Analysis of bacterial load of gut of *Tenualosa ilisha* from Chandpur river

A thesis submitted to the Department of Fisheries, University of Dhaka
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Master of Science (MS) in Fisheries

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Declaration by Student

I hereby declare that the dissertation entitled "**Analysis of bacterial load of gut of *Tenualosa ilisha* from Chandpur river**" submitted to the Department of Fisheries, University of Dhaka for the degree of Master of Science (MS) is based on self-investigation carried out under the supervisions of Mohammad Mamun Chowdhury, Associate Professor, Department of Fisheries, University of Dhaka, Dhaka- 1000, Bangladesh and Dr. Md. Abdul Karim, Professor, Department of Botany, University of Dhaka, Dhaka- 1000.

I also declare that this or any part of this work has not been submitted for any other degree anywhere. All sources of knowledge used have been duly acknowledged.

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Certificate

We certify that the research work embodied in this thesis entitled "**Analysis of bacterial load of gut of *Tenualosa ilisha* from Chandpur river**" submitted by Md. Foysal Hossain, roll number: 813, session: 2015-16, registration number: 2010-313-000 has been carried out under our supervision.

This is further to certify that it is an original work and suitable for the partial fulfillment of the degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka.

We wish every success in his life.

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Abstract

Hilsha (*Tenualosa ilisha*) is a popular, tasty fish found in the rivers of Bangladesh round the year.It is now generally recognized that the digestive tract of fish particularly the intestine contains a large number of bacteria. Sample were collected from Meghna river in Chandpur for gut microbial analysis. During the study sample were collected in two different sesson *viz*. summer and winter.

Four types of bacteriological culture media *viz*. EMB agar medium for coliform, XLD agar for *SalmonellaShigella*, PYG media for heterotrophic bacteriaand TCBS for *Vibrio* were used to assess the quantitative and qualitative study of bacteria associated with the fresh hilsa fish collected from Meghna river in Chandpur. The bacterial load of fresh fish samples was found to be ranged between 11.6 x $10^8$to 1.3 x10^4cfu/g, 3.56 x10^6 to 4 x 10^3cfu/g, 5 x $10^8$to 2.7 x10^4cfu/g and 9.5x10^5 to 5.4x10^3in PYG agar, EMB agar, and TCBS agar, XLD agar respectively.

Highest bacterial count 11.6 x 10^8, 3.56 x10^6, 5 x 10^8, 9.5x10^5 in PYG agar, EMB agar, and TCBS agar, XLD agar medium. All of the highest bacterial count found in summer.

During this study 123 bacterial colonies were isolated. Among them 27 bacterial isolates were selected for further study. Out of 27 bacterial isolates 22 were Gram positive and 5 were Gram negative. The provisionally identified Gram positive bacteria were *Bacillus cereus* (2), *Bacillus subtilis* (4), *Bacillus coagulans* (3), *Renibacterium salmoniarum* (2), *Bacillus licheniformes* (1), *Bacillus pumilus* (3), *Erysipelothrix rhusiopathiae* (1), *Bacillus alvei* (3), *Bacillus polymyxa* (1), *Renibacterium salmoniarum* (2) and Gram negative bacteria were *Legionella micdadei* (2), *Vibrio nereis* (1), *Legionella pneumophila* (1), *Pasteurella multocida* (1).

The outcome of the study indicates that gut microflora of Hilsa may not only be pathogenic to fish but also for human health which can cause foodborne disease.

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Abbreviations

%	Percent
&	And
^{0}C	Degree Celsius
Cfu	Colony forming unit
Cm	Centimeter
Conc.	Concentration
e.g.	Exempli gratia (for example)
ed.	Edition
Ed.	Editor
Eds.	Editors
et al.	et alibi (with others)
etc.	Etcetera
Fig.	Figure
g.	Gram
h.	Hour
hrs.	Hours
i.e.	Id est (that is)
Inc.	Incorporation
L or l	Liter
M.R	Methyl Red
mg	Milligram
min.	Minute
ml	Milliliter
NA	Nutrient agar
NaCI	Sodium Chloride
nm	Nanometer
P	Phosphorus
pH	Negative Logarithm of hydrogen ion concentration
pp.	Pages
Sec.	Second
sp.	Species (Singular)

spp.	Species (plural)
SS	Salmonella-Shigella
V.P	Voges-Proskauer
Viz.	Videli (namely)
Vol.	Volume

Chapter 1

Introduction

1.1 Background

Fish is one of the important sources of quality animal proteins and availability and affordability is better for fish in comparison to other animal protein sources. Fish serves as a health-food for the affluent world owing to the fish oils which are rich in polyunsaturated fatty acids (PUFAs), especially ω-3 PUFAs and at the same time, it is a health-food for the people in the other extreme of the nutritional scale owing to its proteins, oils, vitamins and minerals (Mohanty, 2010).

The hilsa shad, commonly known as Hilsa (*Tenualosa ilisha*, Hamilton 1822) referred in the literature as an anadromous (earlier) Clupeid of the Bay of Bengal and Indian Ocean, now established as a diadromous ascends in the rivers flowing into the Bay of Bengal, Arabian Sea and Persian Gulf. Hilsa belongs to the subfamily Alosinae, family Clupeidae, order Clupeiformes, and is one of the most important tropical fishes of the Indo-Pacific region. It is a fast swimming euryhaline known for its cosmopolitan distribution in brackish water estuaries and marine environment. Naturally hilsa is in great demand globally, specifically in the oriental world and enjoys high consumer preference. Its high commercial demand makes it a good forex earner. This is an important migratory species in the Indo-Pak sub-continent, especially in Bangladesh, India and Myanmar. It is the national fish of Bangladesh and the largest single species fishery contributing 75% of total catch in this region (Raja, 1985) that accounts nearly half of the total marine catch and about 12-13% of total fish production of the country (Haldar, 2008).

Bangladesh is known as the land of rivers, which is endowed with very considerable, marine, estuaries and inland water having great fisheries potential. Fisheries sector is contributing 2.46% to the total export earning, 4.39% to GDP and 22.76% to agricultural sector (DoF, 2013). The hilsa fishery was declining tremendously over the last decades for increasing fishing pressure and environmental degradation from the inland open water although the total marine production remains more or less static. In an investigation, Haldar and Rahman (1998) found that hilsa landing at Chandpur (a major landing center) has lost about 25.8% from 1978-88 to 1989-94 due to loss of freshwater discharge from the upstream international river. Construction of cross dam and flood control dam has destroyed a commercial hilsa fishery of about 500 MT/yr (Haldar et al., 1992).The total annual fish production of Meghna River is 66783 MT (DoF, 2007).

Table 1.1 Production trends of Hilsha for the last 15 years in Bangladesh (source: DoF2015)

Year	Production (MT)			Increase rate (%)
	Inland Capture	Marine Capture	Total	
1999-00	79165	140367	219532	2.34
2000-01	75060	154654	229714	4.64
2001-02	68250	152343	220593	-3.97
2002-03	62944	136088	199032	-9.77
2003-04	71001	184837	255839	28.54
2004-05	77499	198363	275862	7.83
2005-06	78273	198850	277123	0.46
2006-07	82445	196744	279189	0.75
2007-08	8990	200100	290000	3.87
2008-09	95970	200951	298921	3.08
2009-10	114768	198574	313342	4.82
2010-11	114520	225325	339845	8.46
2011-12	114475	232037	346512	1.96
2012-13	98648	252575	351223	1.36
2013-14	127514	257626	385140	9.99

The gut micro biota usually refers to a very complex and dynamic microbial ecosystem that colonizes the gastrointestinal tract of an animal (Sukanta, 2010).It is now generally recognized that the digestive tract of fish particularly the intestine contains a large number of bacteria. Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers favorable conditions for the microorganisms. However some bacteria which possess the ability to tolerate the low pH in gastric juices resist the action of bile acids, lysozyme secreted in intestines, immune responses and adheres to the mucus or enteric wall surface could persist for a relatively long time and eventually make intestinal micro flora specific to each host animal (Olsson et al., 1992). The influence of the gut flora on the host is clearly of great interest in aquaculture, particularly where poor productivity and/or stock losses are

widespread. Within marine and other aquatic animals, the colonization of the digestive system by micro-organisms is influenced by a number of both host and non-host related factors. On the other hand, bacteria producing antibacterial substances were isolated from marine fish intestines (Olsson et al. 1992; Onarheim and Raa, 1990).

1.2 Overview of Gut Microbiota

Although the composition of bacterial communities is strongly determined by properties of the external environments in which they are found (Fierer and Jackson, 2006), the 'environments' provided by eukaryotic hosts are also largely impacted by the microbes that colonize them. For example, gut bacteria often promote nutritional provisioning and nitrogen recycling for their hosts (Douglas 1998; Sabree et al.,2009). In the vertebrate gut, bacteria play important physiological roles, influencing metabolic processes such as the digestion of complex carbohydrates (Turnbaugh et al*.,* 2006) and the regulation of fat storage (Backhed et al.,2004).

Fishes are in association with microbes present in the aquatic environment, and they receive bacteria from the aquatic environment through water and food. Being rich in nutrients, fish gut confers a favorable niche for microorganisms, which in turn is generally colonized by large number of heterotrophic bacteria, and also it have complex microbial ecosystem. Gut microbes of fish have been studied by many researchers (Horsley, 1977; Cahill, 1990; Trust, 1974; Huber, 2004)

The microbial community has an important role in the health and nutrition of the host. (Burr et al., 2005; Sakata 1990; Ringo et al., 1995; Thompson et al., 1999; Verschuere et al., 2000; Suzer et al., 2008). The indigenous microflora of the fish digestive tract has been traditionally investigated by conventional culture-dependent methods including cultivation on selective or non-selective media followed by isolation and phenotypic characterization (Spanggaard et al., 2000; Pond et al., 2006; Hovda et al., 2007; Kim et al., 2007).

Traditionally, studies on fish-associated microorganisms involved culture-dependent techniques of dubious sensitivity, which highlighted only the bacteria (typically the aerobic heterotrophic bacterial component (Montes et al., 1999).The bacterial composition of the fish gastrointestinal tract has been studied previously using culture techniques (Cahill, 1990). However, these methods are time consuming, and only part of

the total bacterial community can be recovered using traditional agar substrates (Romero and Navarrete, 2006; Navarrete et al., 2009).

The non-indigenous contaminate the fish or the habitat one way or the other and examples include *Escherichia coli, Clostridium botulinum, Shigella dynteriae, Staphylococcus aureus, Listeria monocytogens and Salmonella*. The indigenous bacterial pathogens are found naturally living in the fish's habitat for example *Vibrio* species and *Aeromonas* species. The bacteria from fish only become pathogens when fish are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to prevail. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteium, Streptococcus spp., Vibrio spp., Aeromonas spp., Salmonella spp.* and others (Lipp and Rose, 1997). Fish take a large number of bacteria into their gut from water sediment and food (Adedeji et al., 2011). It has been well known that both freshwater and brackish water fishes can harbor human pathogenic bacteria particularly the coliform group. Fecal coliform in fish demonstrates the level of pollution in their environment because coliform are not named flora of bacteria in fish. It has been well known that both freshwater and brackish water fishes can harbor human pathogenic bacteria particularly the coliform group. Fecal coliform in fish demonstrates the level of pollution in their environment because coliform are not named flora of bacteria in fish (Adedeji et al., 2011). *Escherichia coli*, the predominant species of the fecal coliforms, has been found in the intestinal tract of fish (Newman et al., 1972).The isolation of *Salmonella, Shigella, and E.coli* indicate faecal and environmental pollution (Yagoub, 2004). The predominant bacterial species isolated from most of the fish digestive tracts have been reported to be aerobes or facultative anaerobes (Trust, 1974; Bairagi, 2002; Saha, 2006). Roberts (1978) showed that Pseudomonas and Vibrio sp. cause infectious diseases in fish.Salmonella sp. is a potential pathogen for humans and fish (Alcaide et al., 2005)

1.3 Rationale

One of the most important issues in microbial ecology of the gastrointestinal tract is the understanding of how important factors (systems, water, feed, species and age) influence gut microbiota. The gut microbiota usually refers to a very complex and dynamic

microbial ecosystem that colonizes the gastrointestinal tract of an animal (Sukanta, 2010), most fish related food borne illness are traced to *Salmonella*, *Staphylococcus spp.,Escherichia spp*., *Vibrio parahemolyticus, Clostridiumperfringens, Clostridium botulinum E,* and *Enteroviruses*. Extensive papers were published on various aspects of the microbial flora associated with fish eggs, skin, gills and intestine, and on the relationship of the intestinal microbiota to that of the aquatic habitats. The microbial populations within the digestive tract of fish are rather dense with numbers of microorganisms much higher than those in the surrounding water indicating that the digestive tract provides favorable ecological niches for these organisms.

The presence of bacteria in fish could play diverse roles some of which might be beneficial to the fish itself. However, the presence of some bacterial species could lead to post harvest spoilage and adverse health conditions. The intestinal microflora may be significant in fish spoilage and may be involved in spread of fecal contaminants (Geldreich and Clarke, 1966)

1.4 Problem Statement

The composition of the microbiota and its functions may change or be influenced by factors such as diet and environmental conditions (Ringo et al., 2001). Many factors influence the composition of the normal intestinal microflora in fish and must be considered when comparing studies. Some reports suggest that the intestinal microflora of fish reflects the bacterial content of ingested food, while others showed the importance of the rearing water. However, few investigations of the dietary influence on the intestinal flora in fish have been undertaken (Sugita et al., 1988; Strøm and Olafsen, 1990; Ringø and Strøm, 1994). Pelzar et al. (1986) stated that the microflora of caught fish and other aquatic specimens is largely a reflection of the microbial quality of the water where they were harvested.

1.5 Research gap

Water and natural food are the two main sources of microorganisms available to fish, the factors underlying the successful colonization of ingested microbes and the community assembly inside the gut are still poorly understood.

1.6 Objectives

The specific objectives was

1. To determine the bacterial density in gut of fresh hilsa
2. To compare the bacterial load in summer and winter
3. To identify bacteria by biochemical test

Chapter 2

Materials and Methods

2.1Experimental organism

*Tenualosa ilisha*was chosen for the study because of its market demand. Six experimental fish was chosen at random for this study.

2.2 Sample collection

*Tenualosa ilisha*was collectedat harina ghat Meghna river in Chandpur. Samples were returned to the laboratory as early as possible by using ice box which was covered with ice.

2.3 Sampling date

Samples were collected at two different times.

Table 2.1 Sampling number, date and number of sample collected from each sampling for the experiment

Number of sampling	Date of sampling	Number of sample collected
1	October 29, 2016	2
2	November 27, 2016	2

Table 2.2 Length and weight of experimental fishes

Serial no	Number of fish	Length(cm)	Weight(g)
1	Fish-1	33.0	440
2	Fish-2	32.3	400
3	Fish-3	33.0	435
4	Fish-4	34.0	450

2.4 Preparation of physiological saline

Physiological saline (0.9%) was prepared after mixing 0.9 g of NaCl with 100 ml distilled water in a conical flask and it was autoclaved.

2.5 Preparation of petri dish

Required number of petri dishes were sterilized at 160 °C for 2 hours by dry sterilizer (EYELA NDS-450D).

2.6 Processing of fish sample

1.0 g of intestine of sample fish was separated and was taken into a mortar pestle. 10.0 ml of physiological saline was added into the mortar pestle and it was mixed well.

2.7Media and techniques for the enumeration and isolation of bacteria

2.7.1 Selected culture media

PYG (Peptone Yeast extract Glucose agar) medium were used for the enumeration and isolation of aerobic heterotrophic bacteria present in water samples. The pH of the medium was adjusted to 7.2. The pH was adjusted before the addition of agar into the medium.

For the determination and isolation of enteric, pathogenic and related bacteria following selective media were used:

- EMB (Eosine Methyline Blue Agar) agar medium (Difco) and
- XLD (Xylose lysine deoxycholate agar) agar medium (Diagnostic Pasteur)
- TCBS (Thiosulfate Citrate Bile salt Sucrose agar)agar (Difco)

Table 2.3 Media used for the experiment

Serial no	Name of the media	Bacteria
1	Thiosulfate Citrate Bile salt Sucrose agar (TCBS)	*Salmonella* spp., *Vibrio cholera, E.coli, Enterobacter aerogens*
2	Peptone Yeast extract Glucose (PYG)	Heterotrophic
3	Eosine Methyline Blue Agar(EMB)	*E. coli*and *Aerobaacter aerogens*
4	Xylose lysine deoxycholate agar (XLD)	*Salmonella* and *Shigella*

Those selective media used for isolation of *Salmonella* spp., *Vibrio cholera, E. coli,* and Heterotrophic bacteria.

2.7.2 Techniques employed

Dilution plate technique was used for the enumeration and isolation of bacteria.

2.7.2.1 Dilution plate technique

Serial dilution plate technique (Claus, 1995) was used for the isolation of microorganisms. One ml of solution was transferred to 9 ml of sterile water for ten-fold (1:10) dilution and further diluted up to 10^5 dilutions. Plating in duplicate plates was made each diluted sample. One ml of each of the diluted sample was taken in a sterilized Petri plate by sterilized pipette. Then molten agar medium was poured and mixed thoroughly by rotating the Petri plate, first in one direction and then in the opposite direction.

After solidifying the medium the plates were inverted and incubated at 37°C for 24 h in an incubator (Memmert GmbH + Co kg 8540 Sehwabach).

2.8 Enumeration of bacteria

After 24 h of incubation the plates having well discrete colonies were selected for counting. The selected plates were placed on colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) and the colonies were counted.

In case of EMB agar medium, dark, blue-black colonies with metallic green sheen were considered as coliform bacteria while white colonies were considered as non-lactose fermenter. XLD agar medium, black colonies were considered as highly pathogenic.

2.9 Isolation of bacteria

Well discrete aerobic heterotrophic, coliform and related enteric bacterial colonies were isolated immediately after counting. Based on their colonial morphology, different discrete colonies were selected for isolation.

The selected colonies were marked and studied for various characters *viz.* color, form, elevation, margin surface, optical characters etc. (Eklund and Lankford, 1967; Bryan, 1950). Then the marked and observed bacterial colonies were transferred on nutrient agar slant for further studies.

2.10 Maintenance and preservation of isolates

The isolates were then transferred on nutrient agar slant. The slants were kept in polythene bags and preserved as stock culture in a refrigerator at 4°C for further study. Periodical transfers of isolates on agar slants were done for maintaining viability of the organisms.

2.11 Morphological observation of isolated strains

For the identification of selected isolated strains, following morphological characters were studied and recorded.

2.11.1 Colonial morphology

The bacterial colonies on plating medium were morphologically studied as their form, elevation, margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and their rate of growth.

2.11.2 Microscopic examination of isolated strains

Bacterial cells suspension was made by using fresh culture with physiological saline. The prepared suspension was used to make smear. A good quality glass slide was used for this purpose. Thin smear was prepared on the clean and oil free slide. The smear was allowed to dry in air and was fixed by passing the slide over the flame of a spirit lamp. The following two different staining methods were employed to stain the fixed smears.

- Simple staining method
- Differential staining method.

2.11.2.1 Simple staining (Bryan 1950)

Manual of Microbiological Methods (SAB 1957) was followed for simple staining. Basic dyes *viz.* crystal violet, basic fuchsine, cotton blue, safranine, mercurochrome and malachite green were used. The fixed smear was flooded with a dye solution for one minute. The flooded smear was washed off with water and dried in air.

2.11.2.2 Differential staining

Staining procedures that make visible differences between microbial cells or parts of cells were termed as differential staining (Pelczaret al.,1986). Differential staining use a combination of dyes that take advantage of chemical differences among cells (Claus, 1995). The differential stains most frequently used are the simple stain, Gram stain, acid-

fast stain, negative stain and spore stain (Tortora et al., 1998). For this purpose, fixed smear was exposed to more than one dye solution.

In this study, two differential techniques were used *viz.* Gram staining and spore staining.

2.11.2.2.1 Gram staining

This is one of the most important and widely used differential staining technique is considered as one of the important steps in identifying an unknown bacterium. For Gram staining, method described by Claus (1995) was followed.

Fixed smear was treated with the following solutions and after application of each solution slide was gently washed off with water.

Crystal violet solution for 60 sec., Lugol's iodine solution for 60 sec., 95% Ethyl alcohol for 30 sec and Safranine solution for 60 sec. The slide was dried through air and observed under microscope (Nikon MICROPHOT, UFX-IIA, Japan). The results were recorded as Gram positive (blue-violet) and Gram negative (light red).

2.11.2.2.2 Spore staining

The method described by Claus (1995) was applied in spore staining. Smear was made from 24 h old bacterial culture. The fixed smear was flooded with 5% aqueous solution of malachite green and heated over a brass plate for about 15-20 minutes taking care that the dye must not be dried off. Excess dye was then washed gently and basic fuchsine was used as a counter stain for 1 minute. The slide was washed gently, dried and examined under microscope.

Spores were stained green and vegetative cells or sporangia were stained with red color of basic fuchsine. The shape and position of the spores within sporangia were observed. The swelling nature of the sporangium was also observed and recorded**.**

2.11.2.3 Negative staining (SAB 1957)

To introduce a rapid method for demonstrating microorganisms against a dark background and for use in measuring the size of bacteria an unusual staining process are employed. This process is known as Banians' Congo red method.

In this method a drop of 2% Congo red [2 g Congo red (80% dye content) and 100ml distilled water] was placed on a slide and the culture were mixed with a loop and spread out in a thick film. After drying, the film was washed with 1% HCl.

2.12 Physiological and biochemical studies of the isolates

Following Bergey's Manual (Sneath et al.,1986) the physiological and biochemicaltests of the isolated bacteria were carried out. Along with Bergey's Manual several other manuals such as Manual of Microbiological Methods (SAB, 1957), Microbiological Methods (Collins and Lyne, 1984) and Understanding Microbes (Claus, 1995) were also consulted.

2.12.1 Catalase test (Claus 1995)

The microbes produce the enzyme catalase to break the hydrogen peroxide into water and molecular oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2\uparrow$$

Catalase is an enzyme produced by and found in essentially all actively growing microorganisms capable of using oxygen for respiration.

The test for catalase in bacteria was performed by simply placing few drops of hydrogen peroxide directly on some cells on a glass slide.

The evolution of oxygen bubbles indicated the positive result i.e. production of catalase**.**

2.12.2Deep glucose agar test (Hall 1929)

Microorganisms vary widely in their requirements for oxygen. The nature of microbial growth in agar deeps reflects the cells' relative need for oxygen or an oxygen free environment. In relation to free oxygen, organisms are generally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes. A tube of deep glucose agar medium was inoculated in fluid condition approximately at 45°C. The tube was rotated to mix the inoculums with the medium and was allowed to solidify.

After incubation at 37°C for 7 days observation was made to find out whether the organisms grew on the surface and in the upper layer of the medium (strict aerobes), or the organisms grew just a few millimeters below the surface (microaerophiles), or the organisms grew throughout the medium (facultative anaerobes), or the organisms grew deeper in the medium (strict anaerobes).

2.12.3 Oxidase test (Claus 1995)

The enzyme oxidase in certain bacteria catalysed the transport of electron from donor bacteria to the redox dye tetra-methyl-para-phenylene-diamine dihydrochloride. The dye in the reduced state has a deep purple color. To perform this test filter papers were

soaked in 1% aqueous tetramethyl-phenylenediamine dihydrochloride. Fresh young culture was rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds. Blue color indicated a positive result**.**

2.12.4 Methyl red test (Bryan 1950)

Methyl red (M.R.) test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by "Methyl Red" indicator. For this test V.P. broth was inoculated and incubated at 37°C for 5 days.

After incubation, 5 drops of methyl red indicator were added to the culture broth. Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.12.5 Voges Proskauer (V.P) Test (SAB 1957)

For the Voges-Proskauer reaction according to the “Standard Methods” of the APHA (1946), to 1 ml of culture add 0.6 ml of 5% α-napthol in absolute alcohol and 0.2 ml of 40% KOH. It is important to shake for about 5 sec. after addition of each reagent.

A recent modification of Coblenty (1943) is similar to the APHA method but uses a agar slant culture followed by incubation of the broth for 6 hours. Also the 40% KOH has 0.3% of creatine added to it to intensity the reaction. After addition of the reagents the culture is shaken vigorously for 1 minute.

Positive reaction is characterized by an intense rose pink colour developing in a few seconds to 10 min.

Fig. 2.1 (A) icebox with ice, (B-C) weight measurement, (D-E) measuring length

Chapter 3

Results

lt will the status of gut microbiota of *Tenualosa ilisha* of Chandpur river. The present study has been delineated in two distinct phases.

- Quantitative analysis of microorganisms associated with the collected fresh.
- Isolation and identification of the heterotrophic and enteropathogenic bacteria associated with the collected samples.

3.1Enumeration of aerobic heterotrophic bacteria

The heterotrophic bacterial count of fish samples was shown in Table (3.1). During summer season maximum number of bacterial count was 11.6×10^8 cfu/g while minimum number was found in winter season and it was 1.3×10^4 cfu/g.

3.2 Enumeration of enteric and related bacteria

Enteric and related bacterial count on XLD agar medium of fish sample was shown in Table 3.2. During summer season highest (9.5×10^5 cfu/g) growth was found while minimum (5.4×10^3 cfu/g) growth was found during winter season. Enteric and related bacterial count on EMB agar was presented in Table 3.4. The highest bacterial count was 3.56×10^6 cfu/g during summer Season and lowest count was 4×10^3 cfu/g during winter season. Bacterial count of *Vibrio* in TCBS medium was shown in table 3.3. Bacterial count on TCBS agar was ranged between 2.7×10^4 cfu/g and 8.4×10^7 cfu/g. Lowest growth was found in winter season and it was 2.7×10^4 cfu/g and highest growth was found in summer season and it was 8.4×10^7 cfu/g.

Table 3.1 Total bacterial count (cfu/g) in *Tenualosa ilisha* fish sample

Season	Sample	Bacterial load
Summer	Fish 1	$1.6 \times$
	Fish 2	$11.6 \times$
Winter	Fish 3	$9.8 \times$
	Fish 4	$1.3 \times$

The height count of TBC was (11.6×10^8cfu/g) found in Fish 2 and lowest TBC count was (1.3×10^4cfu/g) found in Fish 4.

Table 3.2Total *Shalmonella-Shigella* count (cfu/g)in *Tenualosa ilisha* fish sample

Season	Sample	Bacterial load
Summer	Fish 1	9.5×10^{5}
	Fish 2	7.4×10^{5}
Winter	Fish 3	2.7×10^{3}
	Fish 4	5.4×10^{3}

In table 3.2 shows that highest *Shalmonella-Shigella* count (9.5×10^{5}cfu/g) found in Fish 1 and lowest value (5.4×10^{3}cfu/g) found in Fish 4.

Table 3.3Total *Vibrio* count (cfu/g)in *Tenualosa ilisha* fish sample

Season	Sample	Bacterial load
Summer	Fish 1	8.4×10^{8}
	Fish 2	5×10^{8}
Winter	Fish 3	9.8×10^{4}
	Fish 4	2.7×10^{4}

In table 3.3 shows that height *Vibrio* count (5×10^{8}cfu/g) was found in Fish 1 and lowest *Vibrio* count (2.7×10^{4}cfu/g) was found in Fish 4.

Table 3.4Total EMB count (cfu/g)in *Tenualosa ilisha* fish sample

Season	Sample	Bacterial load
Summer	Fish 1	1.4×10^{5}
	Fish 2	3.56×10^{6}
Winter	Fish 3	2.7×10^{3}
	Fish 4	4×10^{3}

In table 3.3 shows that height EMB count (3.56×10^{6}cfu/g) was found in Fish 2 and lowest EMB count (4×10^{3}cfu/g)was found in Fish 4.

3.3 Isolation and selection of the isolate

During this study a total of 123 colonies were primarily selected. These colonies comprised of all aerobic heterotrophic, enteric and related bacteria. Finally 27 isolates were selected and purified for detail study towards identification. Out of these 27 isolates 13 were aerobic heterotrophic bacteria and 14 were facultative anaerobic. Bacterial colonies developed after applying dilution plate and streak plate techniques and the result were shown in Fig.3.1,3.2,3.3,3.4,3.5,3.6, and 3.7.

3.4 Colonial morphology of the selected isolates

Colonies of the selected isolates were found to be different in there for elevation, margin, surface, color and optical characteristics. The colonial morphology of the selected isolates as observed on PYG agar, EMB agar, XLD agar and TCBS agar were presented in Table 3.5, 3.6, 3.7, 3.8.

3.5 Microscopic observation of the selected isolates

From isolated 27 bacterial strains, 5 were Gram-negative and 22 were Gram-positive. Photomicrograph of the selected bacterial strains were shown in Fig.3.9-3.26.

3.6 Physiological and biochemical characteristics of the selected isolates

Some physiological and biochemical tests of the selected bacterial strains were given in Table 3.9 and rest of the isolates were identified by using selective media *viz.* (1) TCBS agar media, (2) EMB agar media and (3) XLD agar media. Among the 96 isolates from selective media 20 were *Vibrio parahaemolyticus*identified as green color in TCBS media, 12 were *Vibrio cholerae* identified as yellow color in TCBS media, 31 were *Escherichia coli*identified as golden metallic sheen or dark color in EMB media, 9 were *Pseudomonas aeruginosa*identified as pink color in EMB media, 6 were *Salmonella* identified as black color in XLD media, 18 were *Shigella*identified as yellow color in XLD media.

3.6.1 Physiological and biochemical characteristics of the selected isolates

The result of the biochemical tests was given in Table 3.9 and showed in Fig.3.8. All the tested strains were catalase positive except only one stain. All the strain were V.P. positive.Among the 27 tested strains 1, 10, 11, 12, 13, 14, 15, 17, 20, 22, 24 were oxidase negative. Except 22 stain, other showed positive result in methyl red test.

Among 27 bacterial strains five stains found as gram negative and remain 22 stains found as gram positive.

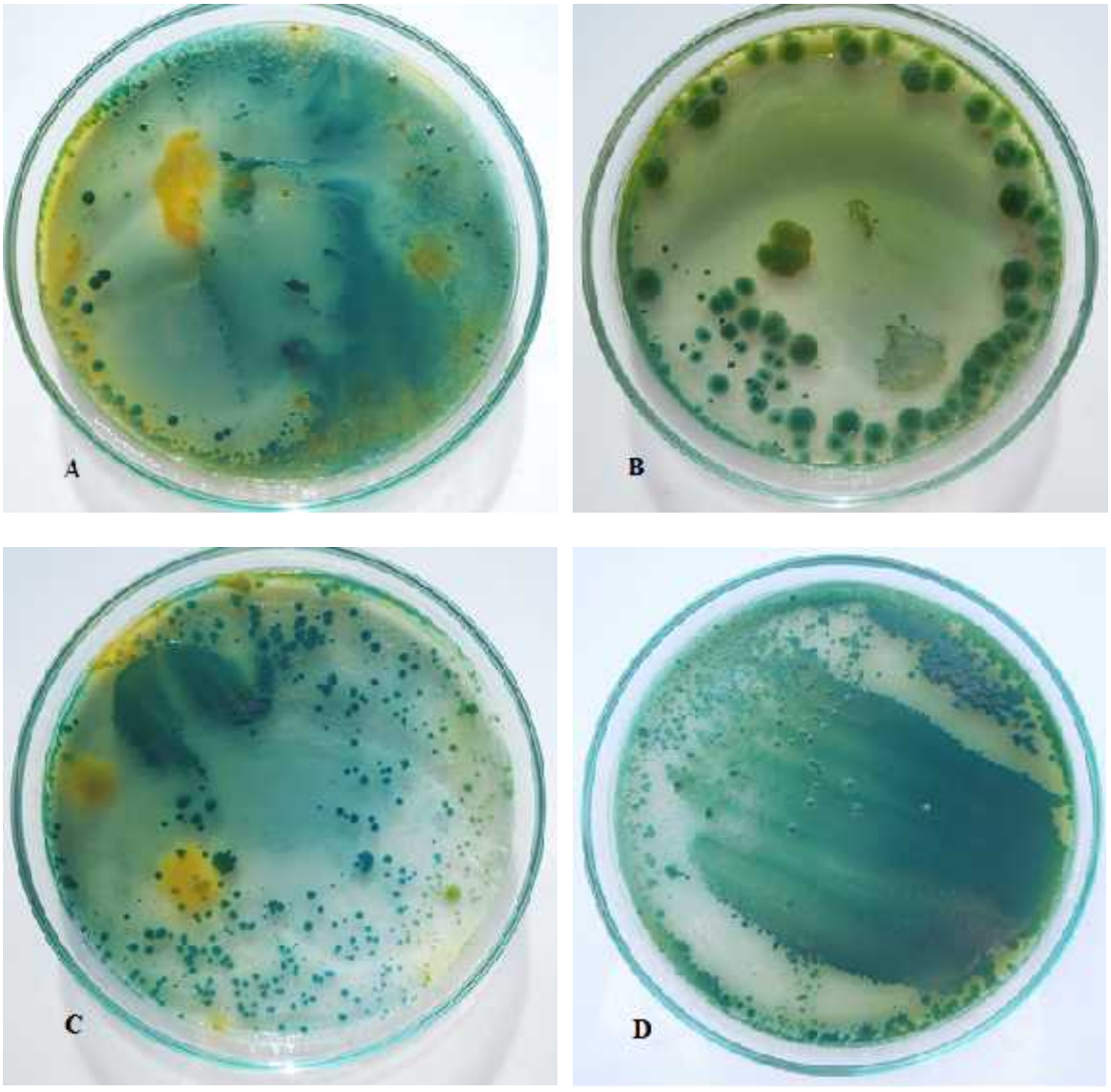

Figure 3.1 (A-D) Bacterial colonies development in TCBS agar media in sample 1

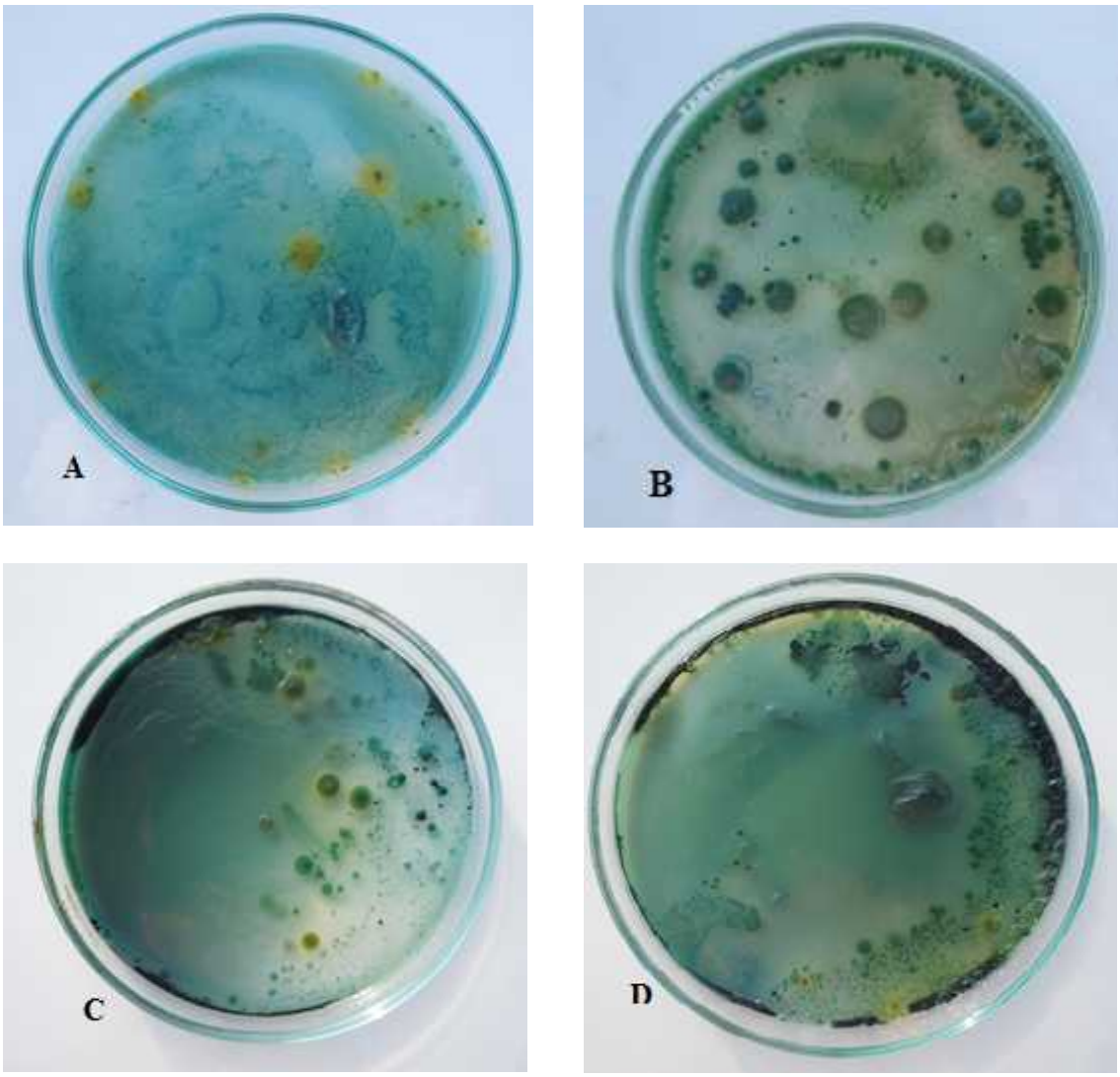

Figure 3.2 (A-D) Bacterial colonies development in TCBS agar media in sample 2

Table 3.5 Colony morphology of the selected isolates on TCBS agar media

Number of isolates	Form	Margin	Elevation	Surface	Optical Density	Diameter (cm)	Pigmentation	Provisional identification
1/5/1	Irregular	Curled	Raised	Smooth	Opaque	1.5	yellow	*Vibrio parahaemolyticus*
1/5/2	Circular	Entire	Raised	Smooth	Opaque	0.6	green	*Vibrio parahaemolyticus*
1/1′ /1	Circular	Entire	Raised	Smooth	Opaque	0.8	Lite green	*Vibrio parahaemolyticus*
1/1′ /2	Circular	Entire	Convex	Smooth	Opaque	0.5	green	*Vibrio parahaemolyticus*
2/5/1	Irregular	Curled	Flat	Smooth	Opaque	1.1	yellow	*Vibrio cholerae*
2/5/2	Irregular	Curled	Raised	Smooth	Opaque	0.5	green	*Vibrio parahaemolyticus*
2/5/3	Circular	Entire	Flat	Smooth	Opaque	1	yellow	*Vibrio cholerae*
2/5/4	Circular	Entire	Raised	Smooth	Opaque	0.4	green	*Vibrio parahaemolyticus*

Table 3.5 Colony morphology of the selected isolates on TCBS agar media (cont.)

2/4/1	Circular	Entire	umbonate	Smooth	Opaque	0.3	Green	*Vibrio parahaemolyticus*
2/4/2	Circular	Entire	Flat	Smooth	Opaque	1.3	Yellow with green center	*Vibrio cholerae*
2/4/3	punctiform	Curled	Raised	Smooth	Opaque	1.2	Green	*Vibrio parahaemolyticus*
2/4/4	Circular	Entire	Umbonate	Smooth	Opaque	0.3	Green	*Vibrio parahaemolyticus*
2/3/1	Circular	Entire	Flat	Smooth	Opaque	0.8	Yellow	*Vibrio parahaemolyticus*
2/3/2	Irregular	Curled	Flat	Smooth	Opaque	0.8	Yellow	*Vibrio cholerae*
2/2/1	Circular	Entire	Flat	Rough	Opaque	0.2	Green	*Vibrio parahaemolyticus*
2/1/1	Circular	Entire	Undulate	Smooth	Opaque	0.3	Green	*Vibrio parahaemolyticus*
2/5′ /1	Circular	Entire	Flat	Rough	Opaque	2.2	Yellow	*Vibrio parahaemolyticus*

Table 3.5 Colony morphology of the selected isolates on TCBS agar media (cont.)

2/5′ /2	Circular	Entire	Umbonate	Smooth	Opaque	0.4	Yellow with green center	*Vibrio cholerae*
2/5′ /3	Circular	Entire	Raised	Smooth	Opaque	0.3	Green	*Vibrio parahaemolyticus*
2/5′ /4	Circular	Entire	Convex	Smooth	Opaque	0.4	Green with yellow edge	*Vibrio parahaemolyticus*
2/5/5	Circular	Entire	Umbonate	Smooth	Opaque	0.5	Yellow with green center	*Vibrio cholerae*
3/1′ /1	Circular	Entire	Flat	Smooth	Opaque	0.1	Yellow	*Vibrio cholerae*
3/1′ /2	Circular	Entire	Flat	Smooth	Opaque	0.3	Green	*Vibrio parahaemolyticus*
3/1/1	Circular	Entire	Raised	Smooth	Opaque	0.5	Yellow	*Vibrio cholerae*
3/2 ′ /1	Circular	Entire	Raised	Smooth	Opaque	0.6	Green	*Vibrio parahaemolyticus*
4/2 ′ /1	Circular	Entire	Raised	Smooth	Opaque	0.2	Yellow	*Vibrio parahaemolyticus*
4/1′ /1	Irregular	Curled	Raised	Smooth	Opaque	0.5	Green	*Vibrio cholerae*
4/1′ /2	Circular	Entire	Raised	Smooth	Opaque	0.3	Yellow	*Vibrio cholerae*

Table 3.5 Colony morphology of the selected isolates on TCBS agar media (cont.)

4/1/1	Circular	Entire	Raised	Smooth	Opaque	0.7	Green	*Vibrio parahaemolyticus*
4/1/2	Circular	Entire	Raised	Smooth	Opaque	0.4	Lite green	*Vibrio cholerae*
4/2/1	Circular	Entire	Raised	Smooth	Opaque	1.1	Yellow	*Vibrio cholerae*
4/2/2	Circular	Entire	Raised	Smooth	Opaque	0.7	Green	*Vibrio parahaemolyticus*

Table 3.6 Colony morphology of the selected isolates on EMB agar media

Number of isolates	Form	Margin	Elevation	Surface	Optical Density	Diameter (cm)	Pigmentation	Provisional identification
1/1′ /1	Circular	Entire	Raised	Smooth	Opaque	0.4	Dark purple	*Escherichia coli*
1/1′ /2	Circular	Entire	Raised	Smooth	Opaque	0.7	Purple	*Escherichia coli*

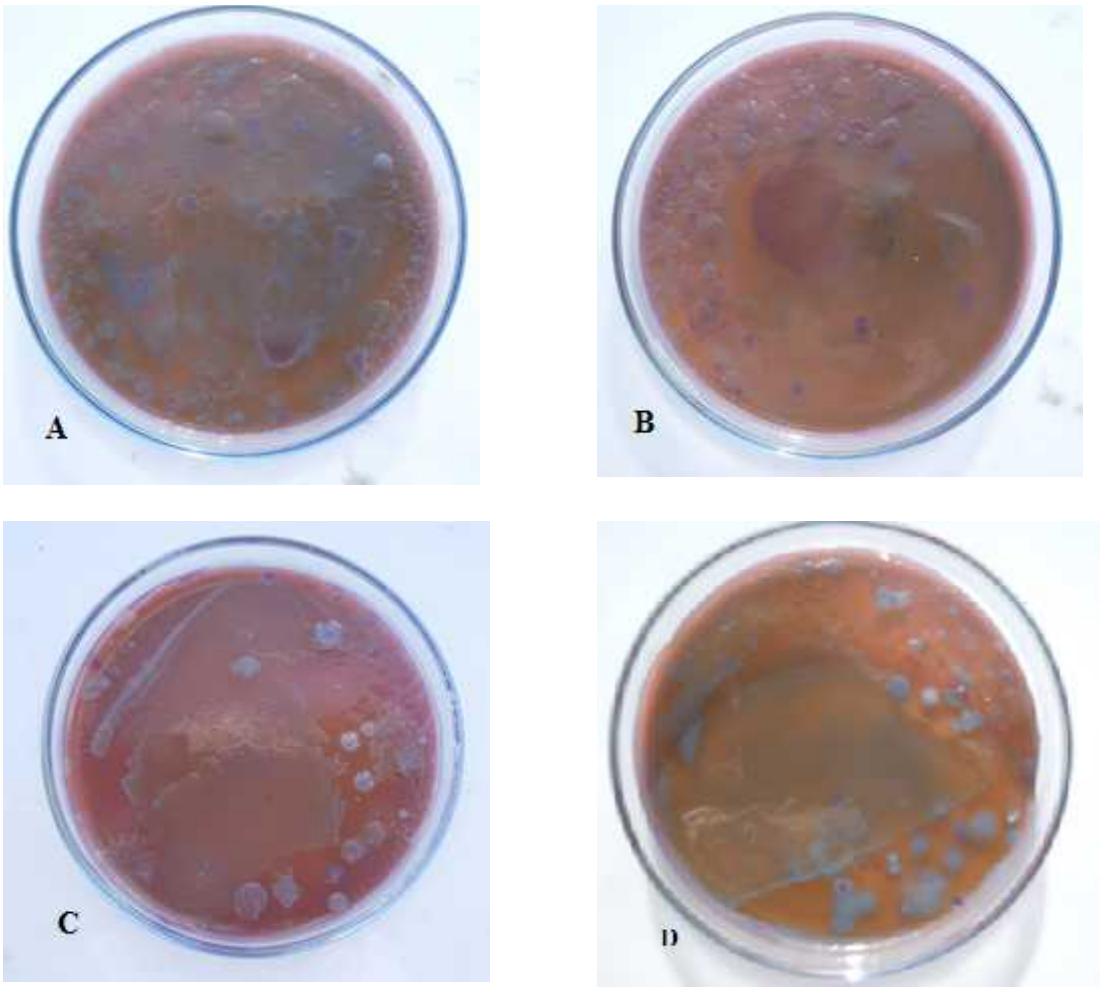

Figure 3.3 (A-D) Bacterial colonies development in EMB agar media in sample 1

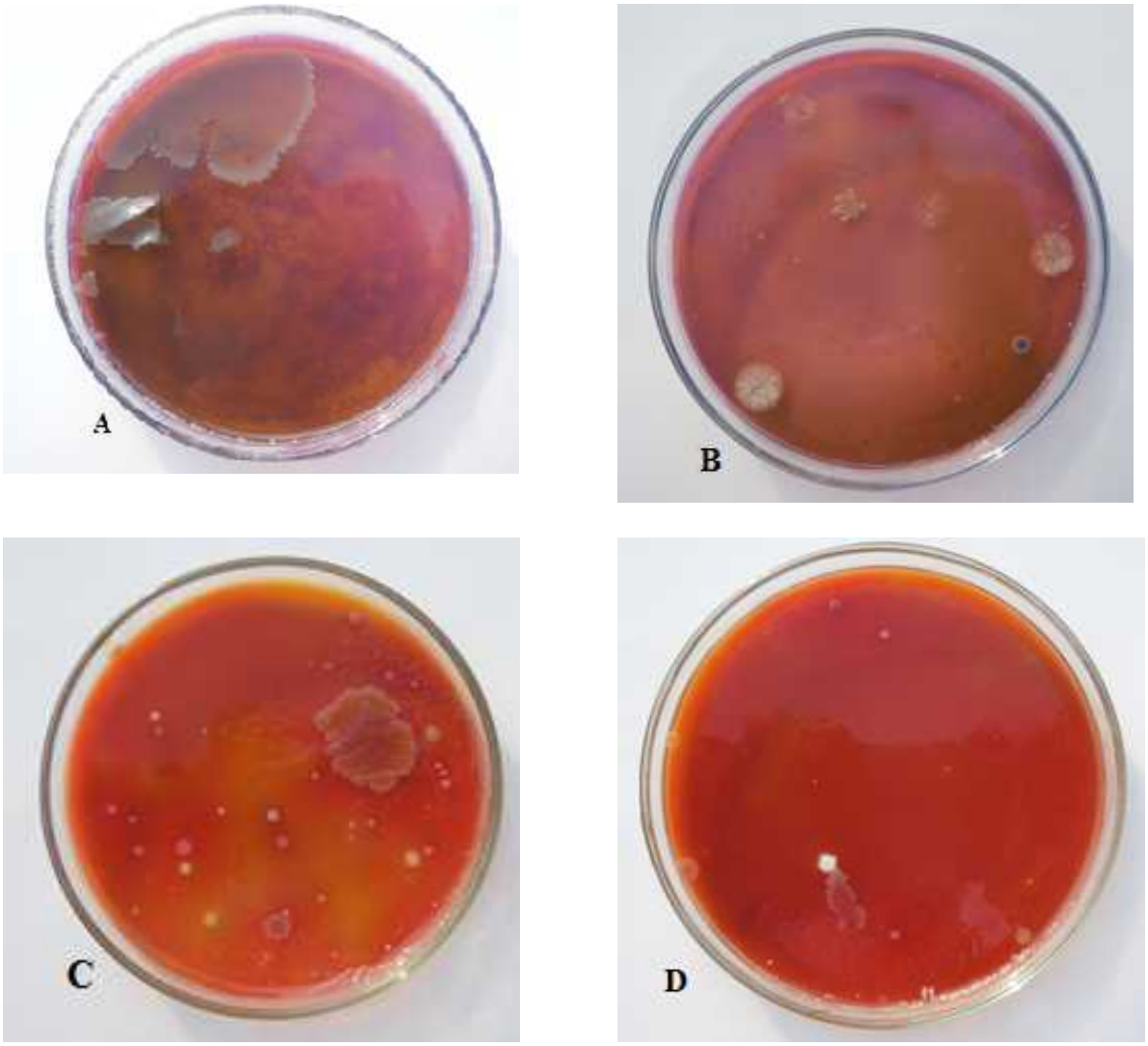

Figure 3.4 (A-D) Bacterial colonies development in EMB agar media in sample 2

Table 3.6 Colony morphology of the selected isolates on EMB agar media (cont.)

1/1′ /3	Circular	Entire	Raised	Smooth	Opaque	0.3	Dark	*Escherichia coli*
1/1′ /4	Circular	Entire	Raised	Smooth	Opaque	0.3	Dark purple	*Escherichia coli*
1/4/1	Circular	Entire	Convex	Smooth	Opaque	0.5	Metallic sheen	*Escherichia coli*
1/3 ′ /1	Irregular	Curled	Convex	Smooth	Opaque	1.4	Pink with dark edge	*Escherichia coli*
1/3 ′ /2	Circular	Entire	Raised	Smooth	Opaque	0.8	Purple	*Escherichia coli*
1/ 2 ′ /2	Irregular	Curled	Flat	Rough	Transparent	2.8	Dark	*Escherichia coli*
1/ 2 ′ /2	Circular	Entire	Convex	Smooth	Opaque	0.8	Dark	*Escherichia coli*
1/2 ′ /3	Circular	Entire	Convex	Smooth	Opaque	0.5	Yellow with green center	*Escherichia coli*
1/2/1	Irregular	Curled	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*
1/2/2	Circular	Entire	Flat	Rough	Opaque	1.1	Dark	*Escherichia coli*
1/2/3	Circular	Entire	Flat	Smooth	Opaque	0.7	Dark	*Escherichia coli*
1/2/4	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*

Table 3.6 Colony morphology of the selected isolates on EMB agar media (cont.)

2/3 ´ /1	Irregular	Curled	Convex	Rough	Opaque	0.9	Lite pink	*Pseudomonas aeruginosa*
2/3 ´ 2	Irregular	Curled	Raised	Smooth	Opaque	0.6	Lite pink	*Pseudomonas aeruginosa*
2/4/1	Circular	Curled	Flat	Smooth	Opaque	0.3	Lite pink	*Pseudomonas aeruginosa*
2/2/1	Circular	Entire	Convex	Smooth	Opaque	0.5	Dark pink	*Pseudomonas aeruginosa*
2/2/2	Irregular	Curled	Convex	Smooth	Opaque	0.8	Dark pink	*Pseudomonas aeruginosa*
2/2/3	Circular	Entire	Flat	Smooth	Opaque	0.4	Dark pink	*Pseudomonas aeruginosa*
2/5/1	Circular	Entire	Umbonate	Smooth	Opaque	0.3	Dark pink	*Pseudomonas aeruginosa*
2/1 ´ /1	Irregular	Curled	Convex	Smooth	Opaque	0.4	Dark pink	*Pseudomonas aeruginosa*
2/1 ´ /2	Circular	Entire	Convex	Smooth	Opaque	0.5	Dark pink	*Pseudomonas aeruginosa*

Table 3.6 Colony morphology of the selected isolates on EMB agar media (cont.)

3/2 ′ /1	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*
3/2 ′ /2	Circular	Entire	Flat	Smooth	Opaque	0.4	Dark	*Escherichia coli*
3/2/1	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*
3/2/2	Circular	Entire	Raised	Smooth	Opaque	0.4	Dark	*Escherichia coli*
3/2/3	Circular	Entire	Raised	Smooth	Opaque	0.4	Dark	*Escherichia coli*
3/3/1	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark with center point	*Escherichia coli*
3/5 ′ /1	Irregular	Curled	Flat	Smooth	Transparent	0.9	Violet	*Escherichia coli*
3/5 ′ /2	Circular	Entire	Raised	Smooth	Opaque	0.3	Dark	*Escherichia coli*
3/1′ /1	Circular	Entire	Flat	Smooth	Opaque	0.2	Dark	*Escherichia coli*
3/1′ /2	Circular	Entire	Raised	Smooth	Opaque	1.1	Dark	*Escherichia coli*
3/1/1	Circular	Entire	Raised	Smooth	Opaque	0.3	Dark	*Escherichia coli*
3/1/2	Circular	Entire	Raised	Smooth	Opaque	0.4	Dark	*Escherichia coli*
3/3 ′ /1	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*

Table 3.6 Colony morphology of the selected isolates on EMB agar media (cont.)

3/4/1	Circular	Entire	Raised	Smooth	Transparent	0.5	Dark	*Escherichia coli*
4/1/2	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*
4/2/1	Circular	Entire	Raised	Smooth	Opaque	0.5	Dark	*Escherichia coli*
4/1′ /1	Irregular	Curled	Raised	Smooth	Transparent	5.1	Dark	*Escherichia coli*

Table 3.7 Colony morphology of the selected isolates on XLD agar media

Number of isolates	Form	Margin	Elevation	Surface	Optical Density	Diameter (cm)	Pigmentation	Provisional identification
1/3/1	Circular	Entire	Raised	Smooth	Transparent	0.4	Off white	*Shigella*
1/3/2	Circular	Entire	Raised	Smooth	Transparent	0.3	Off white	*Shigella*
1/1/1	Circular	Entire	Flat	Smooth	Opaque	0.2	Black	*Salmonella*
1/1/2	Irregular	Curled	Flat	Smooth	Opaque	0.6	Black	*Salmonella*
1/1′ /1	Punctiform	Curled	Flat	Smooth	Opaque	0.8	Black	*Salmonella*

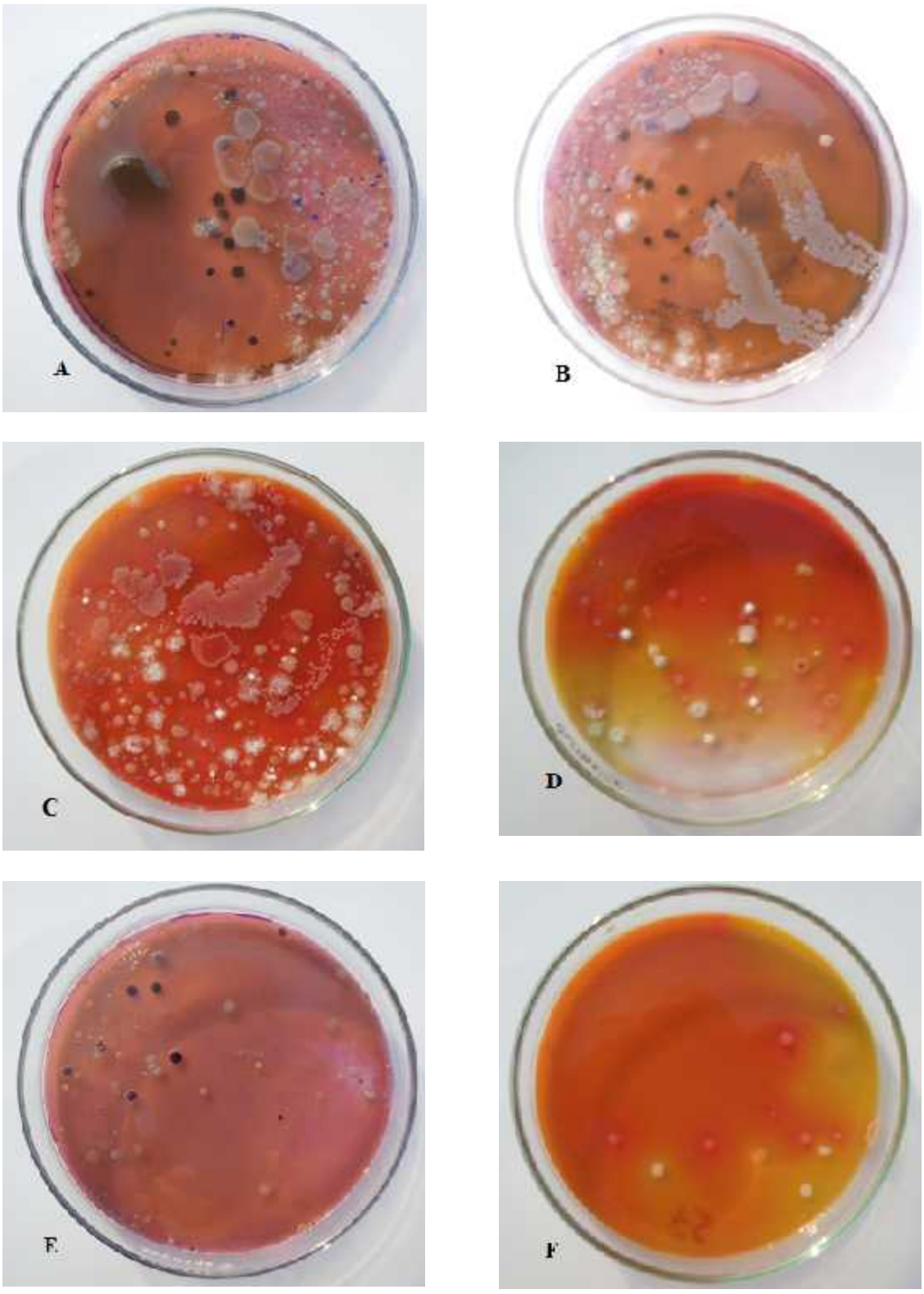

Figure 3.5 (A-D) Bacterial colonies development in XLD agar media in sample 1 and (E-F) in sample 2

Table 3.7 Colony morphology of the selected isolates on XLD agar media (cont.)

1/1 ′ /2	Irregular	Curled	Flat	Smooth	Opaque	1.8	Black	*Salmonella*
1/1 ′ /3	Irregular	Curled	Flat	Smooth	Opaque	0.9	Black	*Salmonella*
1/3′ /1	Circular	Entire	Raised	Smooth	Transparent	1.2	Yellow	*Shigella*
2/3/1	Punctiform	Curled	Raised	Smooth	Transparent	0.4	Yellow	*Shigella*
2/3′ /1	Circular	Entire	Flat	Rough	Opaque	1.3	Yellow with green center	*Shigella*
3/3/1	Circular	Entire	Raised	Smooth	Opaque	1.2	Green	*Shigella*
3/1 ′ /1	Circular	Entire	Raised	Smooth	Transparent	0.3	Yellow	*Shigella*
3/1/1	Rhizoid	Lobate	Raised	Smooth	Opaque	0.8	Yellow	*Shigella*
3/2 ′ /1	Circular	Entire	Raised	Smooth	Opaque	0.8	Yellow	*Shigella*
3/2/1	Circular	Entire	Raised	Smooth	Opaque	0.2	Yellow	*Shigella*
3/2/2	Circular	Entire	Raised	Smooth	Opaque	0.3	Yellow	*Shigella*
4/2/1	Circular	Entire	Flat	Smooth	Opaque	0.2	Yellow	*Shigella*

Table 3.7 Colony morphology of the selected isolates on XLD agar media (cont.)

4/1/1	Circular	Entire	Raised	Smooth	Opaque	0.6	Yellow	*Shigella*
4/1/2	Circular	Entire	Flat	Smooth	Opaque	0.2	Dark	*Salmonella*
4/1/3	Circular	Entire	Raised	Smooth	Opaque	0.3	Yellow	*Shigella*
4/2′ /1	Irregular	Curled	Raised	Smooth	Opaque	0.7	Yellow	*Shigella*
4/1 ′ /1	Circular	Entire	Raised	Smooth	Transparent	0.6	Dark	*Shigella*
4/1 ′ /2	Circular	Entire	Flat	Smooth	Opaque	0.2	Yellow	*Shigella*
4/1 ′ /3	Irregular	Curled	Raised	Smooth	Opaque	2.5	Yellow	*Shigella*

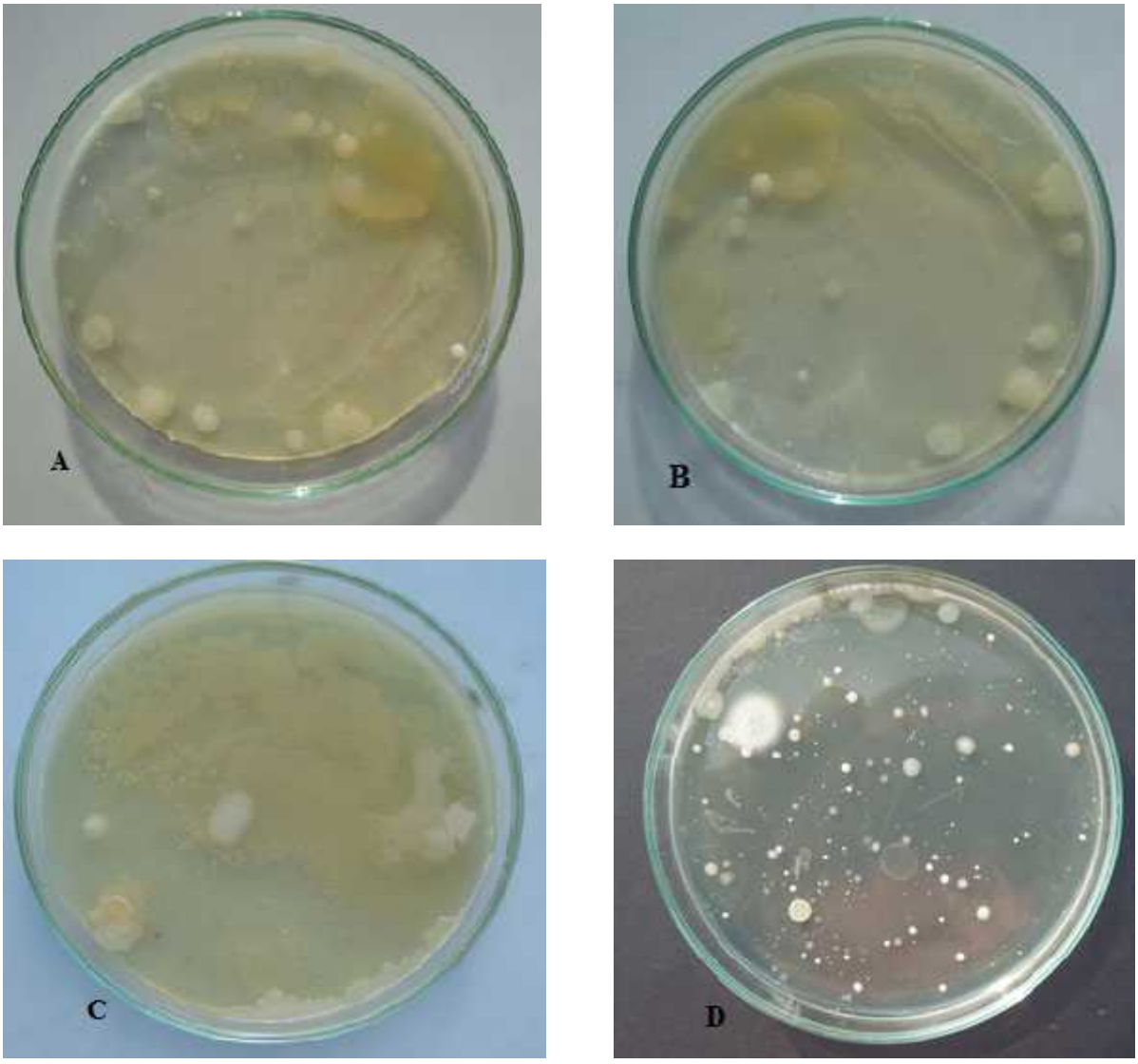

Figure 3.6 (A-D) Bacterial colonies development in PYG agar media in sample 1

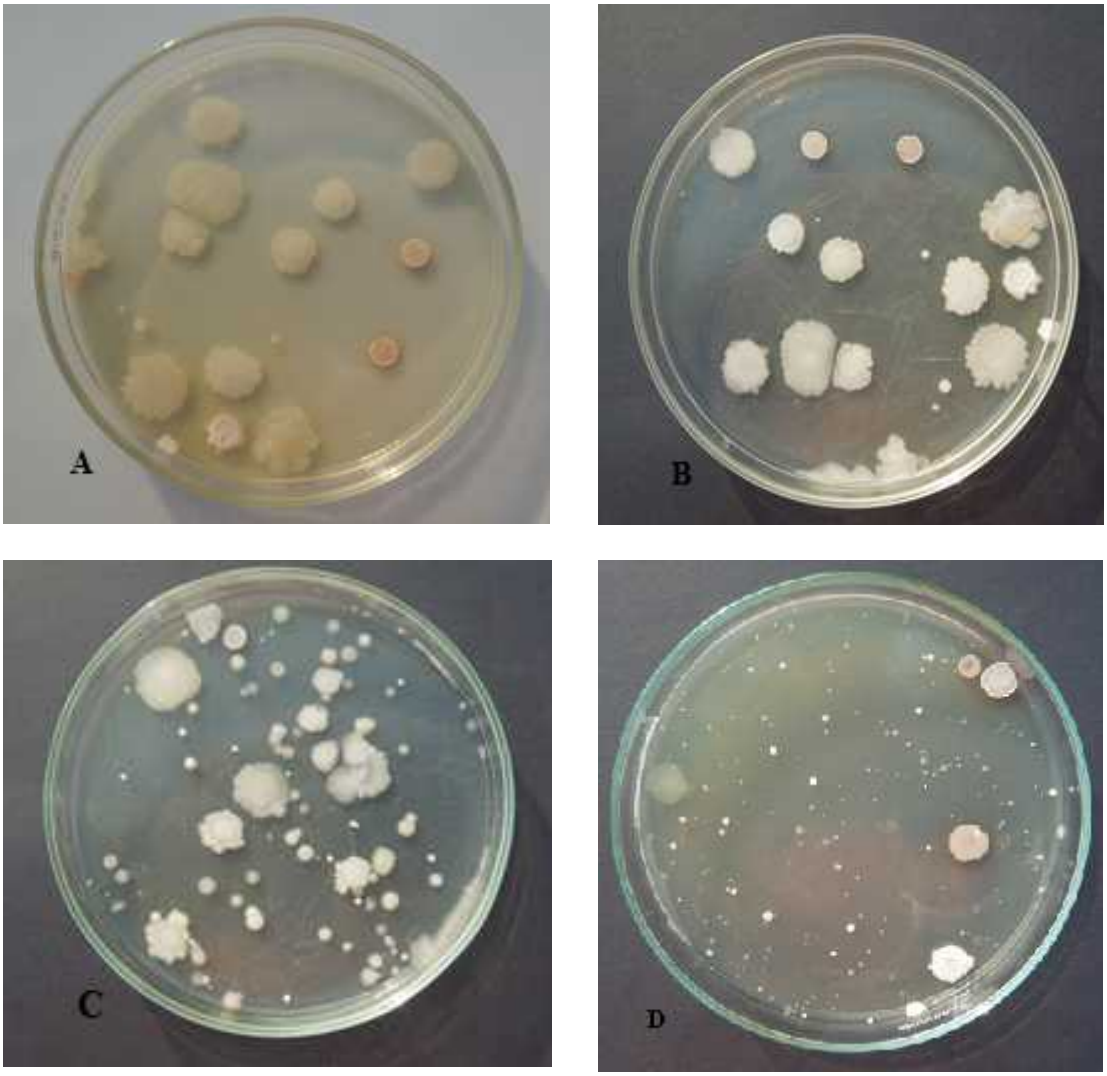

Figure 3.7 (A-D) Bacterial colonies development in PYG agar media in sample 2

Table 3.8 Colony morphology of the selected isolates on PYG agar media

Number of isolates	Form	Margin	Elevation	Surface	Optical Density	Diameter (cm)	Pigmentation
1/2/1	Irregular	Curled	Raised	Rough	Opaque	3.9	Off white
1/3 ′ /2	Irregular	Curled	Raised	Smooth	Opaque	0.9	Off white
1/5′ /1	Circular	Entire	Raised	Smooth	Opaque	0.5	Off white
1/1′ /1	Circular	Entire	Raised	Smooth	Opaque	0.7	Off white
1/3′ /1	Irregular	Curled	Raised	Rough	Opaque	1.1	Off white
3/4/1	Circular	Entire	Raised	Rough	Opaque	1.2	Off white
3/2′ /2	Circular	Entire	Raised	Rough	Opaque	1.0	Off white
3/5/1	Circular	Entire	Flat	Smooth	Opaque	0.5	Off white
3/4/2	Irregular	Lobate	Raised	Rough	Opaque	2.0	Off white
3/2 ′ /1	Circular	Entire	Raised	Smooth	Opaque	1.0	Off white
3/3/2	Irregular	Undulate	Raised	Rough	Opaque	1.2	Off white
4/3/1	Irregular	Undulate	Raised	Smooth	Opaque	2.1	Off white
3/5 ′ /1	Circular	Entire	Raised	Smooth	Opaque	1.5	White

Table 3.8 Colony morphology of the selected isolates on PYG agar media (cont.)

4/2/1	Circular	Entire	Raised	Smooth	Opaque	0.2	Yellow
4/1/2	Irregular	Curled	Flat	Rough	Opaque	0.9	White
4/5/2	Irregular	Curled	Raised	Smooth	Opaque	1.3	White
5/3/1	Irregular	Curled	Raised	Smooth	Opaque	2.9	Off white
5/3 ′ /1	Circular	Entire	Flat	Smooth	Opaque	0.6	White
5/5 ′ /1	Irregular	Lobate	Raised	Smooth	Opaque	3.0	White
5/5/1	Irregular	Curled	Raised	Rough	Opaque	1.5	Brown
6/4/1	Circular	Entire	Raised	Rough	Opaque	0.5	White
6/5 ′ /2	Circular	Entire	Pulvinate	Smooth	Opaque	0.4	White
6/5/1	Circular	Entire	Wrinkled	Rough	Opaque	1.1	Off white
6/5 ′ /1	Circular	Entire	Umbonate	Rough	Opaque	0.9	White with brown center
6/4 ′ /1	Circular	Entire	Raised	Rough	Opaque	0.9	Off white
6/4 ′ /2	Irregular	Curled	Flat	Smooth	Transparent	0.8	White
6/3/1	Rhizoid	Lobate	Raised	Smooth	Opaque	5.0	White with brown edge

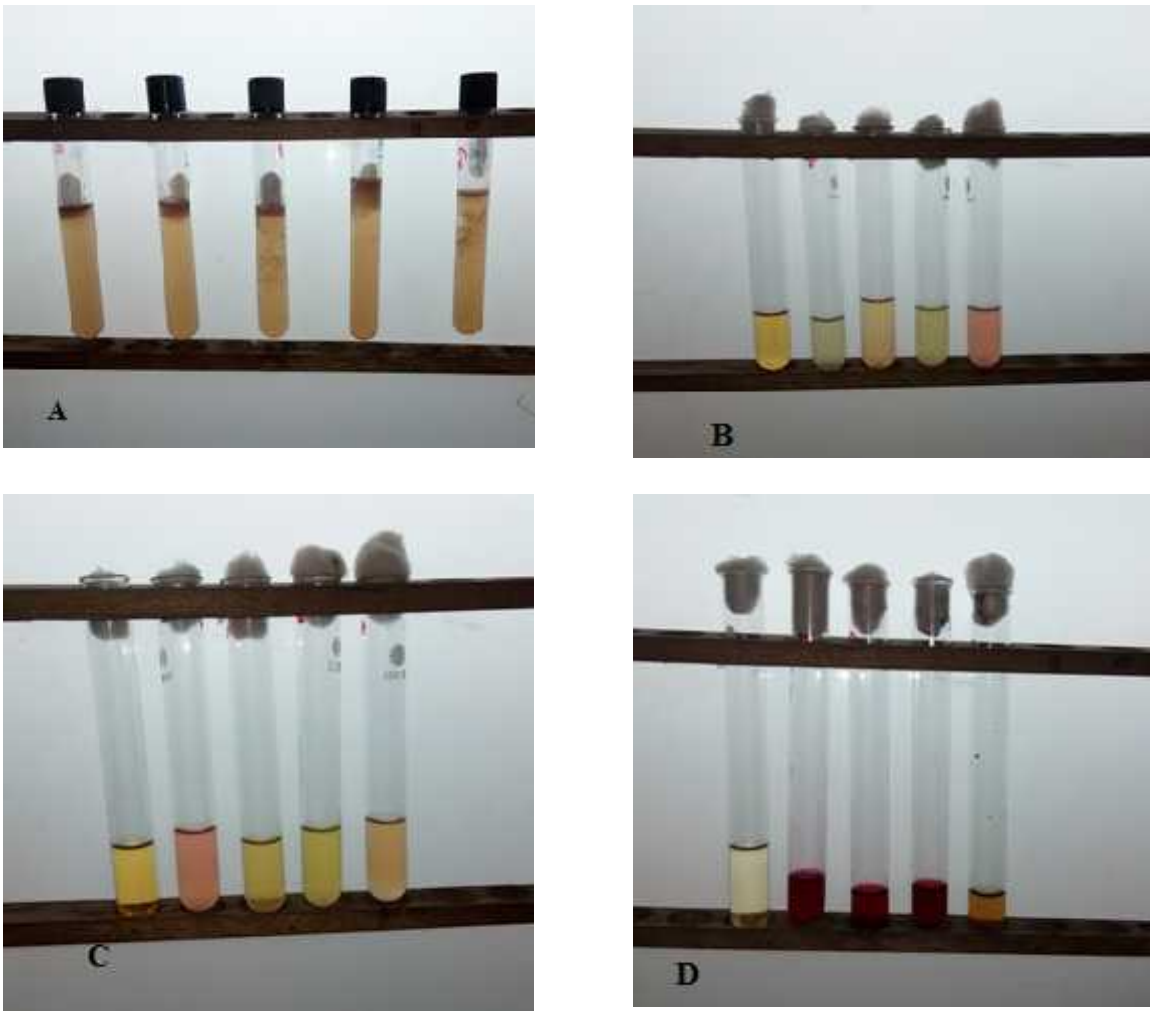

Figure 3.8 Photograph showing (A) growth in deep glucose, (B-C) VP test, (D) MR test

Table 3.9 Physiological and biochemical characteristics of the selected isolates

Isolate no.	oxidase	Catalase
1/2/1	-	+
1/3 ′ /2	+	+
1/5′ /1	+	+
1/1′ /1	+	+
1/3′ /1	+	+
3/4/1	+	+
3/2′ /2	+	+
3/5/1	+	+
3/4/2	+	+
3/2 ′ /1	-	+
3/3/2	-	-
4/3/1	-	+
3/5 ′ /1	-	+
4/2/1	-	+
4/1/2	-	+
4/5/2	+	+
5/3/1	-	+
5/3 ′ /1	+	+
5/5 ′ /1	+	+
5/5/1	-	+
6/4/1	+	+
6/5 ′ /2	-	+
6/5/1	+	+
6/5 ′ /1	-	+
6/4 ′ /1	+	+
6/4 ′ /2	+	+
6/3/1	+	+

Table 3.9 Physiological and biochemical characteristics of the selected isolates (cont.)

Name of the isolates	Gram stain	Deep glucose agar
1/2/1	Gr (+)	FA
1/3 ′ /2	Gr (–)	SA
1/5′ /1	Gr (–)	SA
1/1′ /1	Gr (+)	SA
1/3′ /1	Gr (–)	FA
3/4/1	Gr (+)	FA
3/2′ /2	Gr (+)	SA
3/5/1	Gr (+)	SA
3/4/2	Gr (+)	FA
3/2 ′ /1	Gr (+)	SA
3/3/2	Gr (+)	FA
4/3/1	Gr (+)	SA
3/5 ′ /1	Gr (+)	SA
4/2/1	Gr (+)	FA
4/1/2	Gr (+)	SA
4/5/2	Gr (+)	FA
5/3/1	Gr (+)	FA
5/3 ′ /1	Gr (–)	SA
5/5 ′ /1	Gr (+)	FA
5/5/1	Gr (+)	SA
6/4/1	Gr (+)	FA
6/5 ′ /2	Gr (+)	SA
6/5/1	Gr (–)	FA
6/5 ′ /1	Gr (+)	SA
6/4 ′ /1	Gr (+)	FA
6/4 ′ /2	Gr (+)	FA
6/3/1	Gr (+)	FA

Table 3.9 Physiological and biochemical characteristics of the selected isolates (cont.)

Name of the isolates	VP	MR
1/2/1	+	–
1/3 ′ /2	+	–
1/5′ /1	+	–
1/1′ /1	+	–
1/3′ /1	+	–
3/4/1	+	–
3/2′ /2	+	–
3/5/1	+	–
3/4/2	+	–
3/2 ′ /1	+	–
3/3/2	+	–
4/3/1	+	–
3/5 ′ /1	+	–
4/2/1	+	–
4/1/2	+	–
4/5/2	+	–
5/3/1	+	–
5/3 ′ /1	+	–
5/5 ′ /1	+	–
5/5/1	+	–
6/4/1	+	–
6/5 ′ /2	+	+
6/5/1	+	–
6/5 ′ /1	+	–
6/4 ′ /1	+	–
6/4 ′ /2	+	–
6/3/1	+	–

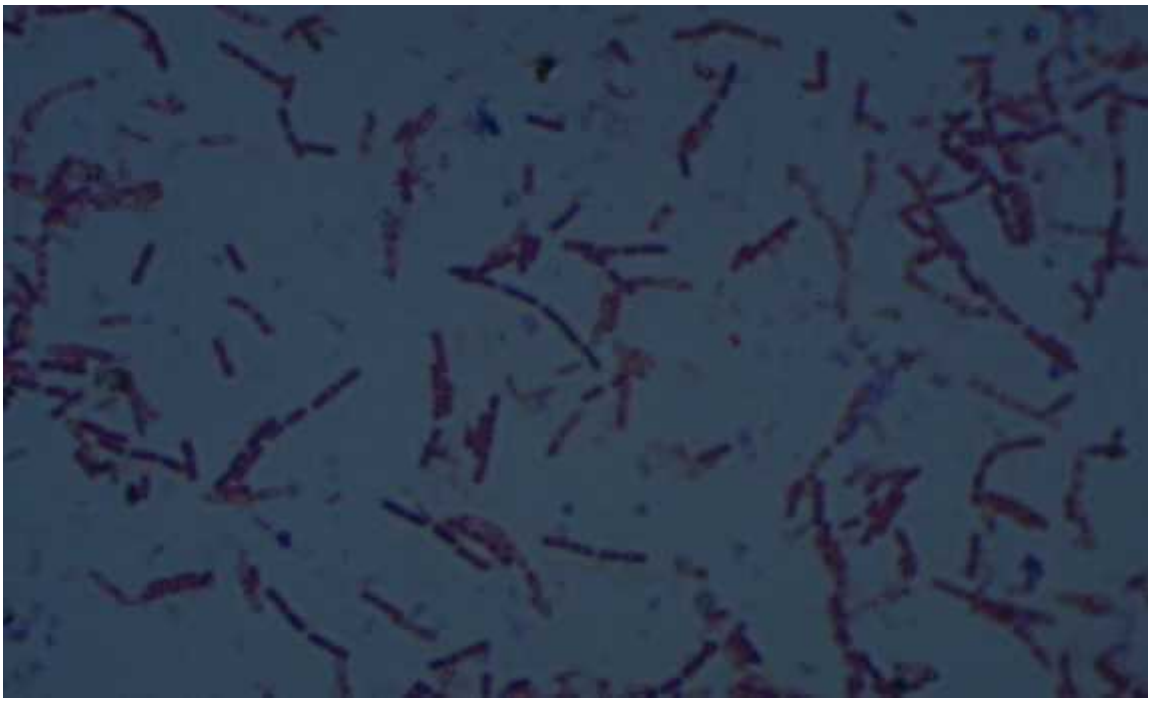

Figure 3.9 Photomicrograph showing gram positive *Bacillus cereus*

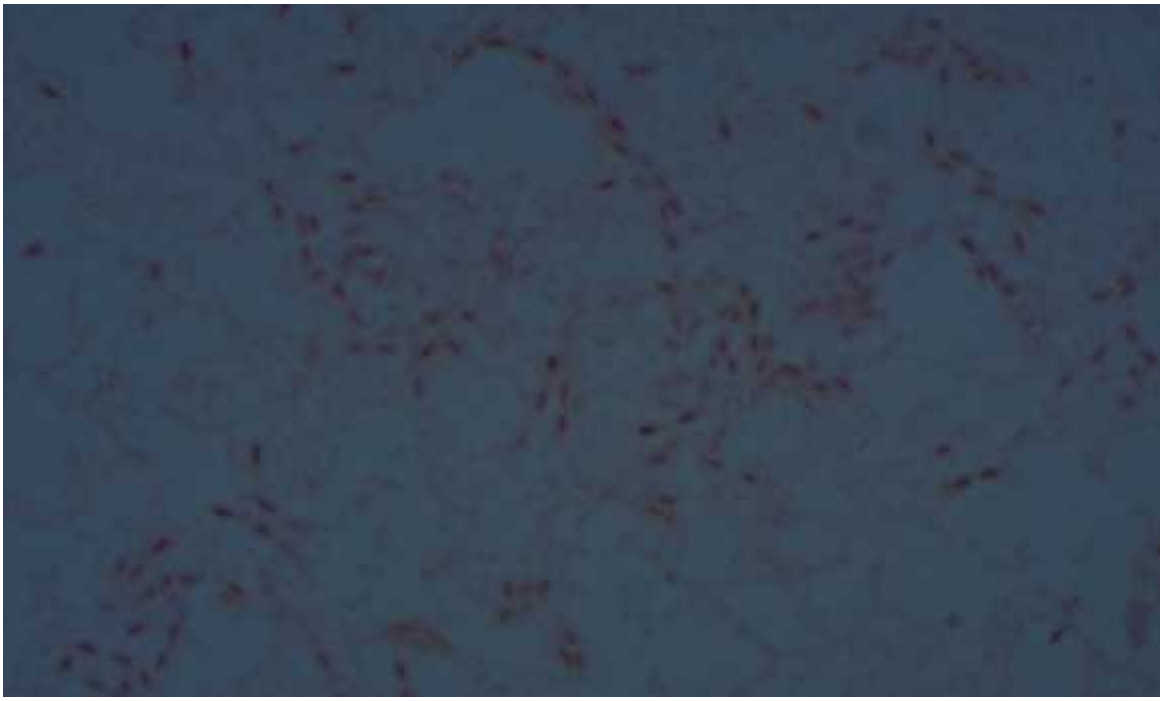

Figure 3.10 Photomicrograph showing gram positive *Bacillus subtilis*

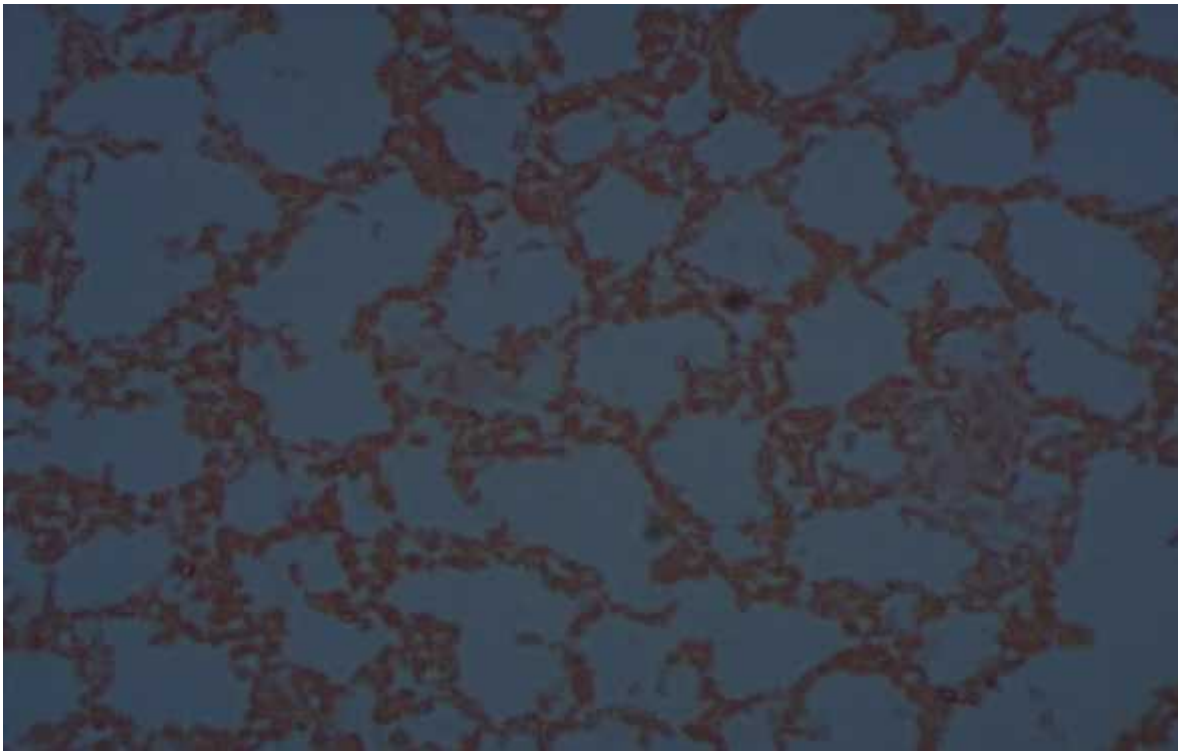

Figure 3.11 Photomicrograph showing gram positive *Bacillus coagulans*

Figure 3.12 Photomicrograph showing gram positive *Renibacterium salmoniarum*

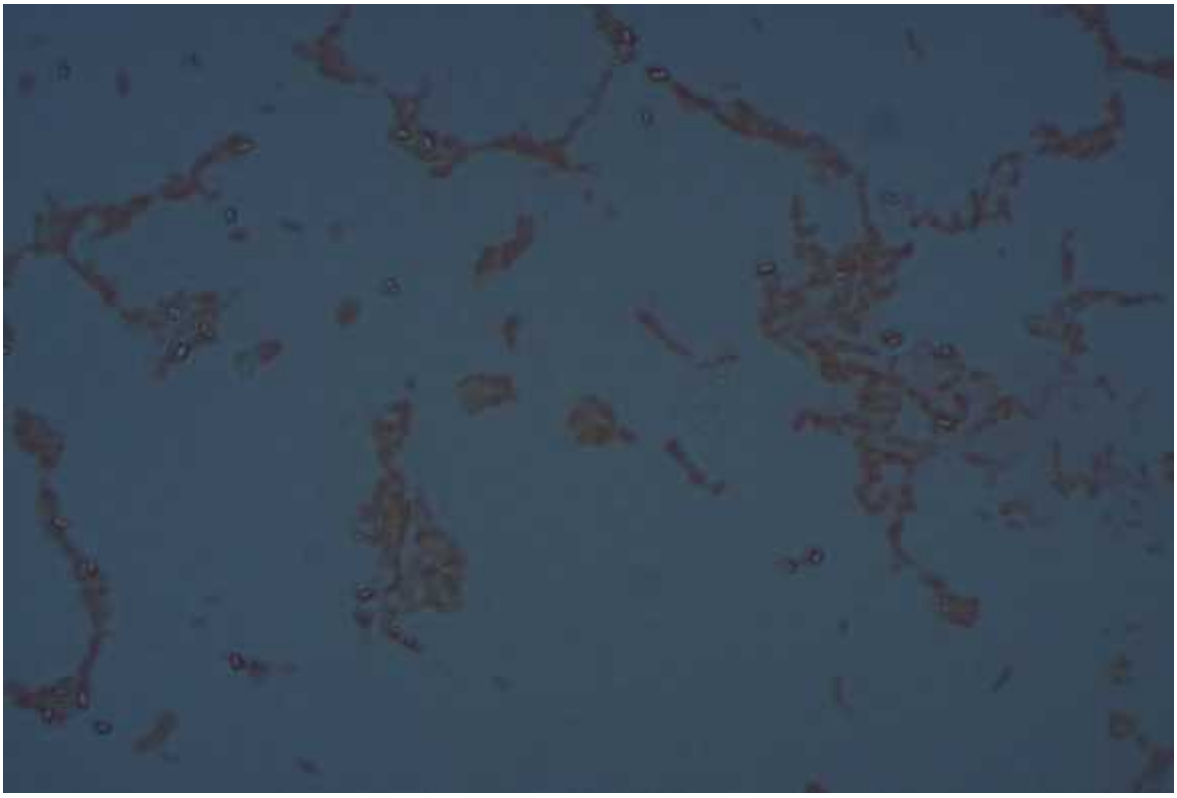

Figure 3.13 Photomicrograph showing gram positive *Bacillus subtilis*

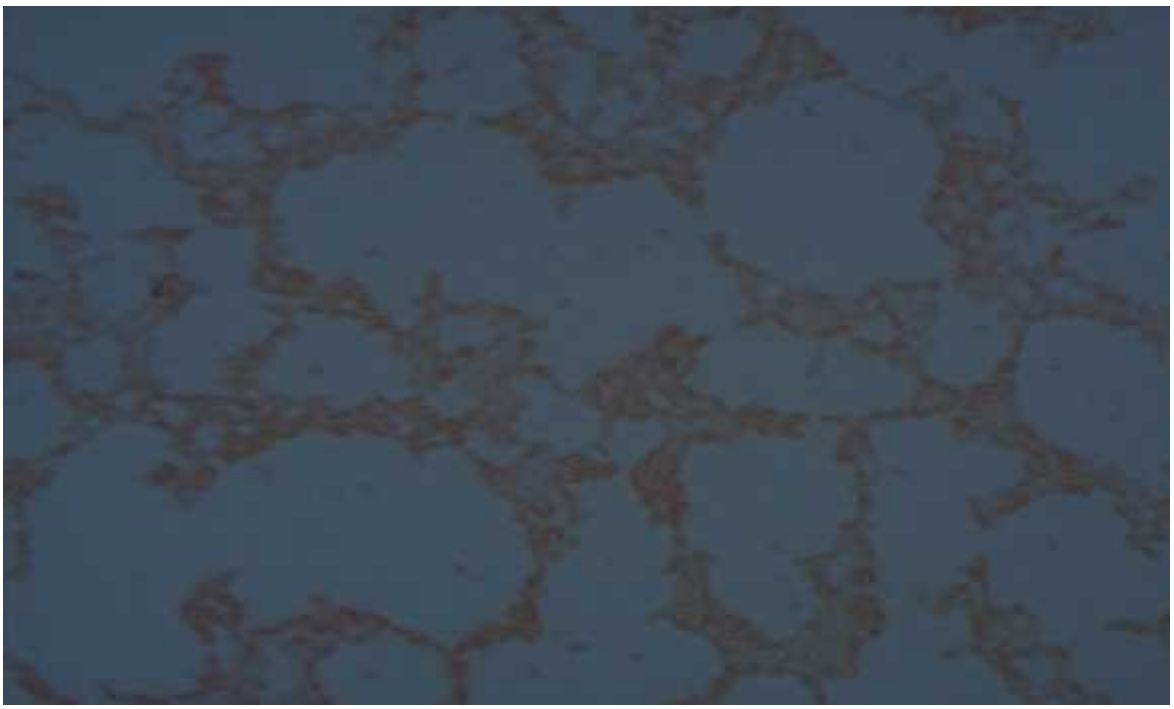

Figure 3.14 Photomicrograph showing gram positive *Bacillus licheniformes*

Figure 3.15 Photomicrograph showing gram positive *Erysipelothrix rhusiopathiae*

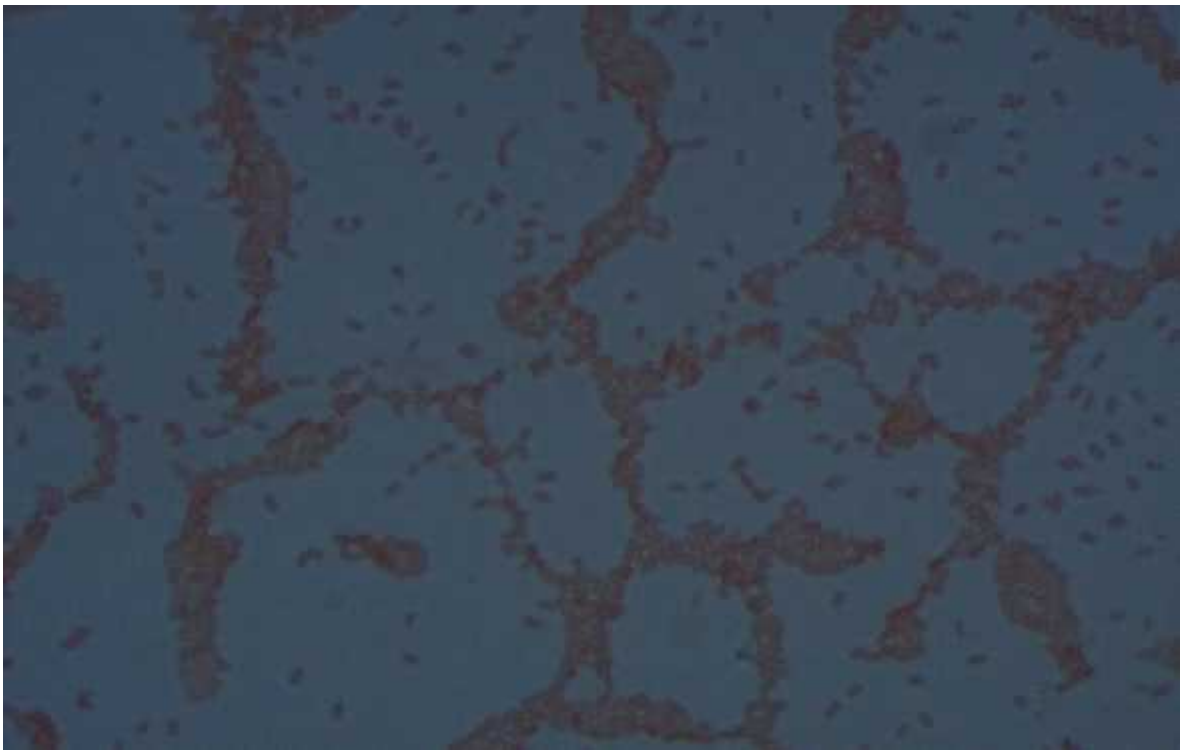

Figure 3.16 Photomicrograph showing gram positive *Bacillus subtilis*

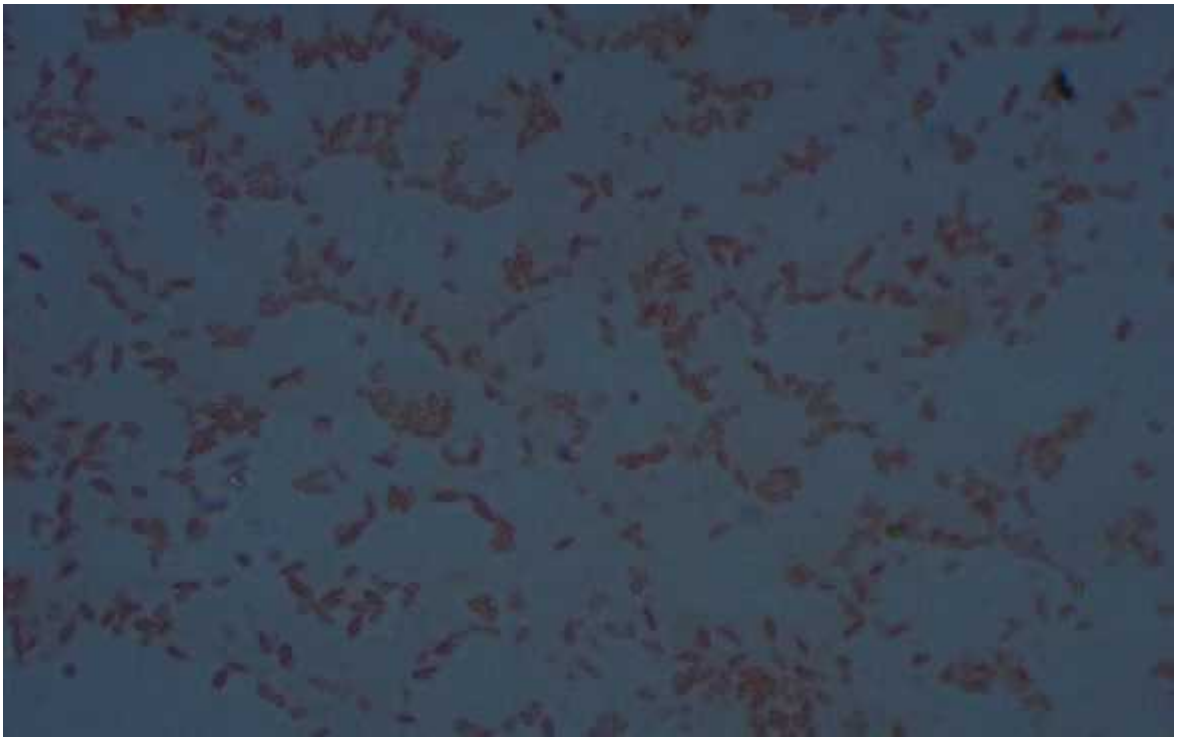

Figure 3.17 Photomicrograph showing gram positive *Bacillus alvei*

Figure 3.18 Photomicrograph showing gram positive *Bacillus pumilus*

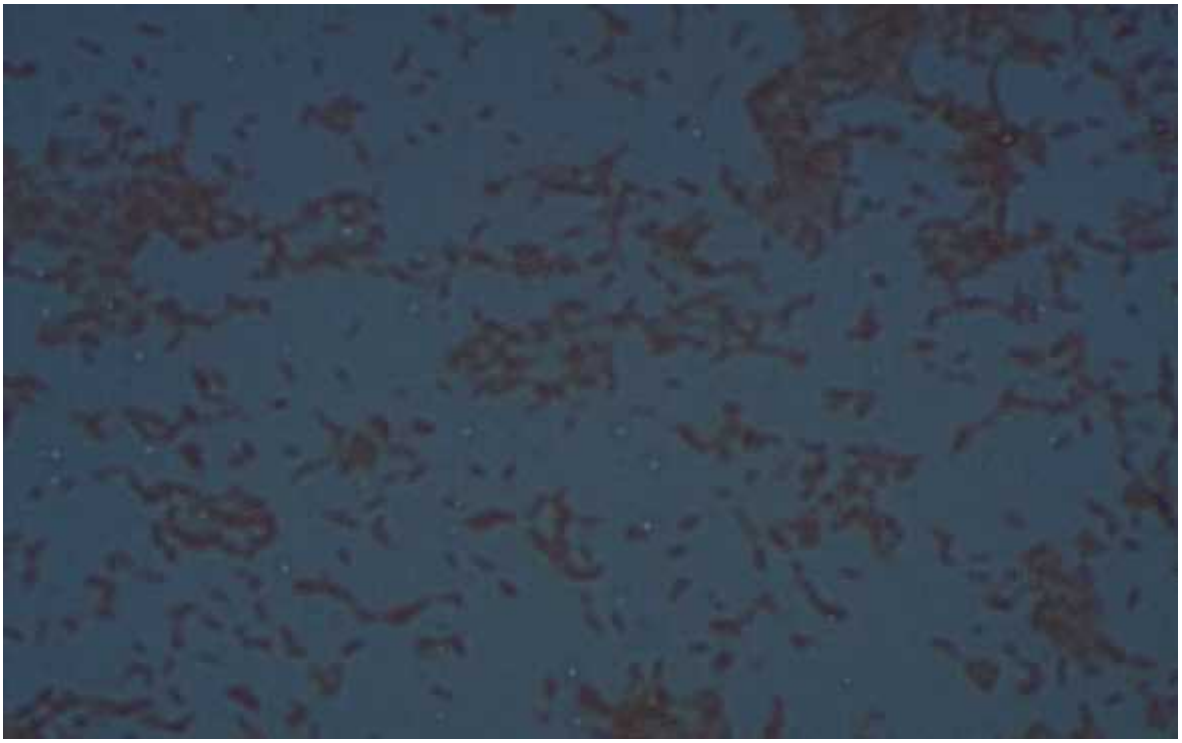

Figure 3.19 Photomicrograph showing gram positive *Bacillus alvei*

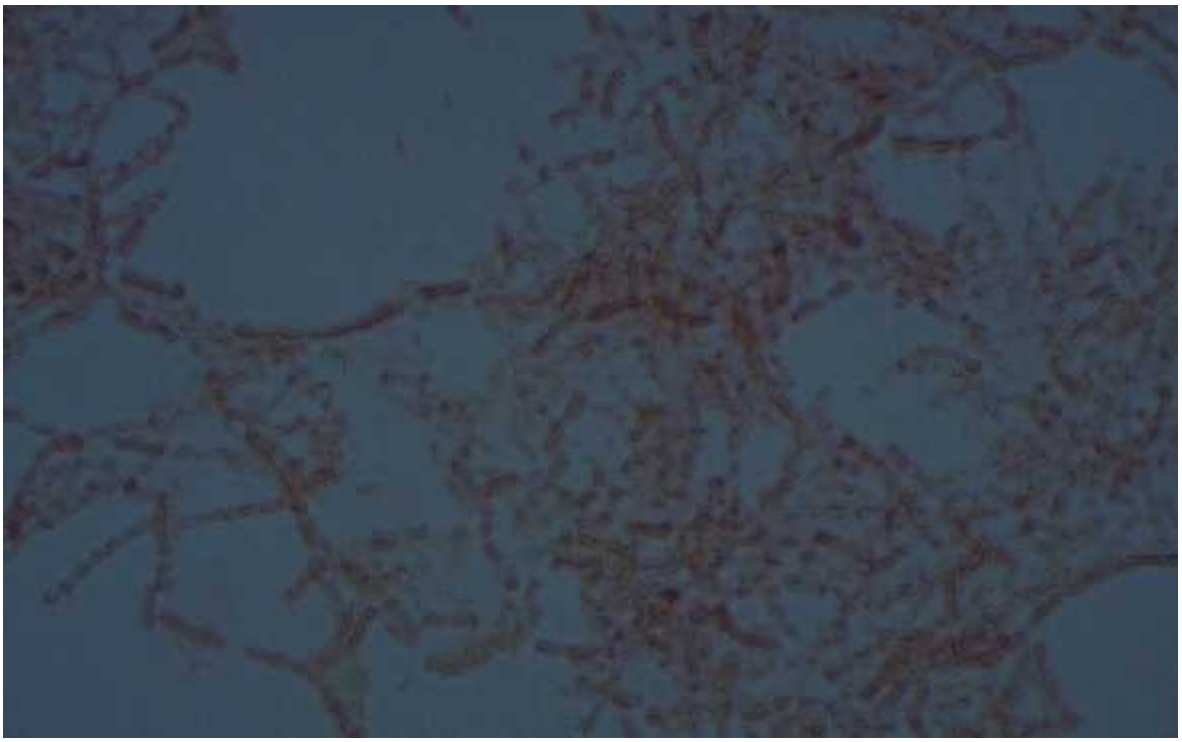

Figure 3.20 Photomicrograph showing gram positive *Bacillus cereus*

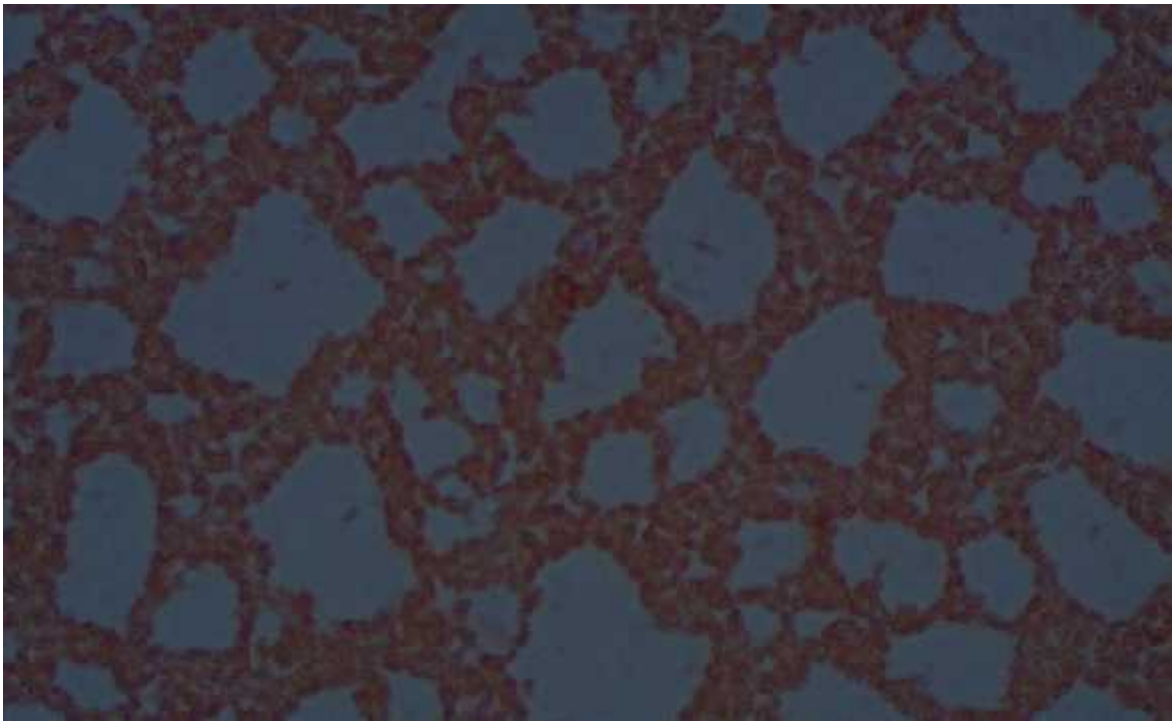

Figure 3.21 Photomicrograph showing gram positive *Bacillus coagulans*

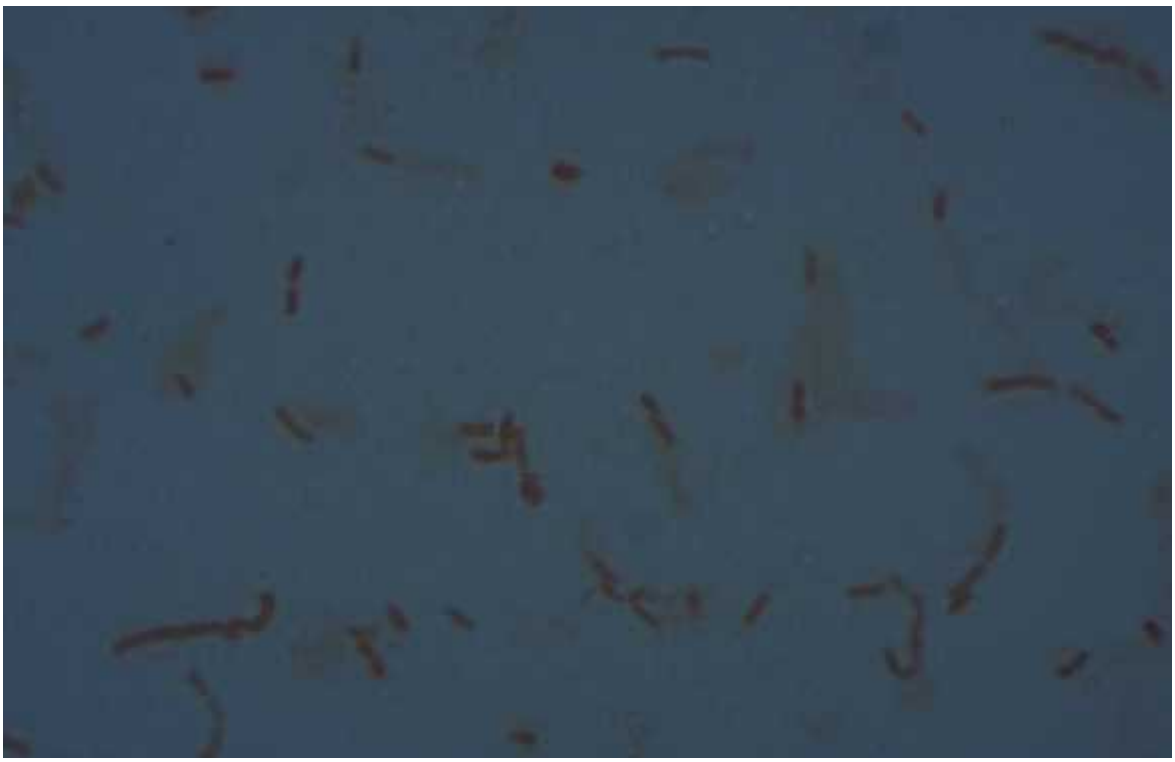

Figure 3.22 Photomicrograph showing gram positive *Bacillus polymyxa*

Figure 3.23 Photomicrograph showing gram negative *Pasteurella multocida*

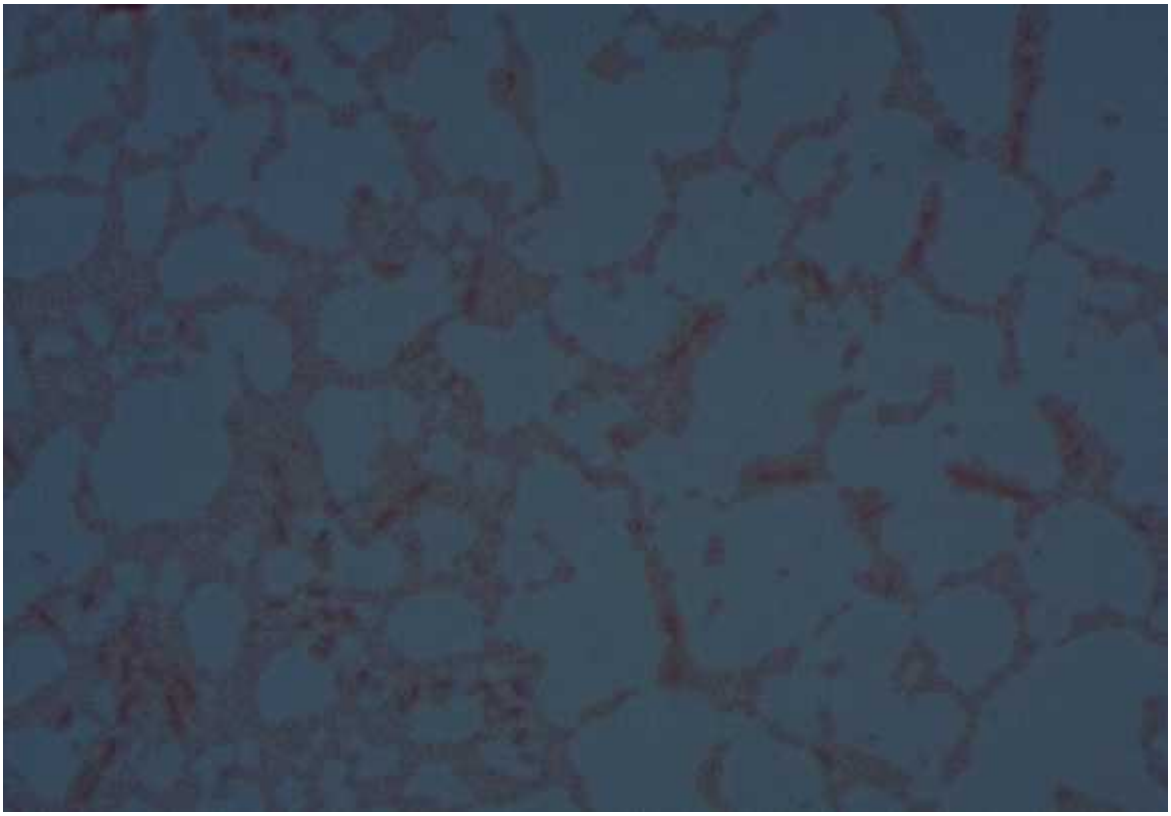

Figure 3.24 Photomicrograph showing gram negative *Legionella micdadei*

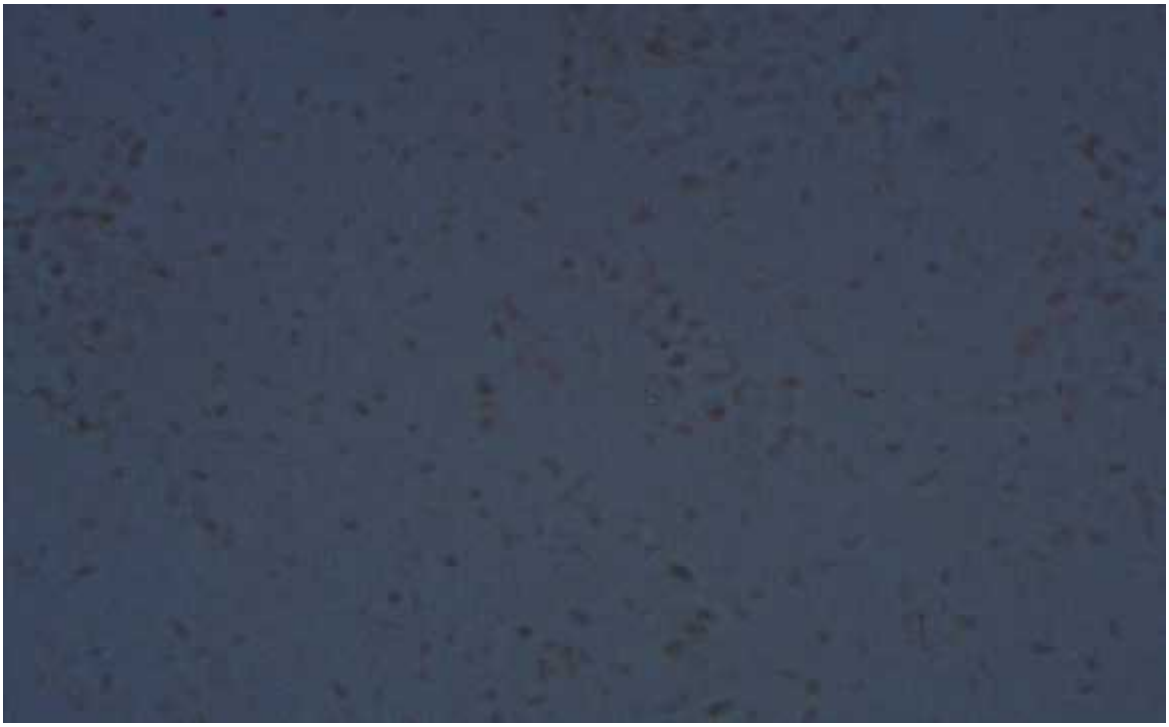

Figure 3.25 Photomicrograph showing gram negative *Vibrio nereis*

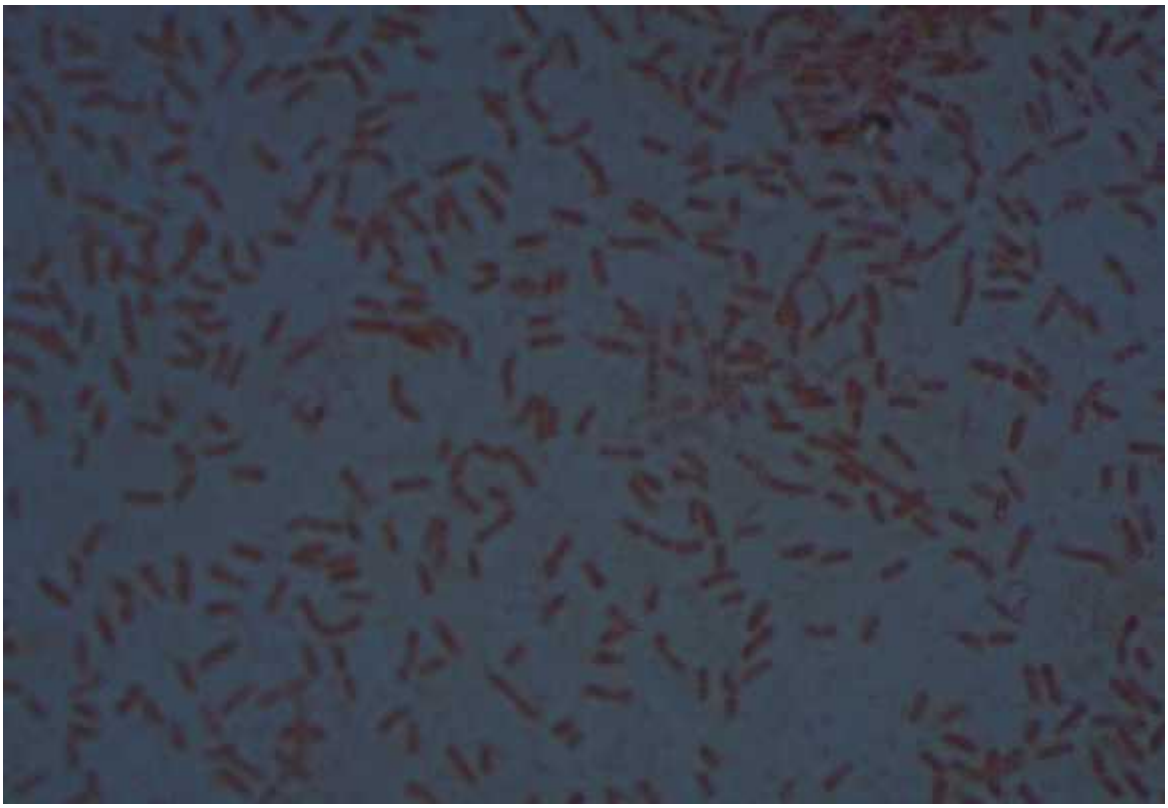

Figure 3.26 Photomicrograph showing gram negative *Legionella pneumophila*

3.7 Identification of the selected isolates

Consulting all observed and tested characters of the selected bacterial isolates, identifications were done. For the purpose of identification Bergey's Manual of Systematic Bacteriology, Vol. 2 (Sneath et al., 1986) was followed for the aerobic heterotrophic bacteria. Manuals of WHO (1987), APHA (1989), Bergey's Manual of Systematic Bacteriology, Vol. 1 (Krieg and Holt, 1984) and Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) were consulted for Gram-negative bacteria.

Table 3.10 Provisional identification of the selected Gram-negative isolate

Number of isolate	Provisionally identified names
1/3 ′ /2	*Legionella micdadei*
1/5′ /1	*Legionella micdadei*
1/3′ /1	*Vibrio nereis*
5/3 ′ /1	*Legionella pneumophila*
6/5/1	*Pasteurella multocida*

Table 3.11 Provisional identification of the selected Gran-positive isolate

Number of isolate	Provisionally identified names
1/2/1	*Bacillus cereus*
1/1′ /1	*Bacillus subtilis*
3/4/1	*Bacillus coagulans*
3/2′ /2	*Renibacterium salmoniarum*
3/5/1	*Bacillus subtilis*
3/4/2	*Bacillus licheniformes*
3/2 ′ /1	*Bacillus pumilus*
3/3/2	*Erysipelothrix rhusiopathiae*
4/3/1	*Bacillus subtilis*
3/5 ′ /1	*Bacillus pumilus*
4/2/1	*Bacillus alvei*
4/1/2	*Bacillus pumilus*
4/5/2	*Bacillus alvei*
5/3/1	*Bacillus alvei*
5/5 ′ /1	*Bacillus cereus*
5/5/1	*Bacillus pumilus*
6/4/1	*Bacillus coagulans*
6/5 ′ /2	*Bacillus subtilis*
6/5 ′ /1	*Bacillus pumilus*
6/4 ′ /1	*Bacillus polymyxa*
6/4 ′ /2	*Bacillus coagulans*
6/3/1	*Renibacterium salmoniarum*

Chapter 4

Discussion

In simple terms, the quality of a food can be defined as those characteristics which make it acceptable to the consumers. There are a number of parameters and standards (physical, chemical, microbial etc.) for the assessment of freshness quality of fresh fish. Bacteriological quality is of public health importance as it directly relates to spoilage of fish.

During this study microbial abundance and types were studied into important categories *viz*.

- Heterotrophic bacteria
- Gram negative, enteric and related bacteria

Raw fishes are highly perishable protein source that contain normal bacterial flora from their environments in addition to the contaminants occurred during harvesting and handling of the products. Coliform may be absent or present in very low density and *Salmonella, Shigella* and other enteric pathogens are usually not found as these are not he normal flora of fishes or of their environment (FAO, 1979).The bacterial flora on newly caught fish depends on the environment rather than on the fish species (Shewan, 1961). Another source of contamination of harmful microorganism could be fishing vessel (Waheb et al.,2003).

The average number of total bacteria was 8.9×10^8 bacteria g^{-1} of intestinal content (Paola et al., 2010) which will coincide with our rest.

The total bacterial load in fresh fish was 11.6×10^8in gut which is beyond the acceptable limit according to the ICMFS (ICMFS, 1998; FDA, 2001).This might be due to contamination of source water fromwhere the fishes were caught or might be due to secondary contamination during the time of handling as well as storage of fishes in ice made from contaminated water (Hatha et al., 2003).

All samples including summer and winter were observed having high quantity of total coliform exceeding the limit ($>10^2$ cfu/g) suggested by (ICMSF, 1986) and proves sample was low quality fish.The presence of coliform confirms the sewage contagion. It also indicates the contamination during handling and selling process in markets including holding temperature. Moreover, the contamination may also come from the water use for washing or icing (Boyd, 1990).Coliform bacteria are the indicator organisms whose presence in food in large quantity indicates the probability of having pathogenic bacteria.

Salmonella in aquaculture fish products mainly originates from the environment rather than from poor standards of hygiene and sanitation. *Salmonella* has been isolated from fresh, frozen, canned and sun dried marine fish products (Nataranjan et al.,1985). This bacterium is been isolated from different raw hilsa products this study. *Vibrio* spp mainly are found in gut of the fishes.*Vibrio* exhibiting greater prevalence in marine species (Nayak, 2010).

Four types of bacteriological culture media viz. PYG agar for heterotrophic bacteria, EMB agar medium for coliform, XLD agar for *Salmonella-Shigella* and TCBS for *Vibrio* were used to assess the quantitative and qualitative study. The bacteria load of fresh fish samples was found to be ranged between $11.6x\ 10^{8}$to $1.3x10^{4}$cfu/g, $3.56x10^{6}$ to $4x\ 10^{3}$cfu/g,$5x\ 10^{8}$to $2.7x10^{4}$cfu/g and$9.5x10^{5}$to $5.4x10^{3}$on PYG agar, EMB agar, and TCBS agar, XLD agar respectively. All bacterial count in these media shows height count in summer and lowest count found in winter. Also, some differences have been considered to reflect seasonality, i.e., with maximum and minimum counts occurring in summer and winter, respectivelyhas been reported (Yoshimizu et al., 1976) which will support out result.

From the selective media,*Vibrio parahaemolyticus*identified as green color in TCBS media *Vibrio cholerae* identified as yellow color in TCBS media *Escherichia coli*identified as golden metallic sheen or dark color in EMB media*Pseudomonas aeruginosa*identified as pink color in EMB media*Salmonella* identified as black color in XLD media *Shigella*identified as yellow color in XLD media. Amande and Nwaka (2013) reported that Bacterial Isolates Obtained from intestine are *Salmonella* sp., *Proteus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Escherichia coli*, *Shigella* sp., *Vibrio* sp. Which will coincide with our result.

27 heterotrophic bacterial strains were selected on the basis of colonial morphology and growth response on PYG agar media. The organisms were compared with the standard description in the Bergey's Manual (Sneath et al., 1986) and isolated strains were provisionally identified.

Among 27 heterotrophic bacterial isolate, 22 were Gram-positive (1/2/1, 1/1′ /1, 3/4/1, 3/2′ /2, 3/5/1, 3/4/2, 3/2 ′ /1, 3/3/2, 4/3/1, 3/5 ′ /1, 4/2/1, 4/1/2, 4/5/2, 5/3/1, 5/5 ′ /1, 5/5/1, 6/4/1, 6/5 ′ /2, 6/5 ′ /1, 6/4 ′ /1, 6/4 ′ /2, 6/3/1)and 5 wereGram-negative (1/3 ′ /2, 1/5′ /1, 1/3′ /1, 5/3 ′ /1, 6/5/1).

The isolated bacterial strains had some minor differences in biochemical characters from those sited in the Bergey's Manual of Systematic Bacteriology Vol. 1 and 2 and Bergey's Manual of Determinative Bacteriology (9^{th} Ed.).

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Considering the results presented in the study this may be concluded that the abundance of total heterotrophic bacteria fluctuated between seasons. Summer is the favorable season for bacterial growth in *Tenualosa ilisha.* Most of the bacteria found in the gut are potential pathogens indicating that fish gut is a reservoir of many opportunistic pathogens which may predispose the fish to bacterial epizootics.

5.2 Recommendations

- Current study was conducted only in very short periods, so further studies are required for better understanding of microorganisms associated with the fish samples.
- Need to find out the antibiotic resistivity of the identified isolates.

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Appendices

Composition of the media and reagents used in this study are as follows.

1. Congo red solution (SAB 1957)

Congo red (80% dye content)	2.0 gm
Distilled water	100 ml

2. Deep glucose agar medium (Hall 1929)

Beef extract	3.0 gm
Peptone	5.0 gm
Glucose	10.0 gm
Agar	15.0 gm
Distilled water	1000 ml
TTC (1%)-1 ml in each 200 ml	

3. EMB agar medium

Peptic digest of animal tissue	10 gm
Dipotassium phosphate	2 gm
Yeast extract	5 gm
lactose	5 gm
Sucrose	5 gm
Eosin-Y	0.4 gm
Methylene blue	0.065 gm
Agar	13.5 gm
Distilled water	1000 ml

4. Methyl Red/Voges-Proskauer broth medium (SAB 1957)

Peptone	7.0 gm
Glucose	5.0 gm
NaCl	5.0 gm
Distilled water	1000 ml
pH	6.5

5. Methyl red solution (Bryan 1950)

Methyl red	0.1 gm
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

6. α-Napthol solution (Bryan 1950)

α –Naptho	l5.0 gm
Ethyl alcohol (95%)	100 ml

7. Nutrient agar medium (Pelczar 1993)

Beef extract	3.0 gm
Peptone	5.0 gm
NaCl	5.0 gm
Agar	15.0 gm
Distilled water	1000 ml

8. Nutrient broth medium (Pelczar 1993)

Beef extract	3.0 gm
Peptone	5.0 gm
Nacl	5.0 gm
Distilled water	1000 ml

9. Oxidase test reagent (Claus 1995)

Tetramethyl-p-phenylene-diamine	
Dihydro-chloride	1.0 gm
Distilled water	100 ml

10. Physiological saline

Sodium chloride	0.85 gm
Distilled water	100 ml

11. PYG medium (Atlas 1997)

Glucose	10.0g
Peptone	5.0g
Yeast extract	5.0g
Agar	15.0g
pH	8.5

12. Safranin solution (SAB 1957)

Safranin	0.5 gm
Distilled water	100 ml

13. TCBS agar

Peptone	10.0 gm
Yeast extract	5.0 gm
Sodium citrate	10 gm
Sodium thiosulfate	10 gm
Iron (III) citrate	1.0 gm
Sodium chloride (NaCl)	10 gm
Dried bovine bile	8.0 gm
Sucrose	20.0 gm
Bromothymol blue	0.04 gm
Thymol blue	0.04 gm
Agar	8.0 gm to 18.0 gm
Water	1000 ml

14. XLD agar (Diagnostic Pasteur)

Beef extract	5 gm
Enzymatic Digest of casein	2.5 gm
Enzymatic Digest of animal tissue	2.5 gm
Lactose	10 gm
Bile Salts	8.5 gm
Sodium Citrate	8.5 gm
Sodium Thiosulfate	8.5 gm
Ferric citrate	1 gm
Brilliant Green	0.00033 gm
Neutral Red	0.025 gm
Agar	13.5 gm