ISOLATION OF LACTIC ACID BACTERIA FROM FISH GUT OF *LABEO ROHIA(*HAMILTON&BUCHANAN,1822*)* IN HINTHADA

Win War War Kyaw[1],Yi Yi Win[2], Nilar Soe[3], Sa Soe Shwe[4] , Kyaw Myo Naing[5]

Abstract

Isolation of Lactic acid bacteria (LAB) from fish gut of *Labeo rohita* from fishery pond near Hinthada Township, Ayeyarwady Region. Study period lasted from March to August, 2020. Isolation of lactic acid bacteria were undertaken by serial dilution method on deMan Rogosa Sharpe (MRS) medium, supplemented with 0.5% $CaCO_3$. Lacid acid bacteria were purified by using streak plate method. They were identified based on colony morphology, cell morphology, gram staining and acid fast staining. Isolated bacteria tested in this study were eight different bacteria. They are micromorphological different Lr-1 and Lr-6 were cocci and Lr-2 to Lr-8 were bacilli. They showed clear zone on deMan Rogosa Sharpe (MRS) agar medium and they were lactic acid bacteria. This findings were useful in probiotic production.

Key words: Isolation, *Labeo rohita*, Lactic acid bacteria

INTRODUCTION

Microorganisms affect our daily lives and influence overall quality of life. Applications of microbiology are especially important in medicine, industry, agriculture, and environmental management (Atlas, 1997).Lactic acid bacteria (LAB) are the most common types of microbes used as probiotics which are safely applied in medical and veterinary function (Divakara *et al.*, 2010).Many lactic acid bacteria (LAB) are proved to function as probiotics, which are benefit to host health, when ingested in sufficient quantities.

The beneficial role played by this microorganism in humans and animals, including effect on the immune system has been extensively reported (Perdigo´n, *et al*.,1992).The lactic acid bacteria are present in the intestine of most animals. The colonization of the gut by probiotic bacteria prevents growth of harmful bacteria by competition exclusion and by the production of organic acid and antimicrobial compounds.

Lactic acid bacteria were not dominant population in fish. It has been well documented in several investigations that lactic acid bacteria are a part of the native microbiota of aquatic animals from temperate regions (Ringø, 2004). Lactic acid bacteria (LAB) are a group of gram positive, cocci or rods, catalase negative and fastidious organisms.

Lactic acid bacteria have attained major attention for probiotic activity and have generally been considered as good probiotic organisms (O'Sullivan *et al*., 2002).Probiotic bacteria would be found to be useful not only as food but also as biological controllers of fish disease and activators of nutrient regeneration.

Nowadays, the focus is not merely on bacteria collected from fermented dairy products but also bacteria collected from the intestine (Sanders, 1999).Fish intestinal tract is considered

[1] Assistant Lecturer, Department of Zoology, University of Hinthada
[2] Professor, Dr, Department of Zoology, University of Hinthada
[3] Lecturer, Dr, Department of Zoology, University of Hinthada
[4] Lecturer, Dr, Department of Zoology, University of Hinthada
[5] Lecturer, Dr, Department of Zoology, University of Pathein

to be valuable waste and a good source for LAB isolation. LAB are a group of Gram-positive, non-motile, cocci or rods, catalase negative. Various authors have shown that lactic acid bacteria are also part of the normal intestinal flora of fish (Ringø *et al.*,1998). Nowadays, the use of probiotic in Bangladesh for both humans and animals has increased.

Fish diseases are major problem for the fish farming industry and among those bacterial infections are considered to be a major cause of mortality fish (Gomez-Gill *et al.*, 2000). The use of LAB as probiotics for improving disease resistance, growth of fish and in enhancing fish immune response has been developed (Balcazar *et al.*, 2007). Keeping this in view, the present study was undertaken with the following objectives: to isolate the lactic acid bacteria from fish gut of *Labeo rohit*a (Nga myit chin), to study the colony morphology of isolated LAB and to investigate the cell characters and staining reactions of isolated bacteria species.

MATERIALS AND METHODS

Study Period

This research was conducted at the laboratory of Zoology Department, Pathein University from March to August, 2020.

Collection of samples

Fish sample of *Labeo rohit*a was collected from the fishery pond near Hinthada Township, Ayeyarwady Region (Site-I, 17°41′ 46″ N and 95°25′ 37″ E) and (Site -II, 17°41′ 46″ N and 95°25′ 38″ E) (Fig. 1). The samples were put into sterilized polythene bag and carried to the laboratory of Zoology Department, Pathein University, by using ice-box. Samples were stored in the refrigerator at 4°C for further study.

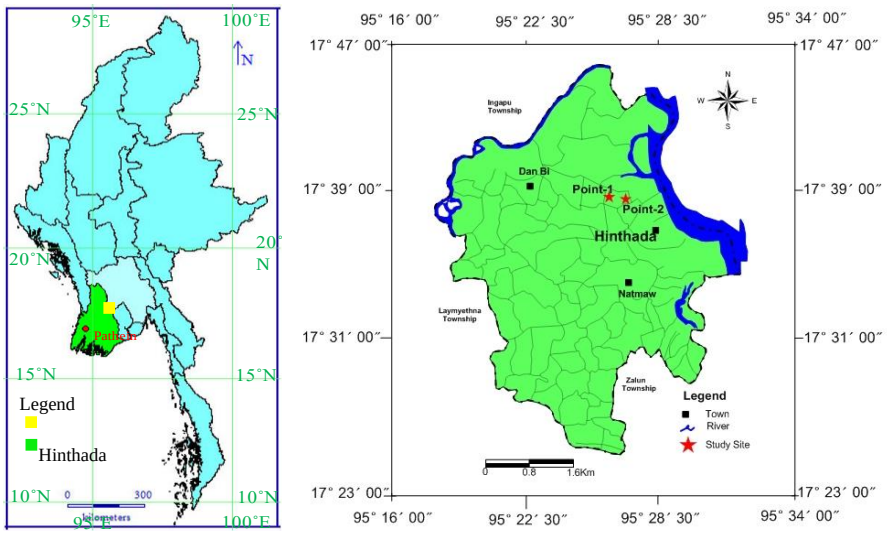


Fig. 1. Map of sample collected area (Source: DIVA-GIS)

A. A fishery pond of *Labeo rohita*

B. A sample fish of *Labeo rohita*

Culture of bacteria

The length and weight of the fishes were measured before dissection. The skin was washed with 70% ethanol before opening the ventral surface with sterile scissors. The fish were dissected to remove the gut, one gram of intestine was taken from each fish sample.

Isolation of pure culture

Then the gut surface was sterilized with ethanol and washed with distilled water for three times and cut into small pieces with sterilized scissors. These pieces were mixed 9 mL of sterile saline diluent (0.85% NaCl). This mixture was shaken agitatedly. Then, it was serially diluted to 10 fold dilutions and plated on deMan Rogosa Sharpe (MRS) media (Himedia, India) containing 0.5% $CaCO_3$. Then, 20 µL of bacterial suspensions from each dilution was inoculated onto MRS agar and incubated at 31℃ for three days. Clear zone forming colonies on MRS agar were selected as lactic acid bacteria. Streak plate method was used to purify these selected bacteria.

For pure culture from plate to test tube, about 100 mL of culture medium were separately distributed into test tubes. The test tubes were plugged with cotton wool and sterilized by autoclaving at 1.05 kg per cm^2 (15 lb per in^2) for 15 minutes at 121℃. The sterilized media were cooled down. Each of separate colonies on petri dishes was taken out to streak on the slant medium to obtain pure culture (Atlas, 1997).

Study of morphology

Colony and cell characteristics were studied according to size, shape, colour, opacity, elevation, margin, morphology, gram staining, acid fast staining nature.

de Man Rogosa Sharpe agar medium (Himedia, India)

Proteose peptone	10g
Meat extract B	10g
Yeast extract	5g
Dextrose(Glucose)	20g
Polysorbate 80	1g
Ammonium citrate	2g
Sodium acetate	5g
Mangnesiumsulphate	0.1g
Manganese sulphate	0.05g
Dipotassium hydrogen phosphate	2g

Agar	12g
Distilled water	1000mL
pH	6.5

Nutrient agar medium (Atlas, 1997)

Peptone	0.5 g
Beef extract	0.3 g
Sodium chloride (NaCl)	0.8 g
Agar	2 g
Distilled water	100mL
pH	6.8

The ingredients were mixed with distilled water and sterilized by autoclaving 121℃ for 15minutes.

Gram staining

A drop of normal saline was placed on clean grease free slide. A small loop of isolated bacteria was smeared on the slide and allows it to dry. The smear was fixed by passing the dried slide three or four times rapidly over a flame. Cover the slide with crystal violet stain and allow it chemical reaction for one minute. Then, the slide was rinsed with distilled water for a few seconds. The slide was covered with fresh iodine solution and allowed for about one minute. Add the alcohol drop by drop and stop adding alcohol when no more colour flows out from the smear. As a counter stain, the smear was covered with safranin for about 20-30 seconds and washed with distilled water. Then the slide was dried. The stained slide was examined under the oil immersion objective of the microscope.

Acid fast staining

Bacterial was smeared on clean and grease free slide. Allow smear to air dry and then heat it. Cover the smear with carbol fuchsin stain. Heat the stain until it vapours and rises. Allow the heated stain to remain on the slide for 5 minutes. After five minutes, wash off the stain with distilled water. As a carbol fuchsin, the smear was covered with acid alcohol for 2 minutes and washed with distilled water. Finally, methylene blue was used to flood the smear for about 30 seconds and washed with distilled water. The slide was dried and then the stained slide was observed microscopically using 100x oil immersion objective.

Catalase test

Two to three drops of 3% H_2O_2 were placed on a clean dry glass slide. A bacterial colony was picked using a clean sterile straight wire loop and mixed into the 3% H_2O_2. Production of bubbles in 5-10 second is a positive test. No appearance of bubbles or appearance after 30 seconds is considered as negative.

Motility

Motility of the isolated bacteria can be detected in semi-solid agar medium. Ten millilitre of semi-solid agar was dispensed in test tubes. The tubes containing the medium were inoculated by stabbing with straight wire about half the depth of the medium. After incubation, motile bacteria will spread into the medium and non- motile will confine to the stab.

RESULTS

Totally eight strains of lactic acid bacteria were isolated from the fish gut of *Labeo rohita*. Isolated strains were designated as Lr-1 to 8, lactic acid bacteria from fish gut of *Labeo rohita*. They were isolated and identified based on colony morphology, cell morphology, gram staining reactions, acid fast staining (Fig. 1- 2 and Table. 1-2).

Lactic acid bacteria from gut of *Labeo rohita*

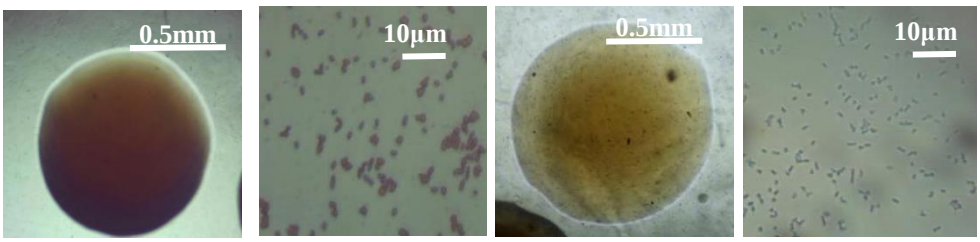
The colony and cell morphology of Lr-1 is circular, yellow, entire and convex. The single colony on MRS medium is 1.3-1.8mm in diameter and on nutrient medium is 1.0-1.5mm in diameter. Cells are cocci shape with 1.35-2.25μm in diameter. Cell morphology of Lr-1 is gram positive, non -acid fast, singly and chain, non -motile and catalase negative. The colony and cell morphology of Lr-2 is circular, yellow, entire and convex. The single colony on MRS medium is 1.2-1.6mm in diameter and on nutrient medium is 1.0-1.5mm in diameter. Cells are rod shape with 1.8-2.7μm in diameter. Cell morphology of Lr-2 is gram positive, non -acid fast, singly and pair, slightly motile and catalase positive.

The colony and cell morphology of Lr-3 is circular, brown- yellow, entire and convex. The single colony on MRS medium is 1.0-1.4mm in diameter and on nutrient medium is 1.0-1.5mm in diameter. Cells are short in rod shape with 1.35-2.25μm in diameter. Cell morphology of Lr-3 is gram positive, non -acid fast, singly and pair, slightly motile and catalase positive. The colony and cell morphology of Lr-4 is circular, yellow, entire and convex. The single colony on MRS medium is 1.2-1.5mm in diameter and on nutrient medium is 1.0-1.5mm in diameter. Cells are short rod shape with 1.35-2.25μm in diameter. Cell morphology of Lr-4 is gram positive, non- acid fast, singly and pair, non -motile and catalase negative.

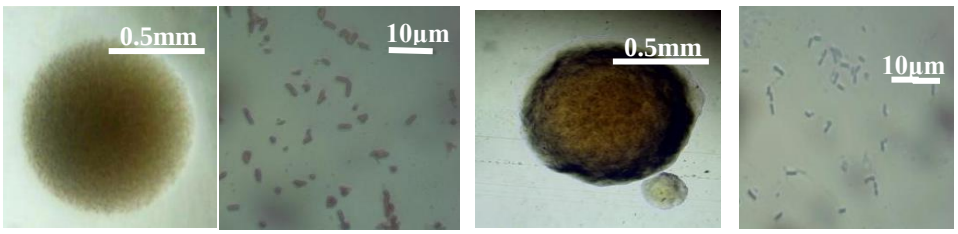
The colony and cell morphology of Lr-5 is circular, white, entire and flat. The single colony on MRS medium is 1.2-1.4mm in diameter and on nutrient medium is 1.3-1.9mm in diameter. Cells are short rod shape with 0.9-1.35μm in diameter. Cell morphology of Lr-5 is gram positive, non -acid fast, singly and pair, non- motile and catalase negative.

The colony and cell morphology of Lr-6 is circular, brown-yellow, entire and flat. The single colony on MRS medium is 1.0-1.4mm in diameter and on nutrient medium is 1.2-1.6mm in diameter. Cells are cocci shape with 0.9-1.35μm in diameter. Cell morphology of Lr-6 is gram positive, non- acid fast, singly and chain, non -motile and catalase negative. The colony and cell morphology of Lr-7 is circular, brown-yellow, entire and convex. The single colony on MRS medium is 1.3-1.5mm in diameter and on nutrient medium is 1.0-1.6mm in diameter. Cells are short rod shape with 0.9-1.35μm in diameter. Cell morphology of Lr-7 is gram positive, non -acid fast, singly and pair, non -motile and catalase negative.

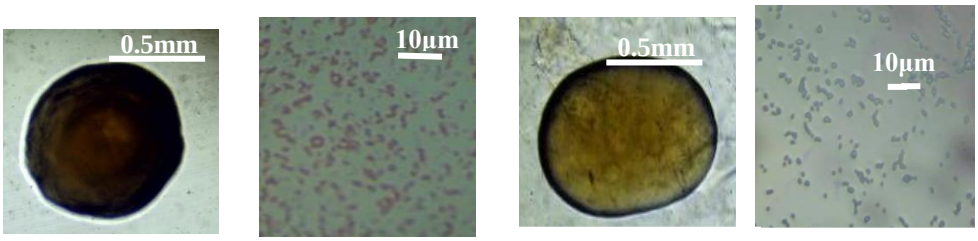
The colony and cell morphology of Lr-8 is circular, yellow, entire and convex. The single colony on MRS medium is 1.2-1.4mm in diameter and on nutrient medium is 1.3-1.9mm in diameter. Cells are short rod shape with 1.35-2.25μm in diameter. Cell morphology of Lr-8 is gram positive, non- acid fast, singly and pair, non- motile and catalase negative.



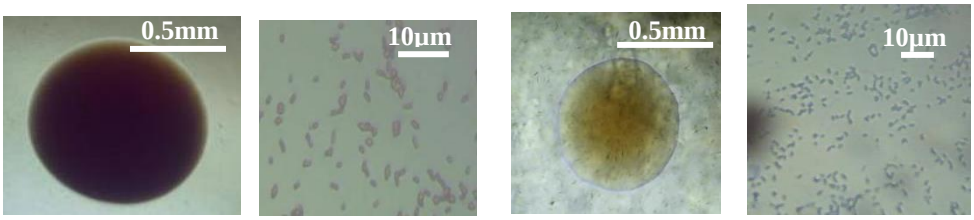
A. Colony of Lr-1 on MRS medium | B. Gram staining of Lr-1 | C. Colony of Lr-1 on nutrient medium | D. Acid fact staining of Lr-1



A. Colony of Lr-2 on MRS medium | B. Gram staining of Lr-1 | C. Colony of Lr-2 on nutrient medium | D. Acid fact staining of Lr-2



A. Colony of Lr-3 on MRS medium | B. Gram staining of Lr-3 | C. Colony of Lr-3 on nutrient medium | D. Acid fact staining of Lr-3



A. Colony of Lr-4 on MRS medium | B. Gram staining of Lr-4 | C. Colony of Lr-4 on nutrient medium | D. Acid fact staining of Lr-4

Fig. 1. Colony and cell morphology of isolated bacteria

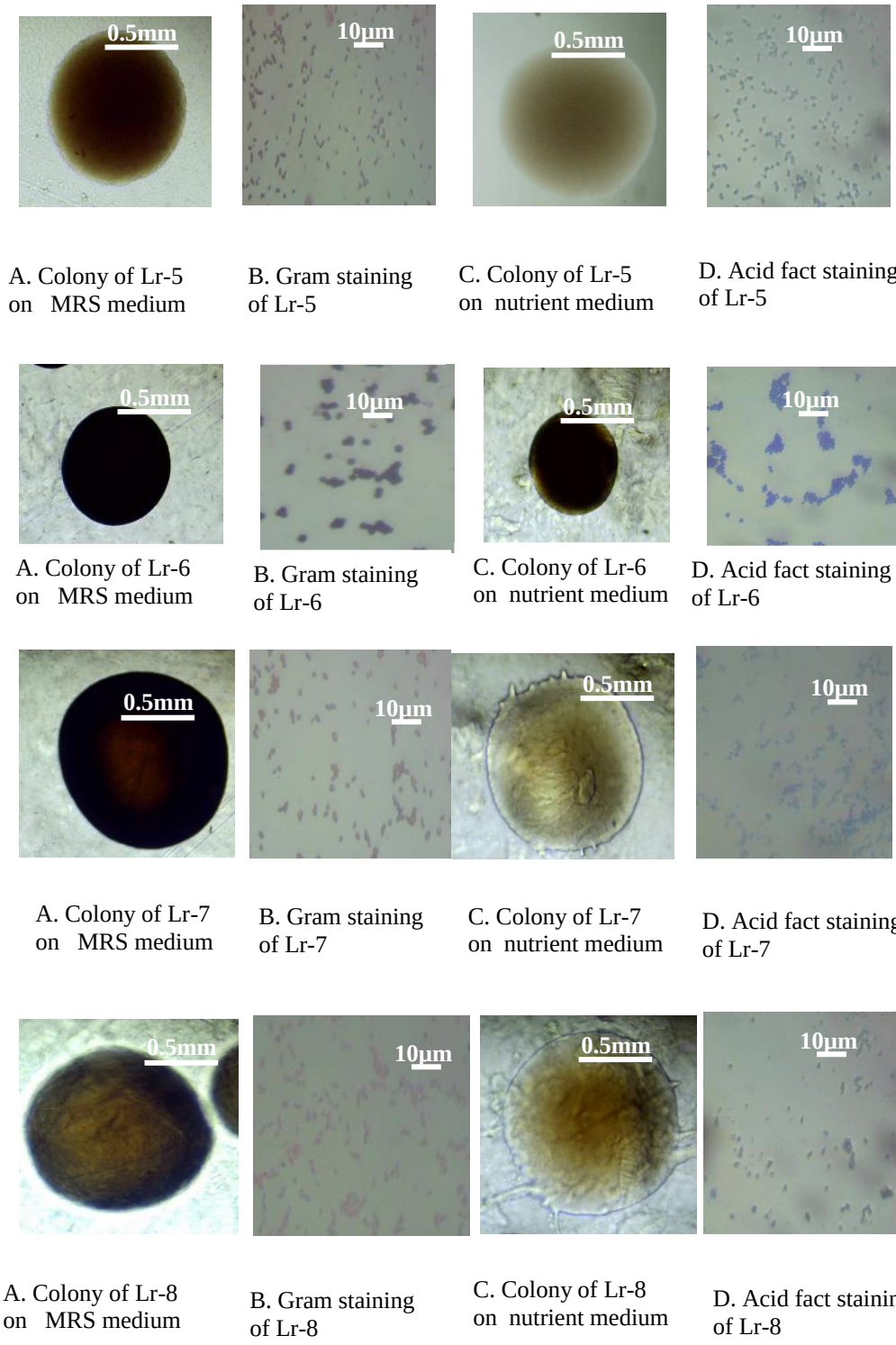


Fig. 2. Colony and cell morphology of isolated bacteria

Table 1. Colony morphology of isolated LAB strains

LAB strains	Size (mm)	Shape	Colour	Margin	Elevation
Lr-1	1.3-1.8 mm	Circular	Yellow	Entire	Convex
Lr-2	1.2-1.6 mm	Circular	Yellow	Entire	Convex
Lr-3	1.0-1.4 mm	Circular	Brown-Yellow	Entire	Convex
Lr-4	1.2-1.5 mm	Circular	Yellow	Entire	Convex
Lr-5	1.2-1.4 mm	Circular	White	Entire	Flat
Lr-6	1.0-1.4 mm	Circular	Brown-Yellow	Entire	Flat
Lr-7	1.3-1.5mm	Circular	Brown-Yellow	Entire	Convex
Lr-8	1.2-1.4 mm	Circular	Yellow	Entire	Convex

Table 2. Cells morphology, staining reactions and motility test of isolated LAB strains

LAB Strains	Cell size (μm)	Shape	Arrangement	Gram reaction	Acid fast reaction	Motility	Catalase test
Lr-1	1.35-2.25 μm	Cocci	Singly and chain	Positive	Non-acid fast	Non motile	Negative
Lr-2	1.8-2.7μm	Rod	Singly and pair	Positive	Non-acid fast	Motile	Positive
Lr-3	1.35-2.25 μm	Short rod	Singly and pair	Positive	Non-acid fast	Motile	Positive
Lr-4	1.35-2.25 μm	Short rod	Singly and pair	Positive	Non-acid fast	Non motile	Negative
Lr-5	0.9-1.35 μm	Short rod	Singly and pair	Positive	Non-acid fast	Non motile	Negative
Lr-6	0.9-1.35 μm	Cocci	Singly and chain	Positive	Non-acid fast	Non motile	Negative
Lr-7	0.9-1.35 μm	Short rod	Singly and pair	Positive	Non-acid fast	Non motile	Negative
Lr-8	1.35-2.25 μm	Short rod	Singly and pair	Positive	Non-acid fast	Non motile	Negative

DISCUSSION AND CONCLUSION

The present work was carried out to isolate the lactic acid bacteria from *Labeo rohita* of Hinthada Township. Lactic acid bacteria from the fish gut of *Labeo rohita* were isolated using deMan Rogosa Sharpe (MRS) medium. Identifications of the isolated bacteria were based on cell morphology, colony morphology, basic chemical staining reactions, and growing temperature. In this research, LAB were isolated from the gut of freshwater fishes.

Lactic acid bacteria are present in the gastrointestinal tract of various vertebrates including freshwater fish, cold water fish and marine fish (Ringø *et al*., 1995). Eight isolates of

lactic acid bacteria from *Labeo rohita* showed which production clear zone around their colonies were obtained. The clear zone appearance is due to the dissolution of $CaCO_3$ on MRS medium by acid agent (Panthavee *et al*.,2007).They are very rapidly growing colonies and spreading all over the plate after about 48 hours incubation.

In this study, some species of lactic acid bacteria are gram negative and motile. However, the remaining species of lactic acid bacteria are gram positive and non- acid fast. These results were also in line with the finding of Lawalata *et al.* (2011) who reported that the LAB isolates are gram positive, rod shape, catalase negative and non -motile.

Ghosh and Ringø (2014) reported that culture temperatures of LAB strains were temperature range from 31℃ to 32℃. So in this study, culture temperatures of LAB was 31℃ to 32℃ and are agreed with culture temperatures of above studies. LAB colonies were circular, flat and low convex with entire margin. All members of lactic acid bacteria were gram positive rods or cocci found in chain, singly and in pairs (Bukola *et al.*, 2008).

The nature of lactic acid bacteria was positive gram character, non-motile, and negative catalase (Axelsson, 2004). They can live singly in pairs or irregular cluster and sometimes in chains of varying length. Isolated bacteria in this work are similar with the above statements for lactic acid bacteria. Eight different bacteria were isolated. Out of them, morphological Lr-1 and Lr-6 were cocci, but other bacteria of Lr-2, Lr-3, Lr-4, Lr-5, Lr-7 and Lr-8 were bacilli. Lactic acid bacteria are highly appreciated as a biological enchanter probiotic useful bacteria. Therefore, these isolated bacteria were LAB.

Acknowledgements

We would like to thank Dr Theingi Shwe (Rector, Hinthada University), Dr Yee Yee Than (Pro-Rector, Hinthada University) and Dr Cho Kyi Than (Pro-Rector, Hinthada University), for their permission to conduct this research. We are deeply indebted to Professor Dr Yi Yi Win, Head of Zoology Department, Hinthada University, for her encouragement and invaluable suggestions.Thanks are also due to our colleagues from Zoology Department, Hinthada University, for their help during this work.

References

Atlas, R.M. (1997). *Handbook of Microbiology Media*. 2nd ed. CRC Press; New york, 228 pp.

Axelsson, L. (2004). Lactic Acid Bacteria: Classification and Physiology. In Lactic Acid Bacteria. Microbiological and Functional Aspects. (S Salmine, A.Von Wright & A Ouwehand, Eds London and Tokyo). New Yorkk: Marcel Dekker, Inc. pp.1-67

Balcazar, J.L., D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, O. Girones and J.I., Muzquiz (2007).In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. *Veterinary Microbiology*, vol. 122(3): pp. 373-380.

Bukola, C.A and A.A. Onilude (2008). Screening of Lactic Acid Bacteria Strains Isolated from Some Nigerian Fermented Foods for EPS Production. *World Applied Sciences Journal* vol.4 (5): pp.741-747.

Divakara, R., B.K. Manjunatha and K. Paul (2010). Lactic acid bacteria as Probiotics: Role in human health. *Res. Rev. Biomed. Biotechnol.* vol. 1(1): pp. 1-5.

Ghosh, A.S., D.G., Selvam, C.s., Nethu, A.V., Saramma, and A.A.,Hatha(2014). Diversity and antimicrobial activity of lactic acid bacteria from the gut of marine fish Rastrelliger kanagurta against fish, shrimp and human pathogens, J. Mar. Biol. Ass. India, 55 (2): 22-27

Gomez-Gill, B., A.Roque, and J.F., Turnbull (2000). The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms.Aquaculture, 1(3):259-270.

O'sullivan, L., R.P. Ross and C. Hill, 2002. Potential of bacteriocin-producting lactic acid bacteria for improvements in food safety and quality. *Biochimie*, vol. 84: pp.593-604.

Panthavee, W., S, Pramuan and W. Nisakornn (2007). Identification and evaluation of lactic acid bacteria for PLa-Sorn (fermented fish) Starter. The 2nd International Conference on "Fermentation Technology For Value Added Agricultural Products" at Kosahotel, Kohn-Kaen, Thailand.

Perdigo´n, G and S. Alvarez. (1992). Probiotic and the immune state. In: Fulletarter. The 2nd International conference on "fermentation technology for value Added Agricultural Producrts" May 23-25, at kasahotel, kohr, R. Ed.., Probiotics: the Scientific Basis. Chapman & Hall, London, pp. 145–180.

Ringø, E., and F.J. Gatesoupe (1998). Lactic acid bacteria in fish: a review**.** *Aquaculture* vol.160 (3): pp.177–203. doi: 10.1016/S0044-8486(97)00299-8

Ringø, E. (2004). Lactic acid bacteria in fish faming**.** *In:* Lactic acid bacteria Microbiological and functional Aspect 3rd ed.Revised and Expanded by S Salminen., A.Von Wright, and A. Ouwehand,(eds). Marcel Dekker, Inc. pp. 581-610.

Sanders, M.E. (1999). Probiotics**.** *Food Technol*. vol. 53: pp. 67-77.