salinity resistance 5%; L1.3: temperature 34 ^{o}C, pH= 6.0 – 7.0, cultivation time to get biomass 20 – 24 h, salinity resistance 5%. Both two species L1.2 and L1.3 have some main characteristics: rod shape, no mobile, lactic acid formation, sugar fermentation (glucose, saccharose, sucrose, mantose, manitol, and sorbitol).

Keywords: Isolation, identification, characterization, *Lactobacillus*, black tiger shrimp

1. Introduction
Aquaculture is a worldwide activity and considered as a major economic and food production sector as it is an increasingly important source of protein available for human consumption. Shrimp farming is an aquaculture business; that is, it exists in either marine or freshwater environment, producing shrimp or prawns. Over the past five years, there have been major developments in shrimp farming (Bestha Lakshmi *et al.*, 2013).
The major virulent strains of vibrio's in shrimp are *Vibrio alginolyticus, V. anguillarum, V. harveyi* and *V. parahaemolyticus.* Successful shrimp culture requires a combination of factors including pathogen free larvae, nutritious feed, good aeration, salinity etc. The abuse use of antimicrobial drugs, pesticides, and disinfectants in aquaculture has the evolution of resistant strains of bacteria and concern of the society (Petlu Nitya Jeevan Kuma *et al*. 2013). Serious viral disease outbreaks of shrimp challenge the shrimp industry to be better prepared in the view of a broadened knowledge about shrimps and their pathogens so that disease prevention methods could be improved (S. Mohapatra *et al.*, 2012).
The beneficial effect of the application of certain beneficial bacteria in human, pig, cattle and poultry nutrition has been well documented. However, the use of such probiotics in aquaculture is a relatively new concept (Sirirat Rengpipata *et al.*, 2000; Ali Farzanfar, 2006; Watchariya Purivirojkul *et al.*, 2013). The probiotics are recently used in aquaculture to promote growth rate of cultured organisms or to inhibit growth of pathogen (Jose Luis Balcazar *et al.*, 2006). The investigation of *Lactobacillus acidophilus* as probiotics on growth performance, food conversion ratio and gut microbial load was well investigated (G. Chelladurai *et al*., 2012; C. N. Ariole, G. E. Nyeche , 2013; Natesan Sivakumar *et al.*, 2012; Natesan Sivakumar *et al*., 2014).
The aim of the our research is to isolate, identify and characterize indigenous strains of *Lactobacillus* from the gut of healthy shrimp (*Penaeus monodon*)

2. Material and Method
2.1 Material
We use the samples of black tiger shrimp (*Penaeus monodon*) from Mekong river delta. The isolation samples are derived from gut of shrimp.

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2.2 Research method

2.2.1 Isolation and identification

2.2.1.1 Isolation of Lactobacillus

We conduct the isolation of *Lactobacillus* species from gut of black tiger shrimp on MRS medium. We weigh 5 gram of black tiger shrimp gut, put into PE bag, add 45 ml proliferation broth having dilution 10^{-1}, mix 1 minute in stomacher. Then we incubate it in incubator at normal temperature. After 24 h, we dilute it into different dilutions from 10^{-2} to 10^{-7}. Take 0.1 ml of sample from dilutions 10^{-5}, 10^{-6}, 10^{-7} inoculate on to agar dishes, incubate at 37 ^{o}C in 24-48 hours. After that, we observe morphology and color of colonies to determine species belongs to *Lactobacillus* spp. They are collected and preserved the pure species in agar culture tube.

2.2.1.2 Cultivation and preservation of Lactobacillus species

Culture preservation is very important to maintain its activity. In order to activate the culture, people use rich nutrients such as yeast extract, vitamin, fatty acid. The preservation process is conducted as follows: The isolated *Lactobacillus* species are kept on slant agar medium. We select Petri dishes having pure colonies, inoculate colonies on agar MRS medium, keep at 4 – 6 ^{o}C, transfer inoculation 2-3 months per once. After determining the probiotic characteristics of isolated species, we keep these species in MRS liquid containing 30 – 50% glycerol. The selected *Lactobacillus* species is cultivated in MRS liquid at 28 - 30-C, shaking at 180 cirlces/minutes until the growth curve comes to logarit phase. Take the cultured fluid into eppendoff having glycerol with 30 – 50% volumn, votex keep in 4-6 ^{o}C during 30 minutes before preserving at –70 ^{o}C. By this preservation in glycerol, we can keep these species 6-12 months.

2.2.1.3 Identification of Lactobacillus species having resistant capability to *Vibrio* spp.

Vibrio species is isolated from black tiger shrimp. In preservation medium, *Lactobacillus* and *Vibrio* are kept in MRS (*Lactobacillus),* APW *(Vibrio)* and then activated at 37 ^{o}C. When *Vibrio* reaches 10^4 CFU/ml and *Lactobacillus* reaches 10^5 CFU/ml, *Vibrio* is inoculated on LB medium. Prepare holes (5 mm) on agar medium, take 50 µl of *Lactobacillus* species into these holes. Incubate these dishes at 37 ^{o}C in 24-48 hours, observe these cirlces and determine diameters of the resistant circles. In order to select *Lactobacillus* species resistant to *Vibrio* with high reability, we perform 2-3 rounds of testing.

2.2.2 Characterization of morphology and biochemistry

2.2.2.1 Morphological characteristics

Observe morphological characteristics of bacteria under microscope: *Lactobacillus* species are cultured on MRS medium, shaked 200 rpm at 28 – 30 ^{o}C. After 24 hours, we collect broth to observe bacterial cells. Gram staining: *Lactic* bacteria belong to Gram (+), rod or ovan shape.

2.2.2.2 Biochemical characteristics

- Lactic acid formation: If colonies have decomposition circles, they are able to form lactic acid.
- Catalase reaction: Use loop to inoculate pure colonies on MRS to lame, drop H_2O_2 30% onto colonies. If there is bubble, we confirm there is catalase in cells.
- Mobility: *Lactobacillus* species are inoculated on agar medium having MRS 5-6 cm, incubated at 30 – 35^{o}C in 2 days. If there are colonies along the inoculating trace, we confirm their mobility.
- Sugar metabolism: Prepare medium meat extract 3 g; Pepton 10 g; NaCl 5 g; phenol red 0.03 g; distilled water 1000 ml. Add sugar 0.5%, adjust pH 7.2 ± 0.2. Dispense medium into culture tube, each tube 5 ml. Insert 1 cone Durham to collect CO_2 if bacteria use sugar. Sterilize them within 10 minutes at 121 ^{o}C. Some sugars are verified including glucose, lactose, mantose, manitol, saccarose, sucrose.

2.2.3 Cultivation conditions

2.2.3.1 Growth

Lactobacillus species are cultured on liquid MRS at 20-30 ^{o}C, shaked at 180 rpm. Take the initial samples and after 3 hours to establish growth curve by measuring optical density (ΔOD_{600nm}).

2.2.3.2 Effect of cultivation temperature

Lactobacillus species are cultivated on liquid MRS medium. We verify different cultivation temperature for *Lactobacillus* at 30, 33, 37, 40 ^{o}C. After 12 hours and 24 hours, we measure pH and optical density.

2.2.3.3 Effect of cultivation time

Lactobacillus species are cultivated on liquid MRS medium. We verify different cultivation time for *Lactobacillus* at 0, 8, 12, 16, 18, 22, 26, 30, 34, 38, 42 hours. We measure pH and optical density.

2.2.3.4 Effect of pH in cultivation

Lactobacillus species are cultivated on liquid MRS medium. We verify different cultivation pH 4, 5, 6, 7, 8 for *Lactobacillus.* We measure pH and optical density after sau 0, 4, 8, 12, 16, 20, 24, 28, 32h.

2.2.4 Probiotic characteristics

2.2.4.1 Production of digesting enzyme

Lactobacillus species are cultivated on liquid MRS medium, shaked at 180 rpm, 30 ^{o}C. After 16-24 hours, we collect the broth by centrifugation at 8000 rpm in 15 minutes. Digesting enzyme is examined by diffusion method (casein with enzyme protease and starch with enzyme amylase).

2.2.4.2 Salinity resistance

Lactobacillus species are cultivated on liquid MRS medium with different NaCl concentration, shaked at 180 rpm, 30 ^{o}C in 24 hours. Check the viability of colonies by optical density.

3. Result & Discussion

3.1 Isolation and identification

3.1.1. Isolation of Lactobacillus species from gut of black tiger shrimp

We have isolated *Lactobacillus* from gut of black tiger shrimp on MRS medium at 37^{o}C. We code these species as follows: L1.1, L1.2, L1.3, L1.4, L1.5, L2.1, L2.2, L2.3, L2.4, L2.5, L2.6.

3.1.2 Identification of Lactobacillus species resistant to Vibrio spp

Table 1: Activity of 5 *Lactobacillus* species resistant to 7 *Vibrio* spp. on MRS medium at 37 °C

	Diameter of decomposition circles (mm)						
	V2.1	**V2.2**	**V2.3**	**V2.4**	**C1**	**C7**	**C23**
L1.2	15	14	20	18	15	22	20
L1.3	18	16	14	22	10	24	21
L1.4	12	16	13	12	10	14	17
L1.5	10	15	17	10	12	11	19
L2.2	13	14	16	13	13	10	11

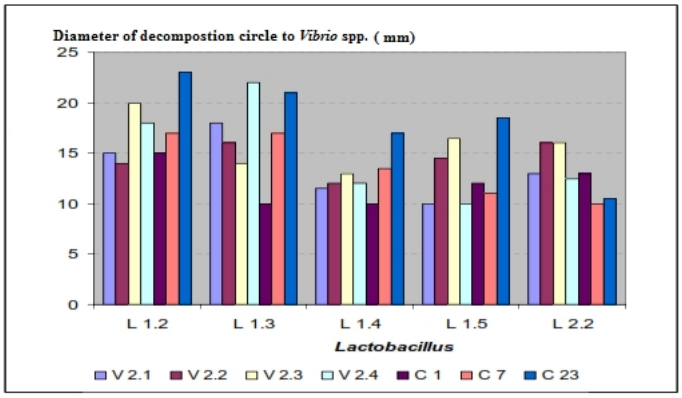

Fig 1: Activity of Lactobacillus species resistant to *Vibrio* spp. on MRS medium at 37 °C.

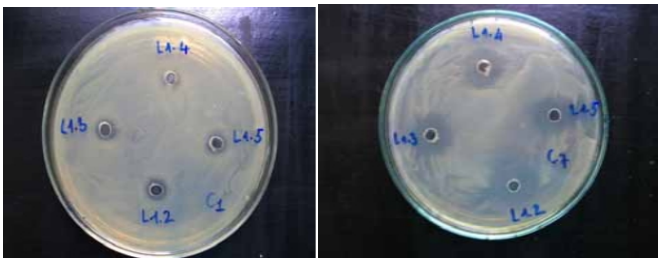

Fig 2: Activity of two *Lactobacillus* species resistant to *Vibrio* spp. on MRS medium at 28-30 °C.

From table 1, five *Lactobacillus* species are able to resistant to *Vibrio* spp. accounting for 45.45%. Among these species, two *Lactobacillus* species L1.2 and L1.3 are able to resist *Vibrio* effectively. So we choose L1.2 and L1.3 for further experiments.

3.2 Morphological and biochemical characteristics

3.2.1 Morphology

3.2.1.1 Morphology of species L1.2

Lactobacillus L1.2 is inoculated on MRS medium with 2% agar, incubated at 37 °C. After 24 hours we observe the colony morphology.

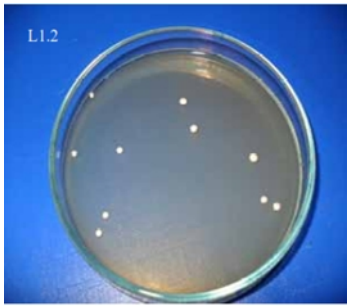

Fig 3: Morphology of *Lactobacillus* L1.2 on MRS medium after 24 h cultivation at 34 °C.

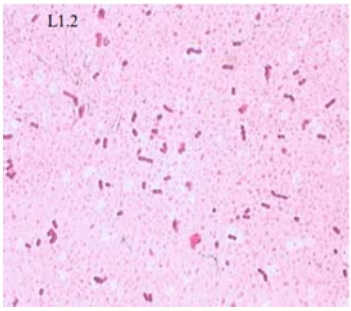

Fig 4: Cell morphology of *Lactobacillus* L1.2 under microscope 100X

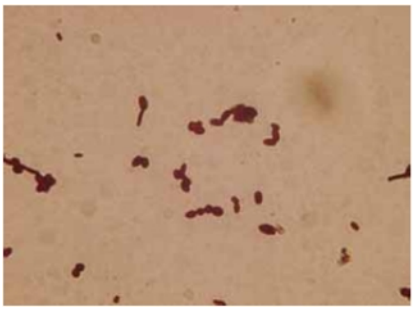

Fig 5: Gram staining of *Lactobacillus* L1.2

Lactobacillus L1.2 colonies have ovan shape, purple color when staining. So we can confirm this bacteria is Gram [+].

3.2.1.2 Morphology of species L1.3

Lactobacillus L1.3 colonies are inoculated on MRS with agar 2%, incubated 37 °C. After 24 hours of cultivation, we observe their morphology.

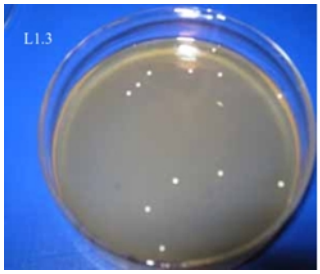

Fig 6: Morphology of *Lactobacillus* L1.3 on MRS medium after 24 h of cultivation at 34 °C

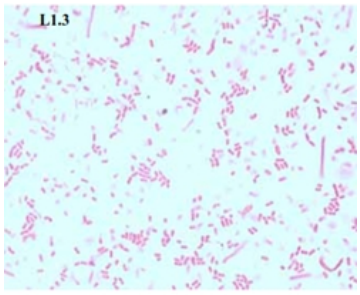

Fig 7: Cell morphology of *Lactobacillus* L1.3 under microscope 100X

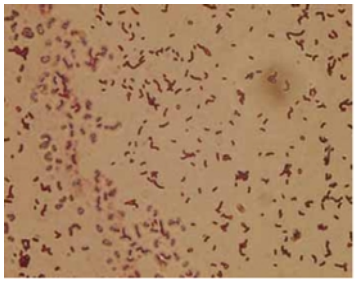

Fig 8: Gram staining of *Lactobacillus* L1.3

Lactobacillus L1.3 colonies have ovan shape, purple color when staining. So we can confirm this species is Gram [+].

3.2.2 Biochemistry

Fig 9: Mobility of *Lactobacillus* L1.2 and L1.3

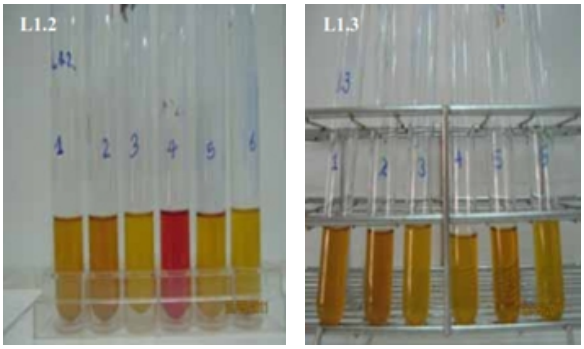

Fig 10: Sugar metabolism of *Lactobacillus* L1.2 and L1.3

Table 2. Characteristics of L1.2 and L1.3

Species	*Lactobacillus* L1.2	*Lactobacillus* L1.3
Lactic acid formation	+	+
Gram	+	+
Mobility	-	-
Catalase	-	-
Sugar metabolism	+	+
Starch	0	0

From table 2, we define that L1.2 and L1.3 are lactic bacteria. These species are selected for further experiments.

3.3 Cultivation conditions and probiotic characteristics
3.3.1 Growth curve of two Lactobacillus species L1.2 and L1.3

a) Lactobacillus L1.2

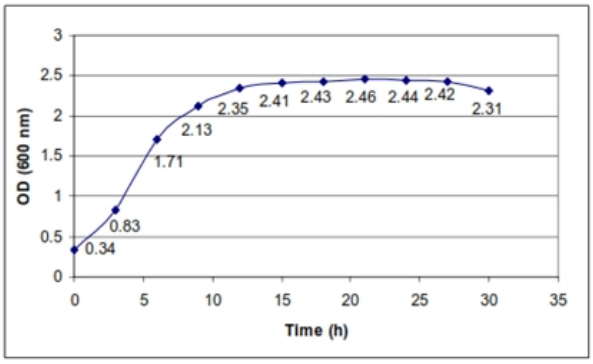

Fig 11: Relationship between cultivation time and optical density of *Lactobacillus* L1.2

b) Lactobacillus L1.3

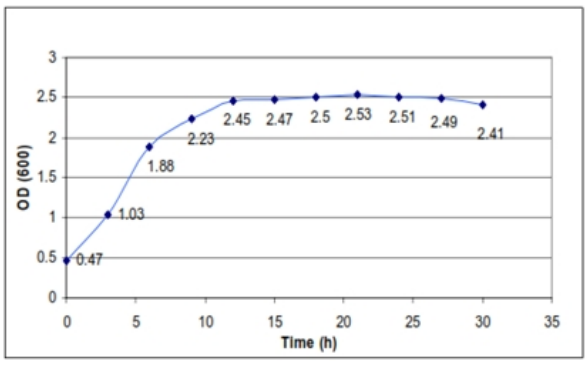

Fig 12: Relationship between cultivation time and optical density of *Lactobacillus* L1.3 From figure 12, *Lactobacillus* L1.3 comes to balance at 15h. We can collect biomass at 18 – 21h.

3.3.2 Effect of cultivation temperature to growth of two Lactobacillus species L1.2 and L1.3

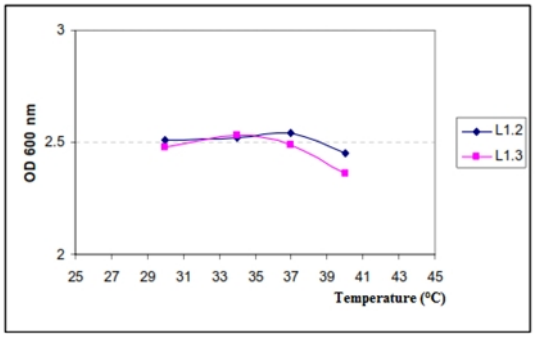

Fig 13: Effect of cultivation temperature to growth of two *Lactobacillus* species L1.2 and L1.3

Both two *Lactobacillus* species L1.2 and L1.3 are thermophilic. Optimal temperature for L1.2 is 35 -37ºC; meanwhile they are inactivated at 37 – 40 ºC. So 37 ºC is suitable for L1.2 growth. Optimal temperature for L1.3 is 33-35 ºC strongest at 34 ºC. So we choose 34 ºC for L1.3 growth.

3.3.3 Effect of cultivation time

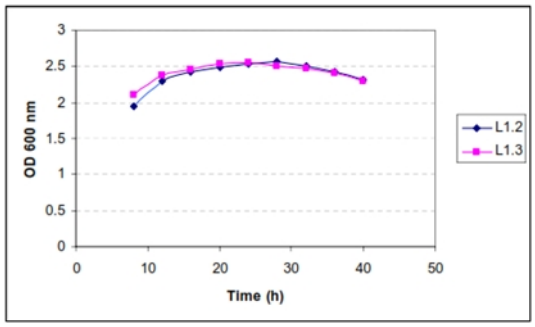

Fig 14: Relationship between cultivation time and living cell density of L1.2 and L1.3 at wave length 600 nm from figre 14, we see L1.2 can grow well and produce biomass after 24 - 28 h; L1.3 can grow well at 20 – 24 h.

3.3.4 Effect of cultivation pH

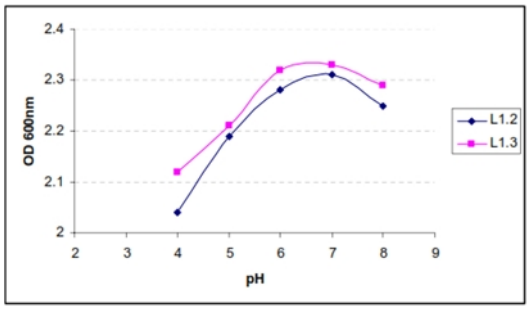

Fig 15: Effect of pH to growth of L1.2 and L1.3

From figure 15, L1.2 is optimal at pH 6.0-7.0. Meanwhile L1.3 is optimal at pH 6.0-7.5. We decide to choose pH 6.5-7.0 for cultivation of L1.2 and L1.3 to get biomass

3.3.5 Salinity resistance of two Lactobacillus species L1.2 and L1.3

Lactobacillus L1.2 and L1.3 are able to withstand NaCl 5%. So it's ideal for probiotic in shrimp farming at brackish region.

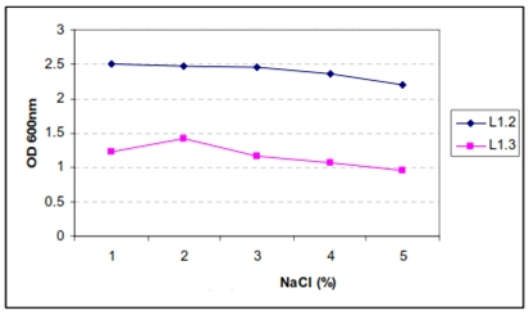

Fig 16: Effect of NaCl concentration to growth of L1.2 and L1.3

4. Conclusion

Shrimp farming is an aquaculture business for the cultivation of marine shrimps or prawns for human consumption and is now considered as a major economic and food production sector as it is an increasingly important source of protein available for human consumption. Intensification of shrimp farming had led to the development of a number of diseases, which resulted in the excessive use of antimicrobial agents, which is finally responsible for many adverse effects. Currently, probiotics are chosen as the best alternatives to these antimicrobial agents and they act as natural immune enhancers, which provoke the disease resistance in shrimp farm. Viral diseases stand as the major constraint causing an enormous loss in the production in shrimp farms. Probiotics besides being beneficial bacteria also possess antiviral activity. Exploitation of these probiotics in treatment and prevention of viral diseases in shrimp aquaculture is a novel and efficient method. This research investigates the isolation, identification and characterization of *Lactobacillus* on black tiger shrimp as probiotics and their role in immune power enhancement towards viral diseases. The application of effective probiotics in shrimp aquaculture is an excellent alternative for chemicals and antibiotics to prevent disease control.

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