Identification, Growth Profile and Probiotic Properties of Autochthonous Intestinal Bacteria of Sagor catfish (*Hexanematichthys sagor*)

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Received 2 April, 2017/Accepted 22 February, 2018

The prevalence of antibiotic resistant bacteria in aquaculture has reached alarming proportions and intensified the search for microbe derived antimicrobial compounds. This study isolated bacteria from the intestine of Sagor catfish (*Hexanematichthys sagor*) and screened it for antagonistic properties. Five out of 334 bacterial isolates inhibited growth of fish pathogens. The 5 bacterial strains included relatives of *Shewanella haliotis, Myroides odoratimimus, Vibrio harveyi, Vibrio alginolyticus* and *Alcaligenes faecalis*. The growth profiles and probiotic properties of these bacteria were examined. The results showed that the isolate 9 (3) 7.5.2.1, whose closest relative was *S. haliotis* exhibited growth and probiotic advantage compared to the other bacterial strains, such as highest doubling time and the ability to survive at all experimental temperatures (18 to 60°C), and bile concentrations (0.01 to 1.00%) and pH (pH2 to 9). While the bacteria with probiotic properties were successfully isolated. Further study is necessary to examine the efficiency of the probiotic candidate bacteria in boosting fish immunity against pathogens.

Key words : Intestinal bacteria / Growth profile / Antagonistic activity / Probiotic properties / *Shewanella haliotis*.

INTRODUCTION

Fish disease outbreaks resulted in huge losses in aquaculture production. Diseases are controlled almost exclusively using chemicals or antibiotic drugs worldwide (Hai, 2015; Anand et al.,2011; Primavera, 2006), and the effect of these on human populations constitute a major public health concern (Lupin 1999).

Alternative to chemicals or antibiotics are necessary. Probiotics are recognized as one of the alternative therapies for fish health management in aquaculture (Ige, 2013; Panigrahi et al., 2007), as these are beneficial to fish health, minimize disease risk in fish and improve aquaculture production (Hai, 2015; Micheal et al., 2014; Hong et al., 2005; Nikoskelainen et al. 2003).

The high potential of microflora in marine fish intestines as a cure for disease has amazed microbiologists (Jayanth et al., 2002; Sugita et al., 1991). It is particularly viable to investigate the probiotic properties of the intestinal flora of the marine catfish due to its carnivorous and predacious feeding behaviour (Parida et al., 2015). This study describes the probiotic properties of bacterial flora isolated from the intestine of the tropical Sagocatfish, *Hexanematichthys sagor*.

MATERIALS AND METHODS

Screening of Bacteria

A total of 20 Sagor catfish (Fig. 1) were captured using hook and line from Mangkabong Bay (6°08’34.4”N 116°12’23.2”E), Malaysia. The fish were dissected right after the fish were delivered to the fish disease laboratory at University Malaysia Sabah.

The abdomen of each fish was cut open and the intestines were cut into pieces (approximately 0.5 cm). About 10 pieces of intestine were randomly selected and homogenized using a sterile mortar and pestle in the presence of 1 ml Phosphate Buffered Saline (PBS) solution (pH7.2). One milliliter of homogenate was
mixed with 9 ml of sterile PBS solution. The dilution was repeated 10 times and 100 µl of mixture was spread evenly on Tryptic Soy Agar (TSA) (Merck, Germany) using a sterile bend rod stick.

All the TSA plates were incubated at room temperature (28°C) and the bacterial growth was monitored at 24 hours intervals. Bacterial colonies exhibiting different shapes or colours were picked and serially isolated until a single colony was obtained (Fig. 2). The isolation of the bacterial colonies was conducted every day consecutively for 15 days. However, newly grown bacteria which exhibited the same shapes and colours were excluded.

The laboratory reference number was labelled based on the number of streaking. For example bacteria 9(3) 7.5.2.1, the 9 means this bacterial was the ninth picked bacteria colony from the first isolation. The second number, 3 meaning that this bacterial was the third colony from second isolation, and so on. There are all together 6 numbers, so this bacterial was screened for 6 times before getting to the pure colony. The bracket at the second number showing that this bacteria is isolated from marine Sagor catfish. This is for the author to differentiate the bacteria isolated from marine and freshwater fish.

Gram staining (Johnson and Case, 2001) was performed to confirm the purity of the bacterial isolates. Bacteria which appeared purple under the light microscope were recorded as Gram-positive; while those which looked pink were noted as Gram-negative. The bacteria were compared based on their cell morphology and the results of the Gram staining (Fig. 3). Strains which did not present the same morphology or colours were subjected to the isolation process again until the pure bacteria were obtained. Subsequently, the bacteria were briefly subjected to oxidase, motility and catalase tests devised by Chauhan (2012). Replicates of each bacterial isolate were subsequently stored at 4°C and -80°C following Lauber et al. (2010). For short term storage, bacteria were cultured at 4°C in semi-solid TSA whose concentration was 3% of the original formula. For long term preservation, overnight bacterial culture was suspended in 70% Tryptic Soy Broth (TSB) and 30% glycerol in cryovial tubes before being stored at -80°C.

Subsequently the bacterial isolates were grown on selective media, including Thiosulphate-citrate-bile
Antagonism Assay

The antagonistic properties of the bacteria were examined using the disc diffusion method utilised by Zaidan et al. (2005). Overnight pathogen cultures (adjusted to OD600 of 0.2) were spread on the TSA using sterile cotton swabs. Then the plates were dried for 5 minutes prior to the antagonistic assay. A total of 100 µl of each filter-sterilized culture fluid was absorbed by a 9 mm diameter sterile disc (Whatman filter paper No.1). These discs were aseptically placed on inoculated TSA. The TSA with blank disc (without bacteria) served as control. Each bacterial sample was examined in triplicate.

Eight bacterial pathogens including Vibrio harveyi, V. parahaemolyticus, V. alginolyticus and Photobacterium damselae, Streptococcus agalactiae, Aeromonas caviae, A. hydrophila and A. salmonicida were tested in this assay. The details of the pathogens used in this study are listed in TABLE 1. The TSA were incubated at 37°C and monitored for 10 days. The inhibition zone was measured using a Vernier caliper at 24 hour intervals and recorded according to the methods of Zaidan et al. (2005).

Polymerase Chain Reaction (PCR)

Extraction and purification of DNA

Bacteria isolated from the intestines of the Sagar catfish were cultured on TSA overnight at room temperature (36°C). The overnight bacteria colonies were collected and dissolved in 0.5 ml of TSB. In order to concentrate the bacteria in the broth, the cultures were centrifuged at 13,000 rpm for 2 minutes and the supernatant discharged.

The extractions of bacterial DNA was conducted using the standard CTAB DNA extraction method as employed by Nishiguchi and Doukakis (2002). A total of 3 ml of bacterial culture was spun down and the supernatant removed. Then, 600 µl of CTAB solution was added and vortexed for a few seconds. After that, the mixture was incubated at 75°C for 5 minutes in the case of the Gram-negative bacteria, and for while 30 minutes in the case of the Gram-positive bacteria. After incubation, the mixture was cooled to room temperature before vortexing and spinning down. Then, 700 µl of chloroform was added to the mixture before vortexing for 25 seconds and centrifuging at 12,000 rpm for 5 minutes. After the centrifugation, the upper aqueous phase of the mixture was transferred into a new 1500 µl tube and then 100 µl CTAB and 900 µl of distilled water were added into the tube. The mixture was mixed well by vortexing before being incubated at 75°C for 5 min. It was then cooled down to room temperature before being centrifuged again at 12,000 rpm for 5 minutes. Then the supernatant in the tube was transferred into a new tube with 300 µl of 95% ethanol. The mixture was mixed by vortexing for 10 seconds before being centrifuged at 12,000 rpm for 5 minutes. Lastly, the supernatant was removed and the remaining white pellet which was sticking on the tube was dried at room temperature (27°C). A total of 50 µl TE buffer was added into the

| Pathogen              | Host / Case                          | Origin         | Strain       | Reference            |
|-----------------------|--------------------------------------|----------------|--------------|----------------------|
| Streptococcus agalactiae | Streptococcosis developed tilapia (Oreochromis niloticus) | Malaysia       | KT 869025    | Nur-Nazifah et al. (2017) |
| Aeromonas caviae       | NA                                   | NA             | ATCC 15468   | Ruimy et al. (1994)   |
| Aeromonas hydrophila   | Japanese Eel (Anguilla japonica)     | Nagasaki University, Japan | A 10        | Kanai and Takagi (1986) |
| Aeromonas salmonicida  | NA                                   | NA             | ATCC 33658   | Ruimy et al. (1994)   |
| Vibrio harveyi         | Seabass (Lates calcarifer)           | Universiti Malaysia Sabah, Sabah, Malaysia | JR 7        | Ransangan and Mustafa (2009) |
| Vibrio parahaemolyticus| Poisoned food related to Shirasu case | Japan          | ATCC 17802   | Ruimy et al. (1994)   |
| Vibrio alginolyticus   | Spoiled horse mackerel fish          | Japan          | ATCC 17749   | Ruimy et al. (1994)   |
| Photobacterium damselae| Marine fish                          | Japan          | ATCC 51805   | Kimura et al. (2000)   |

Note: The full spelling of NA is Not Available. The related information is not found on the internet.
tube and mixed well with the crude DNA extracts (white pellet) before being stored at 4°C.

**Primer and Master Mix**

One µl of each 10 µM forward and reverse oligonucleotide primers (IDT, Singapore) were added into 48µl of Master Mix (Promega, Madison, Wisconsin) (TABLE 5). The PCR was carried out in a thermocycler with a heater lid for 1 cycle at 95°C for 1 minute, then 30 cycles were run at 95°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. Upon conclusion of the thermal cycling, reaction mixtures were held at 72°C for 5 minutes and cooled to room temperature. PCR samples were electrophoresed in a 1.5% agarose gel containing 4.0 µg ml⁻¹ ethidium bromide and visualized on an ultraviolet transilluminator. The PCR products were purified using an AccuPrep® PCR purification Kit (Bioneer Corporation, Korea) in accordance with the manufacturer’s instruction.

**Cloning and Sequencing**

After purification, two microlitres (2 µl) of the PCR product were ligated into a cloning vector pGEM-T easy Vector (Promega, USA) and transformed into the chemically competent Escherichia coli JM109 (Promega, USA) the next day. The recombinant plasmids were purified using a DNA spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Korea). Finally, all plasmids were sent to AIT, Singapore Pt Ltd for bidirectional sequencing to get all the nucleotide sequences of the 16S rRNA genes.

**BLAST analysis**

The homology search for the DNA sequences of the bacterial isolates in this study (against DNA sequences in the public databases) was carried out using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov).

**Growth Profile**

Five intestinal bacteria strains which exhibited strong antagonism were selected for growth profiling for 36 hours. For every selected bacterium, 5 µl of an overnight culture was inoculated in a tube containing 8 ml of Typtic Soy Broth (TSB). The tubes were incubated at room temperature (27°C) and the optical density (OD) was recorded at 600 nm at 1 hour intervals over a 36 hour period. The experiment was carried out in triplicate and the OD readings averaged.

On the next day, the concentration of bacteria were adjusted to the recorded OD before subjected to 10 fold serial dilution. The 10 fold serial dilution was starting with 1 ml of bacteria was transferred into 9 ml of sterile distilled water in the first test tube. The mixture was mix well before another ml of diluted bacteria was transferred to the second test tube contained 9ml of distilled water. The same procedure was repeated until the 10th test tube. After that, 100 µl of diluted bacteria was transferred from each test tube into the TSA. The diluted bacteria was spread evenly using sterile bended rod stick. The TSA was sealed and incubated overnight at room temperature. The colony forming unit in one millilitre (CFU/ml) was calculated on the next day following the formula below:

\[
\text{CFU/ml} = \frac{\text{Number of bacteria colonies X Dilution factor}}{\text{Volume of culture plate}}
\]

**Heat Tolerance**

The heat tolerance assay was conducted according to the method devised by Kim et al. (2001). Prior to this assay, bacteria were grown to log phase on the TSA and kept at room temperature (27°C).

To begin the assay, a colony of bacteria was picked from the TSA and cultured in the TSB until the log phase of the bacteria. The Colony Forming Unit (CFU/ml) of the bacterial culture was determined before it was exposed to various temperatures (18, 25, 37, 45, 50, 55 and 60°C) for an hour. Triplicates of the control and every examined bacterial were exposed at each stated temperature and a TSB without bacteria culture was set as control in this assay. After an hour of heat exposure, the CFU in 1 ml of five selected intestinal bacteria strains was again determined. Increase or reductions in examined bacterial density were determined through observation of changes in the CFU after heat treatment.

**Bile Tolerance**

The bile tolerance test was carried out following the method described by Buntin et al. (2008). A batch of 10 ml TSB tubes containing 0 – 1.0% bile (Sigma, USA) (w/v) was prepared. For each bacteria selected for experimentation, 5 ml of log phase bacteria were inoculated into the TSB. After incubation in different concentrations of bile for 3 hours at room temperature (27°C), the bacteria from the TSB tubes were inoculated on the TSA. Bacterial growth on the TSA the next day indicated a positive result, whereas no bacterial growth indicated a negative result.

**Acid Tolerance**

In order to examine how long the bacterial isolates were able to survive at different pH levels, an acid tolerance test was conducted by inoculating 5 ml of log phase bacteria in 10 ml of TSB containing 0.01 – 1% hydrochloric acid (w/v) (Sigma, USA). To increase the pH value, sodium hydrochloric (NaOH) was added into
the broth. The bacteria were incubated for 1 and 24 hours at room temperature. After incubation, bacteria from the TSB tubes were inoculated on TSA. From observation the next day, the growth of bacteria on the TSA indicated a positive result; while no bacterial growth indicated a negative result.

RESULTS

Bacterial composition
A total of 334 strains of bacteria were isolated from the intestines of Sagor catfish in this study. The isolated bacteria, were found to consist of Gram-positive cocci (39.52%), Gram-positive bacilli (24.25%), Gram-negative bacilli (19.76%) and Gram-negative cocci (16.47%).

Antagonistic Assay
To reduce the number of bacteria, all samples were examined for their antagonistic ability towards fish pathogens. The results show 2.69% of Sagor catfish intestinal bacteria consist antagonistic abilities towards the pathogens in this study. However, only 5 bacteria strains displaying the highest antagonism activity towards pathogens were selected for further analysis. Only the bacteria displaying antagonistic activity towards pathogens (Fig. 4) were listed in TABLE 2. The data shown in TABLE 2 represents the minimum values (mm) derived from the triplicate data.

Characterization of Bacteria
Selected bacteria were examined using Gram staining, biochemical tests and selective media tests. All of these strains were Gram negative. The phenotypes of the selected bacteria are listed in TABLE 3.

Assessment of closest relatives of bacteria sequences
The selected bacteria strains showed high gene similarity to the Shewanella haliots, Myroides odoratimimus, V. harveyi, V. parahaemolyticus and Alcaligenes faecalis strains. The accession number of the bacteria in GenBank, their nucleotide similarity and the closest relatives of the selected bacteria are listed in TABLE 4. The phylogenetic tree for the selected bacteria is shown in Fig. 5.

Growth Profiles
It was noticed that the isolate 9(3)7.5.2.1 multiplied rapidly compared to the other bacteria. Most bacteria reached log phase at the second hour of incubation and reached the stationary phase at sixth hour. Interestingly, the isolate 9(3)7.5.2.1 reached log phase at the first hour and entered stationary phase at the third hour. Nevertheless, the absorbance value (OD) of all the bacteria decreased at the end of the experiment (35th hour) indicating a decline in the growth rate of the bacteria (Fig.6).

In order to minimize the bias in stress examination, the selected bacteria were examined for their growth parameters. The experiment to analyse the growth profile was conducted at room temperature (27°C) over a 36 hours. Each strain of bacteria was prepared in a bijou bottle and the OD was adjusted at 0.2 prior to starting the examination. Based on the result shown in TABLE 6, bacteria 9(3)7.5.2.1 displayed the highest growth rate (0.4) within the first 13 hours with the highest biomass yield (4.9). It also had the shortest doubling time (1.7) compared to the other selected bacteria.

Heat Tolerance
The selected bacteria were examined for their tolerance to different temperatures ranging from 18 to 60°C. Based on the results shown in TABLE 7, four bacterial isolates (9(3)7.5.2.1, 8(3)1.2.1, 6(9)8.4 and 7(3)6.1.2) showed tolerance to temperatures up to 60°C. However, the bacterium 9(2)7.3.2 was only able to survive up to 45°C.

Bile Tolerance
The selected bacteria were examined for their tolerance to bile, whose concentrations ranged from 0.01% to 1.00% (w/v). Bile tolerance results for the selected bacteria are shown in TABLE 8.

pH Tolerance
The selected bacteria were examined for their tolerance to pH ranging from pH2 to pH9. The acid hydrochloric (HCl) was added into the TSB to decrease the pH value; the sodium hydroxide (NaOH) was added to
TABLE 2. Antagonistic activity of the five selected bacteria against fish bacterial pathogens

| Lab reference number | S. agalactiae | A. caviae | A. hydrophila | A. salmonicida | V. harveyi | V. parahaemolyticus | P. damselae | V. alginolyticus |
|----------------------|---------------|-----------|---------------|---------------|------------|----------------------|-------------|-----------------|
| Day 0                |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 10            |           |               |               |            |                      |             |                 |
| 6(9)8.4              |               |           |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               |           |               |               |            |                      |             |                 |
| Day 1                |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 15            |           |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               |           |               |               |            |                      |             |                 |
| 6(9)8.4              |               |           |               |               |            |                      |             |                 |
| 9(3)7.5.2.1          |               |           |               |               |            |                      |             |                 |
| Day 2                |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 16            | 10        | 13            | 10            |            |                      |             |                 |
| 7(3)6.1.2            |               |           |               |               |            |                      |             |                 |
| 6(9)8.4              |               |           |               |               |            |                      |             |                 |
| Day 3                |               |           |               |               |            |                      |             |                 |
| 9(3)7.5.2.1          |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 15            | 14        | 14            | 12            |            |                      |             |                 |
| 6(9)8.4              |               |           |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               | 9         | 19            | 13            | 11         | 13                   |             |                 |
| Day 4                |               |           |               |               |            |                      |             |                 |
| 9(2)7.3.2            |               |           |               |               |            |                      |             |                 |
| 6(9)8.4              |               |           |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               | 13        |               |               |            |                      |             |                 |
| Day 5                |               |           |               |               |            |                      |             |                 |
| 9(3)7.5.2.1          |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 13            | 11        | 12            | 13            |            |                      |             |                 |
| Day 6                |               |           |               |               |            |                      |             |                 |
| 9(2)7.3.2            |               |           |               |               |            |                      |             |                 |
| 6(9)8.4              |               |           |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               | 12        |               |               |            |                      |             |                 |
| Day 7                |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 12            |           |               |               |            |                      |             |                 |
| 9(2)7.3.2            |               | 14        |               |               |            |                      |             |                 |
| 6(9)8.4              |               | 16        |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               | 17        |               |               |            |                      |             |                 |
| Day 8                |               |           |               |               |            |                      |             |                 |
| 9(3)7.5.2.1          |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 18            |           |               |               |            |                      |             |                 |
| Day 9                |               |           |               |               |            |                      |             |                 |
| 9(2)7.3.2            |               |           |               |               |            |                      |             |                 |
| 6(9)8.4              |               | 14        |               |               |            |                      |             |                 |
| 7(3)6.1.2            |               | 20        |               |               |            |                      |             |                 |
| Day 10               |               |           |               |               |            |                      |             |                 |
| 9(3)7.5.2.1          |               |           |               |               |            |                      |             |                 |
| 8(3)1.2.1            | 18            |           |               |               |            |                      |             |                 |

The empty cells represent no antagonism activity of the bacteria towards pathogen.
TABLE 3. The phenotypes of 5 selected intestinal bacteria from Sagor catfish intestine

| Lab reference number | Gram staining | Morphology | Biochemical tests | Selective media tests |
|----------------------|---------------|------------|-------------------|-----------------------|
|                      |               |            | Motility | Oxidase | Catalase | Mac Conkey | TCBS | Pseudomonas isolation agar | TSA |
| 9(3)7.5.2.1          | -             | bacillus   | +        | +       | +        | +          | -    | +                           | +   |
| 8(3)1.2.1            | -             | bacillus   | +        | +       | +        | +          | -    | +                           | +   |
| 9(2)7.3.2            | -             | bacillus   | +        | +       | +        | +          | -    | +                           | +   |
| 6(9)8.4              | -             | bacillus   | +        | +       | +        | -          | +    | +                           | +   |
| 7(3)6.1.2            | -             | bacillus   | +        | +       | +        | -          | -    | -                           | +   |

Note: The + at selective media tests shows growth of bacteria on specific media; - means no growth of bacteria on the specific media.

TABLE 4. Identity of the selected intestinal bacterial strains

| Laboratory reference number | Accession number | Nucleotide similarity | Closest relative species |
|------------------------------|------------------|-----------------------|--------------------------|
| 9(3)7.5.2.1                  | MF 175883        | 99%                   | Shewanella halotis strain CE08 |
| 8(3)1.2.1                    | MF 175885        | 99%                   | Myroides odoratimimus strain PR63039 |
| 9(2)7.3.2                    | MF 175882        | 99%                   | Vibrio harveyi isolate EHP7 |
| 6(9)8.4                      | MF 175884        | 99%                   | Vibrio parahaemolyticus strain BF-1 |
| 7(3)6.1.2                    | MF 175881        | 99%                   | Alcaligenes faecalis strain LZU-52 |

FIG. 5. Phylogenetic tree inferred from the 16S rRNA gene nucleotide sequences by using the neighbor-joining method. Five antagonistic isolates obtained from Segor catfish intestine as well as their closest relatives are included. Scale bar, nucleotide substitutions.
increase the pH value. The pH value was measured using a pH meter. Results for the selected bacteria are indicated in Table 9.

### DISCUSSION

The digestive tracts of vertebrates are colonized by complex assemblages of micro-organisms, collectively

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**TABLE 5.** Chemicals and quantity needed for the master mixture in the DNA extraction

| Chemicals            | Quantity |
|----------------------|----------|
| Bacteria DNA         | 2.0 µl   |
| Forward primer       | 1.0 µl   |
| Reverse primer       | 1.0 µl   |
| dNTP                 | 1.0 mM   |
| Taq polymerase       | 0.5 U    |
| Magnesium chloride   | 3.4 mM   |
| Distilled water      | 31.1 µl  |
| 5 x PCR buffer       | 10.0 µl  |
| **Total volume**     | 50 µl    |

**TABLE 6.** Growth parameters of selected bacteria

| Laboratory reference number | Growth parameters (h⁻¹) | μ  | λ  | A  | t₀ |
|-----------------------------|-------------------------|----|----|----|----|
| 9(3) 7.5.2.1                |                         | 0.4 | 4.7 | 4.9 | 1.7 |
| 8(3) 1.2.1                  |                         | 0.2 | 2.7 | 2.9 | 2.9 |
| 9(2) 7.3.2                  |                         | 0.1 | 1.6 | 1.7 | 4.9 |
| 6(9) 8.4                    |                         | 0.3 | 3.5 | 3.8 | 2.2 |
| 7(3) 6.1.2                  |                         | 0.1 | 1.6 | 1.8 | 4.2 |

Note: μ specific growth rate; λ maximum specific growth rate; A maximum biomass; t₀ doubling time of bacteria

**TABLE 7.** Heat tolerance of the selected bacteria

| Laboratory reference number | Temperature (°C) | 18  | 25  | 37  | 45  | 50  | 55  | 60  |
|-----------------------------|------------------|-----|-----|-----|-----|-----|-----|-----|
| 9(3) 7.5.2.1                |                  | +728| +635| +415| +10584| +216| +364| +1441|
| 8(3) 1.2.1                  |                  | -116| +660| +218513| +74636| +46843| +10757| -209 |
| 9(2) 7.3.2                  |                  | -218| +181| +4405| +3114| X   | X   | X   |
| 6(9) 8.4                    |                  | +33 | +13 | +362 | +1212| +147 | +411 | -154 |
| 7(3) 6.1.2                  |                  | +141| +896| +2946 | +5716| +8069| +145 | +265 |

Note: + indicates increase number of bacteria; - indicates decrease number of bacteria; X indicates no bacteria growth

**TABLE 8.** Bile tolerance of the probiotic bacteria

| Laboratory reference number | Bile concentration (w/v) | 0.01% | 0.10% | 0.20% | 0.30% | 0.40% | 0.50% | 0.60% | 0.70% | 0.80% | 0.90% | 1.00% |
|-----------------------------|-------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 9(3) 7.5.2.1                |                         | +10   | +109  | +51  | +8   | +1   | -6   | -2   | +8   | +12   | +4   | +35   |
| 8(3) 1.2.1                  |                         | +4345 | +107  | +408 | +5   | +215 | +1450| -47  | -59  | +4    | +40  | -6    |
| 9(2) 7.3.2                  |                         | +8   | +2105 | +491 | +2491| +1267| +148 | -2   | +894 | +23   | +8   | +91   |
| 6(9) 8.4                    |                         | +30  | +272  | +741 | -27  | +3   | +145 | -43  | -26  | 0     | +60  | -31   |
| 7(3) 6.1.2                  |                         | +17  | +712  | +16  | +49  | +36  | +8   | +96  | +47  | +11   | -4   | +181  |

+ indicates increase number of bacteria; - indicates decrease number of bacteria
Differences in the intestinal microbiota of the Sagor catfish through screening, growth profiling, antagonism and tolerant abilities of bacteria. A total of 334 bacteria strains were successfully isolated from intestine of Sagor Catfish. The isolation was done on Tryptic Soy Agar (TSA) (Merck, USA). The bacteria was sub-culture everyday for 60 days. Only bacteria appearing in single colour and shape were proceed to the next stage of examination.

To confirm the bacteria are in single and pure colonies, Gram staining was conducted on them. The bacteria appeared in one shape and one colour under light microscope is consider as purified bacteria. Based on the results of Gram staining, most of the bacteria isolated from the Sagor catfish intestine were Gram positive bacilli (132 out of 334 isolated strains). These findings agreed with those of Banerjee et al. (2015) who studied bacterial composition in the gastrointestinal tract of catfish. They found that Gram positive bacilli bacteria are dominant in gastrointestinal tract of catfish.

Then the bacteria were subjected to selective media test, Thiosulfate-citrate-bile salts-sucrose (TCBS), MacConkey and Pseudomonas isolation agar were involving in this test. TCBS agar was originally developed for the isolation of Vibrio spp. pathogenic for humans (West et al., 1982). The results shows that bacteria strains 9(2)7.3.2 and 6(9)8.4 on this study growth on TCBS, this indicates that these bacteria are related to the bacteria Vibrio spp. This results were once again assured by the blasting of sequences which shows that bacteria strain 9(2)7.3.2 and 6(9)8.4 are closest relatives to Vibrio harveyi and Vibrio parahaemolyticus. Meanwhile, MacConkey is a selective media for Gram-negative and bacillus bacteria (Lee et al., 2010). The results shows that only bacteria 9(3)7.5.2.1, 8(3)1.2.1 and 9(2)7.3.2 growth on MacConkey. This is perhaps the crystal violet and bile salts in MacConkey inhibited the growth of bacteria 6(9)8.4 and 7(3)6.1.2. The pseudomonas isolation agar was apply in the selective media test is for target the pseudomonas bacteria which are known to growth on soil and water. However, bacteria 9(3)7.5.2.1, 8(3)1.2.1 and 6(9)8.4 which growth on pseudomonas isolation agar did not have closest relative to the bacteria Pseudomonas.

After the phenotype screening of intestinal bacteria of Sagor catfish, the bacteria were carried on to antagonism examination. Pathogens used in this study are responsible for some common disease affecting Malaysian aquaculture, such as streptococcosis (S. agalactiae), vibriosis (V. harveyi, V. alginolyticus and V. parahaemolyticus), pasteurellosis (Photobacterium damselae) and motile Aeromonas septicemia (MAS) (Aeromonas hydrophila, A. caviae and A. salmonicida) (Amal and Zamri-Saad, 2011; Spanggaard et al. 2000). The details of pathogens in this study was listed at TABLE 1. Results in this study revealed that an average of 2.69% of probiotic bacteria exhibited growth of pathogens. The bacteria with strong antagonistic properties in this study were listed as common gut bacteria by Jayanth et al. (2002). He reported that gut bacteria mostly belong to the genus Bacillus, Micrococcus, Pseudomonas, Vibrio, Flavobacterium, Alcaligenes, Xanthomonas and Achoromobacter. However, only 5 bacteria which display antagonism activities on both freshwater and marine pathogens were chosen for further characterization.

The characterization of probiotic candidates in this study was carried out using Polymerase Chain Reaction (PCR) which is commonly application for bacteria identification in researches. The DNA sequences of selected probiotic candidates were blasted in NCBI blast machine and the closest relatives were listed at TABLE 4. The closest relative of the probiotic candidates were found to be either originally from the fish gut or from the surrounding of fish (sediment and water). According to Chaklader et al. (2014), fish takes in the bacteria into their guts through inhale of the water surrounding them.
The fish intake the bacteria from its surrounding into their gut. Some of the bacteria are found to have probiotic properties through a series of tests in this study. After that, all probiotic candidates were subjected to growth profiling examination for 36 hours. The growth profile of the selected bacteria was examined to minimize the bias due to the multiplying rate of bacteria in different phases. The log phase of bacteria in which the bacteria multiply actively, was used to start every tolerance tests (Kim et al., 2001; Hai et al., 2007). The bacteria strain 9(3)7.5.2.1 shows highest specific growth rate (0.4), maximum specific growth rate (4.7) and maximum biomass (4.9) with the shortest doubling time (1.7) compared to other probiotic candidates in this study. According to Roszak and Colwell (1987), the bacteria will grow when the temperature, pH and nutrient of surrounding is sufficient. Thus, the bacteria strain 9(3)7.5.2.1 is expecting to produce high mass of productivity when apply on fish feed or in the fish gut.

In the search for new, effective and safe probiotic bacteria, several procedures need to be conducted, including isolation and characterization of the bacteria, determination of physiological characteristics, tolerance to the conditions of the digestive tract of host (temperature, pH and bile tests) and growth profiling of the bacteria strains (Corcionivoschi et al., 2010; Aditya et al., 2008).

The stress tolerance of selected bacteria and the temperature of the fishes’ surrounding were examined in order to ensure the survival of bacteria in the gastrointestinal tract of the fish (host) (Allameh et al., 2012). Study of the relationship between the selected bacteria and the diet of the fish is possible in further research. The temperature was examined to test for the survival of bacteria at different temperatures ranging from that of pond water (18°C) (Amal and Zamri-saad, 2011) to that of an oven, 60°C (in the case if the selected probiotic candidate will be subjected to apply on fish feed).

Comparing the strains from this study in terms of their probiotic properties, the isolate 9(3)7.5.2.1 had the highest growth rate, produced the highest biomass and exhibited the shortest doubling time compared to other bacterial strains. The isolate yielded 4.9 OD of 600 nm (2.17 x 10^15 CFU/ml^-1) at the 13th hour. Meanwhile, this bacterium also survived in all the tolerance tests conducted and inhibited the growth of all the fish bacterial pathogens used in this study. It greatly inhibited pathogen growth within the genus Vibri.

However, as Anand et al. (2011) explained all studies to date (including the present study) have not yet determined the main factor in the antibacterial activity of intestinal bacteria. Consequently, further study is necessary to confirm the efficiency of the bacterium in boosting fish immunity and inhibition against pathogens.

ACKNOWLEDGEMENT

This study was financially supported by the Ministry of higher Education Malaysia under the Higher Institutions Centers of Excellent (HiCOE) research grant no. CE00007.

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