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Bacillus tequilensis, a novel unionized ammonia-nitrogen degrading bacterium isolated from tilapia pond soil

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Abstract
The objective of this study was to isolate, characterize and identify unionized ammonia-nitrogen (NH_3-N) degrading bacteria from tilapia pond soil. Eight bacterial colonies were isolated from diluted soil sample based upon colonial characteristics and growth pattern in solid and liquid medium. The isolated colonies were subjected to NH_3-N degrading experiment. In all isolates used, isolate C showed the most reliable result after 72 hours incubation: 99.32%, 4.76%, 56.00%, 54.55% and 40.00% NH_3-N reductions in experimental set-ups with 10, 20, 30, 40 and 50 mg/L Ammonium Chloride (NH_4Cl), respectively. Morphological, biochemical and physiological tests revealed that Isolate C was Gram-positive, rod shape, positive to catalase and citric acid utilization tests, negative to urea hydrolysis and phenylalanine deamination tests, and had the ability to grow in medium supplemented with 6.5% salt. Isolate C was subjected to DNA isolation, purification and sequencing. BLAST analysis of sequences derived from both revealed that the isolate belongs as to genus *Bacillus*. Highest hits indicated that the species is *tequilensis*. This is one of the first studies that describe the ability of the bacterium to degrade or take-up NH_3-N in their cells.

Keywords: Aquaculture, tilapia, pond soil, *Bacillus tequilensis*, unionized ammonia-nitrogen

1. Introduction
Aquaculture is the farming and harvesting of freshwater and marinewater aquatic organisms such as fishes, shellfish, mollusks and aquatic plants within a controlled environment [1]. It is one of the fastest developing sectors in the world and Asia presently contributes about 90% of the global production [2].

In culture system, pond soil plays an important role for the growth and survival of the aquatic organism [3]. Pond soil serves as a biological filter through the adsorption of organic residues of feed, fish excretions and algal metabolites, and provides all the important nutrients in water [4]. Bacteria perform important functions in the soil such as decomposing of organic residues from enzyme release on it [5].

In aquatic environment, ammonia-nitrogen is one of important Nitrogen (N) forms that can be used by phytoplankton, algae, plants, heterotrophic bacteria and nitrifying bacteria [6]. The ammonia-nitrogen in aquaculture systems primarily comes from fertilizers such as urea, ammonia phosphate and ammonia nitrate [7]. fish excretion [8]. diffusion from the sediment [9]. and from aquaculture discharges [10]. Ammonia-nitrogen is considered the next important factor after low dissolved oxygen concentration that limits the amount of fish that can be produced in culture system [9]. The sub-lethal or lethal effects of ammonia in sensitive fish include gill damage, reduction of blood oxygen-carrying capacity, lack and depletion of adenosine triphosphate (ATP) in the brain and liver, and malfunction of kidneys [11].

There are several ways or practices to reduce ammonia-nitrogen concentration in pond water that include adding an acid to lower pH, applying an algicide to lessen phytoplankton and exchanging water in ponds to flush out ammonia-nitrogen [9]. However, such treatments are expensive [9]. One affordable remedy to manage high ammonia-nitrogen in water is the application of chopped hay [8]. This practice, however, requires large amount of organic carbon and increases the oxygen demand [8].

A number of bacteria in ponds are involved in constant cycling of ammonia [8]. Live bacteria as amendments in ponds to lessen ammonia-nitrogen is becoming popular in Asia [12].

This method is acknowledged to be a more economical and efficient [12].

This study was conducted in order to: (1) isolate and culturally characterize bacterial colonies from tilapia pond soil; (2) evaluate the unionized ammonia-nitrogen (NH_3-N) degrading ability of the bacterial isolates; (3) characterize the NH_3-N degrading bacteria using selected biochemical and physiological tests; and (4) identify the NH_3-N degrading bacteria using16S Ribosomal RNA (16 S rRNA) sequencing.

2. Materials and Methods

2.1 Collection of soil samples

Composite soil samples were collected around the pond perimeter using an improvised soil borer at a depth of 5 cm. The collected samples were placed in disinfected plastic bags and transported in the laboratory for immediate analysis.

2.2 Isolation of bacterial colonies

Two series of 10-fold dilutions (10^{-3} and 10^{-4}) of tilapia pond soil was made in sterile distilled water. One hundred microliters of the diluted sample was streaked in Trypticase Soy Agar (TSA) plates. The plates were incubated at 37˚C for 18 to 24 hours.

2.3 Cultural characterization of bacterial colonies

Bacterial colonies grown in TSA plates were sorted based on their appearances on the medium. Cultural characters such as size, shape, margin, elevation and surface texture, and growth pattern in nutrient agar and broth were considered. Colonies that exhibit different cultural characters were used in the next phase of the study.

2.4 NH_3-N degrading experiment

Glass bottles with 250 mL capacity were filled up with 50 mL sterile distilled water and 0.5% carbon substrate (glucose). Five milliliter of bacterial suspension with approximate cell density of 1 x 10^8 cells/mL (turbidity of 0.5 MacFarland standards) was added in each bottle. The Ammonium Chloride (NH_4Cl) concentration in the bottles was adjusted to 10, 20, 30, 40 and 50 mg/L. The control bottle was not provided with bacterial suspension. The concentration of total ammonia nitrogen (TAN) at 0 hour and after 72 hours incubation was determined using the prescribed procedures in the laboratory manual of Aquatic Ecology of the College of Fisheries, Central Luzon State University. An online application was used to compute the NH_3-N provided that the absorbance of TAN, pH and temperature were available. Data on absorbance of TAN, pH and temperature of each glass bottle served as inputs on the spaces provided in the spreadsheet and automatically the NH_3-N concentration was computed. Percent NH_3-N reduction was computed using the below formula:

$$\text{Percent } NH_3\text{-N reduction} = \frac{NH_3\text{-N control} - NH_3\text{-N sample}}{NH_3\text{-N control}} \times 100$$

Bacterial colonies that were consistent in reducing NH_3-N were used in the next phase of the study.

2.5 Gram staining and selected biochemical and physiological tests

2.5.1 Gram staining

The unknown bacterium was streaked on TSA and was incubated at 37 ˚C for 18 to 24 hours. A smear was prepared by mixing a small amount of growth with a drop of distilled water. The smear was air dried and fixed by heat. The glass slide was labeled properly. The dried smear was stained with crystal violet for 1 minute and was rinsed thoroughly with tap water. Afterwards, the smear was covered with Gram's iodine for 1 to 2 minutes and was washed with tap water. The smear was decolorized by dripping 95% ethanol and was washed immediately. Then, the smear was counterstained with safranin for 45 seconds and was washed by tap water. The slide was examined under microscope. Gram-positive bacterium should be colored blue while gram negative bacterium should be colored red. Cell size, shape and arrangement were also noted.

2.5.2 Catalase test

The unknown bacterium was streaked on TSA and was incubated at 30 ˚C for 18 to 24 hours. A loopful of the bacterium was transferred to a clean slide. One to two drops of freshly prepared 3% hydrogen peroxide (H_2O_2)was dropped onto the slide. Bubble formation indicates presence of catalase [13].

2.5.3 Citric acid utilization test

The unknown bacterium was inoculated to Simmon Citrate Agar (SCA) slant using a wire loop by stabbing the butt and streaking on the surface. The slant was incubated at 33 ˚C for 48 hours. Growth and shift of the green color to Prussian blue color means positive utilization of citrate.

2.5.4 Urea hydrolysis

The unknown bacterium was inoculated in Christensen's Medium (CM) Urea broth. The test tube was incubated at 35 ˚C for 4 to 6 hours. A red to violet color means positive test for urea hydrolysis.

2.5.6 Phenylalanine deamination test

The unknown bacterium was inoculated in Phenylalanine Agar (PA) slant. The slant was incubated at 33 ˚C for 18 to 24 hours. Four to five drops of 10% ferric chloride solution was added on the test tubes. The immediate appearance of an intense green color indicates positive phenylalanine deamination.

2.5.7 Growth in 6.5% Sodium Chloride (NaCl)

The unknown bacterium was streaked in TSA plate supplemented with 6.5% NaCl. The plate was incubated at 30 ˚C for 24 hours in clean incubator. The plate was observed for the presence of bacterial growth [13].

2.6 16S rRNA gene amplification and clean-up

Prior to amplification using PCR, each gDNA extract was purified using 0.6x AMPure XP beads. The gene amplification includes the following components: genomic DNA, 27F and 1492R universal 16S primers, Taq buffer, DNA polymerase and DNTP mix. Cycling parameters on thermal cycler were as follows: 95 °C for 5 minutes; 30 cycles of 95 °C for 1 minute, 60 °C for 45 seconds, 70 °C for 1 minute; 72 °C for 10 minutes and hold at 4 °C.

2.7 Capillary sequencing

The components in the incorporation of fluorescently labelled chain terminator ddNTPs include: amplicons, primers and AB BigDye® Terminator v3.1 Cycle Sequencing Kit. The cycling parameters on Bio-Rad T100 Thermal Cycler were as follows:

pre-hold at 4 °C; 96 °C for 1 minute; 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, 62 °C for 4 minutes; and hold at 4 °C. Ethanol precipitation was done to remove the unincorporated ddNTPs, excess primers and primer dimers. Capillary electrophoresis on the ABI 3730xl DNA Analyzer was done using a 50 cm 96 well capillary array, POP7TM polymer and 3730xl Data Collection Software v3.1. Base calling was done on the Sequencing Analysis Software v5.4.

3. Results and Discussions

3.1 Cultural characterization of bacterial colonies

Eight bacterial colonies were isolated from the 10^{-3} and 10^{-4} dilutions of composite soil samples from tilapia grow-out pond. The bacterial colonies were chosen based upon its colonial characteristics and growth pattern in Trypticase Soy Agar (TSA) and Trypticase Soy Broth (TSB) as shown in Table 1.

Table 1: Colonial characteristics and growth pattern in TSA and TSB of the eight bacterial isolates from tilapia pond soil.

Isolate	Size	Shape	Margin	Elevation	SurfaceTexture	Growth Pattern in TSA	Surface Growth Pattern in TSB
A	Medium	L-form	Undulate	Raised	Rough	Filiform	Pellicle
B	Small	Irregular and spreading	Lobate	Raised	Rough	Echinulate	Flocculent
C	Small	Round	Entire	Convex	Smooth	Filiform	Pellicle
D	Small	Irregular and spreading	Lobate	Raised	Rough	Echinulate	Ring
E	Large	Round with raised margin	Entire	Raised	Rough	Filiform	Pellicle
F	Small	Complex	Undulate	Hilly	Dull	Beaded	Pellicle
G	Small	Round	Entire	Flat	Smooth	Beaded	Flocculent
H	Small	Irregular and spreading	Lobate	Raised	Mucoid	Beaded	Pellicle

Majority of the colonies were small (B, C, D, F, G and H) while the remaining isolates were medium (A) and large (E). The shape ranged from irregular and spreading (B, D and H), round (C and G), L-form (A), round with raised margin (E) and complex (F). Three of the isolates (B, D and H) had lobate margin (highly indented irregular edges), two isolates (A and F) with undulate (regular wavy indentations) and three isolates (C, E and G) with entire (smooth, even edges). Colony elevation was either raised, thick growth projecting (A, B, D and E); convex, low at the edges and raised at the middle (C); hilly, irregular growth projecting (F) or flat, height not raised (G). Surface texture was described as rough (A, B, D and E), smooth (C and G), dull (F) or mucoid (H). Growth patterns in TSA include filiform, uniform growth along the line of inoculum (A, C and E); echinulate, margins of growth exhibit toothed appearance (B and D); and beaded, separate colonies along the line of inoculum (F, G and H). Lastly, surface growth pattern of isolate A, C, E, F and H was pellicle (thin growth in the surface), isolate B and C was flocculent (presence of floating adherent masses) and formation of surface ring for isolate D (Table 1).

3.2 NH_3-N degrading experiment

The eight morphologically different colonies were used in the NH_3-N degrading experiment. The computed NH_3-N after 72 hours incubation in experimental set-up with and without bacterial inoculation was provided in Table 2.

Isolate C obtained more consistent reductions in NH_3-N after 72 hours incubation: 99.32%, 4.76%, 56.00%, 54.55% and 40.00% reductions in experimental set-ups with 10, 20, 30, 40 and 50 mg/L NH_4Cl, respectively. Isolate C was used for the next phases of the study which were biochemical and physiological characterization and identification using 16S rRNA sequencing.

Table 2: The computed percent reduction of NH_3-N after 72 hours

Ammonium Chloride Concentration (mg/L)	10	20	30	40	50
A	69.29*	-1847.62	52.00*	33.33	30.00
B	98.57*	-4.76	-4.000	-212.12	-245.00
C	99.32*	4.76	56.00*	54.55*	40.00
D	99.79*	33.33	4.00	21.21	-80.00
E	98.29*	61.91*	28.00	-93.94	-10.00
F	97.18*	-328.57	564.00*	-533.33	-330.00
G	98.93*	-9.52	8.00	66.67*	-1040.00
H	99.32*	-890.47	16.00	-63.64	-32.00

Note: Values with asterisk (*) have 50% reduction in NH_3-N

3.3 Gram-staining and biochemical and physiological tests

To further elucidate the phenotypic characteristics of isolate C, Gram-staining, catalase test, and selected biochemical and physiological tests were done (Table 3). Isolate C was Gram-positive with rod cells when observed under the microscope. The isolate was positive to catalase test or the bacterium possessed catalase enzyme. The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. Therefore, the bacterium had the ability to protect itself from the lethal effect of hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism. The isolate was negative to phenylalanine deamination test and urea hydrolysis indicating the lack of enzymes amino acid oxidase and urease [13]. The bacterium had the capability to consume citrate as carbon source and capacity to live in an environment or medium with 6.5% salt.

Table 3: Results of isolate C on Gram-staining, catalase test and other biochemical and physiological tests

Test	Result
Gram reaction	Positive
Shape	Rod
Cell arrangement	Single
Catalase test	Positive
Phenylalanine deamination test	Negative
Urea hydrolysis	Negative
Citric acid utilization test	Positive
Growth in 6.5% NaCl	Positive

3.4 Identification by 16S ribosomal RNA sequencing

Isolate C was subjected to DNA isolation. DNA sample was used as template for PCR amplification using 16S rRNA specific primers. Successful amplification was obtained as evidenced by the expected 1.5 kb sized Amplicon in agarose gel observation (Figure 1).

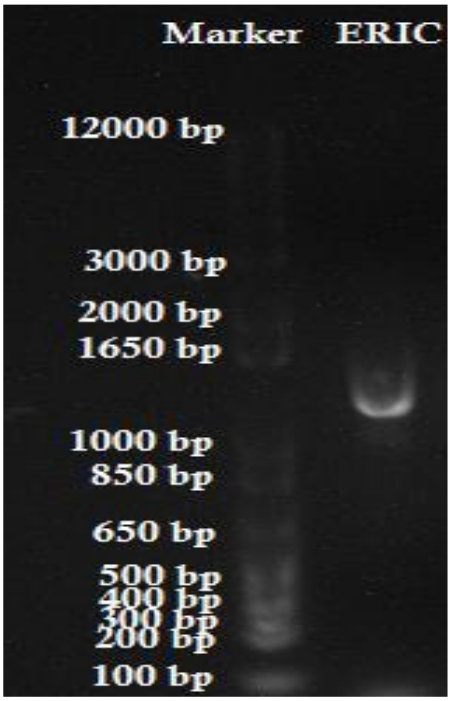

Fig 1: Agarose Gel Electrophoresis of amplified 16S rRNA gene region with ~1,500bp size

PCR products were subjected to sequencing using the same set of primers. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of sequences derived from both revealed that the isolate is of *Bacillus* genus. Highest hits indicated that the isolate belongs to *tequilensis* species.

The genus *Bacillus* is composed of rod-shaped, endospore forming bacteria that are members of the phylum Firmicutes. They are common inhabitants of soil and aquatic sediment; species within the genus are widespread in nature and are found in virtually every environment. The main role of *Bacillus* spp. is carbon and nitrogen cycling. Majority of *Bacillus* spp. are non-pathogenic to humans and animals. Because of this fact, many have been exploited for biotechnological and industrial applications [14, 15, 16, 17].

The isolated and identified NH_3-N degrading bacterium, *B. tequilensis*, can grow in anaerobic condition, motile, positive to oxidase and catalase tests, has the ability to degrade tryptophan and starch but not urea, and can utilize citrate as carbon source. Physiologically, the bacterium can withstand salt concentration as high as 8%. All of the morphological, biochemical and physiological results in this present study have conformed to published reports [18, 19].

B. tequilensis can inhibit the growth of rice blast fungus, *Magnaporthe oryzae*, which suggests the presence of secondary metabolites. It was found out that *B. tequilensis* can produce cellulase, protease, gelatinase, indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate deaminase [19]. In one study, *B. tequilensis* FR9 strain has demonstrated to produce antilisterial Subtilosin A, thus, it has an essential characteristics of a potential probiotics and might be incorporated into human and animal food supplements [20].

This bacterium was first identified and described in 2006 only, thus, published studies on the ability of this bacterium to degrade or take-up NH_3-N in their cells were very limited or none at all. This is one of the first studies that describe the ability of the bacterium to degrade or take-up NH_3-N in their cells.

4. Conclusion

Eight bacterial colonies were isolated from diluted pond soil sample based upon colonial characteristics and growth pattern in solid and liquid medium. The isolated colonies were subjected to NH_3-N degrading experiment. In all isolates used, isolate C showed the best result on the reduction of NH_3-N. Morphological, biochemical and physiological tests revealed that Isolate C was Gram-positive, rod shape, positive to catalase and citric acid utilization tests, negative to urea hydrolysis and phenylalanine deamination tests, and had the ability to grow in medium supplemented with 6.5% salt. Isolate C was subjected to DNA isolation, purification and sequencing. BLAST analysis of sequences derived from both revealed that the isolate is of *Bacillus* genus. Highest hits indicated that the isolate belongs to *tequilensis* species.

5. Acknowledgement

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