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ISOLATION AND CHARACTERIZATION OF SOIL PROBIOTIC BACTERIA IN CAUVERY RIVER BASIN AND THEIR APPLICATION IN AQUACULTURE

ABSTRACT

The research of probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture. The probiotics were defined as live microbial feed supplements that improve health of man and terrestrial livestock. The gastrointestinal micro biota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. The need for increased disease resistance, growth of aquatic organisms, and feed efficiency has brought about the use of probiotics in aquaculture practices. In the past few years, there is an emerging application of the probiotics in the field of aquaculture. There is documented evidence that probiotics can improve the digestibility of nutrients, increase tolerance to stress, and encourage reproduction. Unlike the previous publications, the present study focuses on the role of soil probiotic bacteria isolated from the Cauvery river basin in Tiruchirapalli district. The isolated micro biota – Nitrosococcus and Neisseria was tested for its specific role in aquaculture mainly in Tilapia fish (Oreochromis sp.) This study also enhances the bioremediation property of the isolated soil bacteria which was proved by testing the water quality parameters in the probiotic applied water environment. The growth and mortality rate was also measured in the fish. It was clearly observed that the application of the isolated soil probiotic from cauvery river basin promoted life sustainability in the tilapia fish sp. Thus, the culture and application of soil probiotic bacteria in aquaculture is a promising approach in current science.

KEYWORDS: Probiotics, aquaculture, Cauvery river, Nitrosococcus, Neisseria, Tilapia (Oreochromis sp.) bioremediation property, water quality, life sustainability

1. INTRODUCTION

Aquaculture is viewed as an important food security sector for a growing global human population, and has rapidly developed due to intensified culture methods. An indiscriminate use of chemical additives and veterinary medicines as preventative and curative measures for diseases has resulted in antimicrobial resistance among pathogenic bacteria, and degraded environmental conditions. Consequently, serious loss because of the spread of diseases has been increasingly recorded. This is a significant constraint on aquaculture production and trade, and negatively affects economic development in many countries. Several alternative methods have been considered to improve the quality and sustainability of aquaculture production. Of those methods, probiotics have been shown to have an important role in aquaculture. Although probiotics offer a promising alternative to chemicals and antibiotics in aquatic animals (Rekiel *et al*. 2007), and as an aid in the protection of aqua cultured species, the ways that probiotics are used in aquaculture need to be considered to avoid producing negative results. As aquatic animals interact

with a diverse range of micro-organisms within animals and their habitat, a screening probiotic process for particular fish species plays a vital role to make them species specific for obtaining desired results, in which in vitro and in vivo tests need to be carried out carefully. In addition, choosing appropriate administration methods leads to the creation of favourable conditions, in which probiotics are able to perform well. Probiotic administrations have been widely applied via water routine or feed additives with either single or a combination of probiotics or even a mixture with prebiotics or other immune stimulants.

Aquaculture is emerging as one of the fastest growing and most promising industry for providing animal protein and food security to the growing population. Due to its lucrativeness it is surpassing the agriculture sector also. The expansion of culture area and intensification of culture practices are leading to high stocking densities. The intensification of aquaculture practices has given rise to stressful conditions for both the aquatic animals and the environment. As a result, disease outbreaks are being progressively documented as one the most important threats to the aquaculture industry. The diseases and deterioration of environmental conditions often occur and result in serious situation. During the last decades, antibiotics used as traditional strategy for fish diseases management and also for the improvement of growth and efficiency of feed conversion. An alternative approach to manage fish and shrimp health, that is fast gaining attention in aquaculture industry is, “probiotics”, a microbial intervention approach for disease International Journal of Current livelihood and food security for the ever increasing population. Accordingly there is expansion, intensification and diversification of aquaculture practices to obtain enhanced production. This has resulted in increased environmental and aquatic animal stress, finally to disease out bursts, reduction in production and threat to sustainability of aquaculture. Further, these are considered as major thrust areas of potential research for disease control in aquaculture. Generally, probiotics refers bacteria belonging to gram positive especially Lactobacillus sps, Bifidobacterium sps and Streptococcus, Bacillus sps, Lactococcus sps., Micrococcus sps., Carnobacterium sps., Enterococcus sps., Lactobacillus sps., Streptococcus sps. and Weissella sps. Microalgae (Tetraselmis) and Yeasts (Debaryomyces), Phaffia and Saccharomyces (Gatesoupe, 1999). There are many types of probiotics. There include lactobacilli, bifido bacteria and some yeast. Different probiotics have diverse effects. The present paper summarizes the current knowledge of the use of probiotics in aquaculture. The world “probiotics was coined by Parker (1974), and defined as “Organisms and substances that give to intestinal microbial balance. Fuller (1989) revised the definition as “live microbial feed supplement which beneficially affects host animal by improving its intestinal microbial balance”. Probiotics are often termed as “friendly”, “beneficial”, “good” or “helpful” bacteria, because they help keep the gut healthy. More recently, the probiotics are defined as “live microorganisms” that when administered in adequate amounts confer a health benefit on the host (FAO / WHO, 2001). Types of probiotics There are three types of

probiotics, as mentioned below: Water probiotics: these are marked in 2 forms i) dry forms ii) liquid forms. Liquid forms give positive results in lesser time, when compared to the dry and spore form bacteria, through they are lower in density (Nageswara and Babu 2006). These play major role in improving the water quality of culture pond. Soil probiotics: Bacteria like Nitrobacter, Nitrosomonas and sulphur reducing bacteria clean the bottom of aqua ponds. Feed / gut probiotics: Lactic acid bacteria. Probiotics act as a microbial dietary medicine that benefits the host health condition by reducing mucosal and systemic immunity and improving the physiological and nutritional actions. These enhance the fish and shrimp feed efficiency by stimulating digestive enzyme and maintain the balance of intestinal microbes, resulting in improved nutrient absorption, utilization and ultimately the survival, growth of fish and shrimp. Role of probiotics Three types of probiotic bacteria can be directly applied to soil, water of the farming pond and also as an additive to feed. Various commercial probiotics are available in the market in different combinations and bacterial counts. Reports inform that use of probiotic bacteria reduces mortality rate. However, the quantity of cells present in the probiotic, given with feed plays a major role in the survival of animals. The potential effect of probiotic relies on the source from which the bacteria are isolated and the way of application.

2. MATERIALS AND METHODS

2.1: ISOLATION AND CHARACTERIZATION OF THE SOIL PROBIOTIC BACTERIA

The soil sample is collected from the Cauvery river basin in Tiruchirapalli district. The sample was dried and then checked for the presence of microbes.

Chemicals and reagents

Nutrient Agar medium, Nutrient broth, was purchased from Himedia, India. Whatman filter paper No. 1, Gentamicin antibiotic solution, test samples, test tubes, beakers conical flasks, spirit lamp, double distilled water, petri-plates, TrisHcl, Phenol chloroform, Methyl red, Kovac's reagent, KOH and Alpha napthol.

Enumeration of soil sample

The 0.1g of test sample was dissolved in 10ml of sterile double distilled water and 1 hours of incubation at room temperature. The filter the supernatant was used for the serial dilutions from 10^1 to 10^8. The 10^2 and 10^6 dilution was used to bacterial isolation.

Nutrient Agar Medium

The medium was prepared by dissolving peptone- 0.25gm, NaCl- 0.25 gm, yeast extract- 0.1gm, beef extract- 0.05gm and agar powder- 0.875gm of the commercially available Nutrient Agar Medium (HiMedia) in 50ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten. The 10^2 and 10^6dilution was plated on the nutrient agar medium by spread plate method and the plate was incubated at 37° C for 24 hrs. After incubation, bacterial colonies were isolated and plated in to a fresh plate.

Nutrient broth

Nutrient broth was prepared by dissolving peptone- 0.25gm, NaCl- 0.25 gm, yeast extract- 0.1gm, beef extract- 0.05gm and agar powder- 0.875gm of the commercially available Nutrient Agar Medium (HiMedia) in 50ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Screening and identification of microbes

The medium was prepared by dissolving peptone- 0.25gm, NaCl- 0.25 gm, yeast extract- 0.1gm, beef extract- 0.05gm and agar powder- 0.875gm of the commercially available Nutrient Agar Medium (HiMedia) in 50ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten. Three different colonies were isolated from 10^2 and 10^6 two different colonies isolated from dilution of test sample products. Five different colonies were streaked in a single petri plate.

Gram staining

Procedure

A loop full of bacterial culture was spread in the glass slide. The slide was smeared in front of the flame. The slides were stained with crystal violet dye and kept it for 1 min and washed the slide in a distilled water. Gram’s iodine was added and incubated for 1 minute, then rinsed with distilled water. The decolorizing agent was added and kept for 1 min and then safronin strain was added, after a minute it was

washed using distilled water. The slides were observed under the Trinocular microscope the purple colors indicated gram positive bacteria and the pink color indicates gram negative organism.

HANGING DROP METHOD

The hanging drop technique is a well-established method for examining living, unstained, very small organisms. The traditional procedure employs a glass slide with a circular concavity in the centre into which a drop of fluid, containing the 'microorganisms', hangs from a coverslip. Cavity slides are expensive and coverslips are fragile so some pupils can find them fiddly to work with. Here we offer an alternative technique that is easy to use in the classroom. The simple substitute of a transparent film can lid, blu-tak and two microscope slides gives a cheap and practical option which allows pupils to look at living cultures easily and effectively. Algae and protozoa are of sufficient size for pupils to view them successfully using a standard school microscope. Examination of such 'hanging drops' can lead to useful discussions of size, variety, characteristics and importance of microorganisms and to consideration of differences between 'plant' and 'animal' cells.

The aim of this activity is to allow pupils to experience the magnificence of the microworld that can exist in a hanging drop and to observe some of the microorganisms that populate fresh water. When viewing a correctly focused hanging drop preparation of the recommended mixed algae, using lenses to give x 100, x 200 or x 400 magnification, pupils are able to watch a wide variety of (mostly unicellular) algae of different sizes. Some are motile and swim across the field of view with amazing rapidity. Others, such as the desmids, which possess three perfect planes of symmetry, exhibit interesting and remarkable shapes, as do diatoms whose individual cells demonstrate astonishing intricate architecture.

This method is used to study microorganisms in a 'bought-in' preparation, in pond water or in 'home-grown' cultures. We recommend mixed algal and mixed protozoal cultures obtained from Sciento. (We have found these Sciento cultures to be of consistently high quality both in variety and numbers of organisms.) In the case of the algae, make sure that the drop contains some obvious green material to allow observation of a variety of different cells and non-motile organisms (although a drop of 'clear' liquid may contain a limited variety of motile organisms such as Chlorella). When removing a sample of protozoa from the jar, it is important to select some of the solid material as the protozoa are likely to be feeding there. All algae can carry out photosynthesis. This activity can, therefore, lead into discussions of the importance of the algae in carbon fixation in rivers, lakes (or lochs), seas and oceans and also of plant plankton as the producers at the start of food webs in water environments. Diatoms are thought to be an important constituent in the formation of oil deposits.

2.2: APPLICATION IN AQUACULTURE:

2.2.1. ACCLIMATIZATION OF THE EXPERIMENTAL ANIMAL:

The experimental animal selected for present study was Tilapia fish (Oreochromis mossambicus). Tilapia weighing about 5 - 8 gms and average length of about 8-15 cms, were collected in a nearby pond in Allithurai, Tiruchirappali and was acclimatized to laboratory conditions for about 10 days in large plastic tubs previously washed with potassium permanganate, to free walls from any microbial growth. The food was supplied regularly in the form of pellets. The fishes were aerated well and non-chlorinated tap water was used.

2.2.2. Estimation of Chloride:

10 ml of water sample was taken in a conical flask and 2 drops of 5 % potassium chromate solution was added as indicator. The resulting lemon yellow colour solution was titrated against silver nitrate solution taken in the burette. Care was taken to shake the contents of the conical flask vigorously after the addition of each drop of silver nitrate in order to avoid the dumping of the precipitate. The end point was the appearance of a persistent brick red colour. The titration was repeated for constant concordant values. Estimation was done for various samples and the readings were calculated.

2.2.3. Estimation of Nitrite:

To 10 ml of sample, blank and standard 0.2 ml of EDTA solution was added and stirred well. 0.2 ml of sulphanilic acid was added and mixed thoroughly. After 10 minutes, 0.2 ml of sodium acetate and 0.2 ml of α – naphthylamine hydrochloride solution was added to all the tubes and mixed thoroughly. After 10 minutes, the colour developed was compared visually and the optical density was read at 530 nm. The estimation was repeatedly done.

2.2.4. Estimation of Nitrate:

1 ml of the sample, standard and blank were taken separately in test tube. 0.5 ml of brucine sulphanilic acid solution was added to all the tubes and mixed well. In a second series of test tubes, 5 ml of sulphuric acid solution was added. The contents of the first series of the test tubes was added to each of the second series which contain sulphuric acid and mixed well. These tubes were kept in the dark for 10 minutes. OD was measured at the wavelength of 510 nm and the procedure was followed for all the samples.

2.2.5. Estimation of Dissolved Oxygen:

(**Modified Winkler's Method)**

Dissolved oxygen (DO) levels in natural and wastewater depend on the physical, chemical and biochemical activities in the water body. The analysis for DO is a key test in water pollution and waste treatment process control. Two methods for DO analysis are available: the Winkler's or Iodometric method and its modifications and the electrometric method using membrane electrodes.

Aim: To estimate the amount of DO present in the given water samples.

Principle: It is based on the addition of divalent manganese solution, followed by strong alkali, to the sample in a glass – stoppered bottle. DO rapidly oxidizes an equivalent amount of the dispersed divalent manganous hydroxide precipitate to hydroxides of higher valency states. In the presence of iodide ions in an acidic solution, the oxidized manganese reverts to the divalent state, with the liberation of iodine equivalent to the original DO content. The iodine is then titrated with a standard solution of thiosulfate. The titration end point is detected visually with starch indicator.

The azide modification is used for most wastewater, effluent and stream containing more than 50µ NO_2^- - N/L and not more than 1 mg ferrous iron/L.

Reagents:

1. **Manganous Sulphate reagent:** 480 g Mn SO_4.4 H_2O or 400g Mn SO_4.2 H_2O or 364 Mn SO_4. H_2O was dissolved in distilled water, filtered and diluted to 1 litre.
2. **Alkaline – Iodide – azide reagent:**
   - **a.** 500 g NaOH or 700 g KOH and 135 g NaI or 150 g KI were dissoleved in distilled water and diluted to 1 litre.
   - **b.** 10 g NaN_3 was dissolved in 40 ml distilled water.
   - **c.** Both solution (a) and (b) were mixed together to give alkali – iodide azide reagent.
3. **Concentrated H_2SO_4**
4. **Starch indicator:**

   0.5 g starch powder was mixed with distilled water to get paste. This paste was transferred into 100 ml boiling water and then cooled.
5. **Standard Sodium Thio Sulphate Solution (0.025N):**

6.205g $Na_2S_2O_3$. $5H_2O$ was dissolved in distilled water (previously boiled and cooled) and diluted to 1L. It was standardized against standard potassium dichromate solution. (Note – The equivalent weight of sodium thiosulfate penta hydrate is its molecular weight).

6. **Standard Potassium Dichromate Solution:** (0.1N)

   4.904 g $K_2Cr_2O_7$ was dried at 103^0C for 2 hrs, dissolved in distilled water and diluted to 1000 ml.

Procedure:

1. **Standardization of Sodium Thio Sulphate solution:** 40 ml cold, recently boiled distilled water was placed in a 250 ml conical flask, 1.200g iodate – free potassium iodide and 800mg pure sodium bicarbonate were added and shaken well until the salts dissolved. 2.4 ml Conc.HCL was added slowly whilst the flask was rotated gently. 10.0ml 0.1N $K_2Cr_2O_7$ solution was added and the contents were mixed well. Sides of the flask were washed with a little amount of distilled water. The flask was covered with a small watch glass and allowed to stand in the dark for 5 minutes in order to complete the reaction. The watch glass was rinsed and the solution was diluted with about 120ml boiled cold water. The liberated iodine was titrated with the sodium thio Sulphate whilst constantly rotating the liquid until the colour changed to yellowish green. At that stage, 2 ml starch solution was added and the titration was continued adding the thiosulphate dropwise until 1 drop changed the colour from greenish blue to light green. From this titration, the exact normally of thiosulphate was calculated.

To the sample collected in a BOD bottle, 2 ml manganous sulphate solution and 2 ml alkali – iodide- azide reagent were added (reagents were added well below the surface of the sample water). The bottle was stoppered without any air bubble and mixed by inverting the bottle several times. The precipitate was allowed to settle down at the bottom of the bottle. The stopper was carefully removed and 2 ml conc. H_2SO_4 was added and mixed well by inverting the bottle several times until the dissolution was complete.

50 ml sample from the bottle was pipetted out in to a clean conical flask and titrated against 0.025N thiosulphate solution until it turned to a pale straw color. At that stage, a few drops of starch solution was added and the titration was continued by adding the thiosulphate dropwise to first disappearance of blue colour amount of distilled water. The flask was covered with a small watch glass and allowed to stand in the dark for 5 minutes in order to complete the reaction. The watch glass was rinsed and the solution was diluted with about 120ml boiled cold water. The liberated iodine was titrated with the sodium thio Sulphate whilst constantly rotating the liquid until the colour changed to yellowish green. At that stage, 2 ml starch solution was added and the titration was continued adding the thiosulphate dropwise until 1 drop changed the colour from greenish blue to light green. From this titration, the exact normally of thiosulphate was calculated.

Calculation:

Dissolved oxygen (mg/1)

$$= \frac{V_{THIO} \text{ x } N_{THIO} \text{ x } 8 \text{ x} 1000}{V_2 \ [(V_1 - V) / (V_1)]}$$

Where, V_1 = Total volume of bottle after placing the stopper

V_2 = Volume of the sample taken for titration

V = Total volume of $MnSO_4$ + Alkali iodide

2.2.6. Estimation of free Carbon dioxide:

50 ml of the water sample was taken in a conical flask. A few drops of Phenolphthalein was added as indicator. This solution was titrated against 0.01N sodium hydroxide. The end point is the appearance of pale permanent pink colour. The titration was repeated for constant concordant values and it was estimated for few samples. Dissolved CO_2 has marked effects on the properties of water, if forms a weak carbonic acid solution that changes the pH increases, alkalinity and hardness of water by dissolving minerals.

Principle:

Free CO_2 reacts with NaOH or Na_2CO_3 to form Na $(HCO_3)_2$, the completion of the reaction is indicated by the appearance of pink color in the presence with phenolphthalein indicator as pH of 8.3

Reagents:

- **Std. NaOH solution (0.02 N):** Dissolve 200 ml of stock solution to 1000 ml with distilled water.
- **Stock NaOH solution:** Dissolved 4 g NaOH in 1000 ml distilled water.
- **Phenolphthalein indicator:** Dissolve 0.5 g of phenolphthalein powder in 50ml of 95% C_2H_2OH and add 50ml distilled water.

Procedure:

- Take 50 ml of sample in a conical flask.
- Add 4 to 5 drops of phenolphthalein indicator:
  If the sample is pink color does not appear titrate the sample against standard 0.02 N NaOH and until the pale pink color develop and remain for 30 sec.
- Note down the burette reading

Calculation:

$$\text{Free } CO_2 \text{ (mg/l)} = \frac{V \times N \times 44 \times 1000}{\text{Volume of sample}}$$

V = Volume of 0.02 N NaOH

N = Normality of NaOH

2.2.7. Determination of Oxygen consumption and Ammonia excretion:

The experiments were done once in a week just before the next water exchange. For the measurement of O_2 consumption and ammonia excretion, the sealed vessel method ***(Brett, 1964)*** was followed. Each respirometer was first half filled with water. Fisheries from the experimental and control were randomly collected before feeding, weighed and introduced into the respective respirometers. The mouth of the respirometer was closed and it was filled with water, after which both the inlets and outlets were closed. The fishes were then allowed to acclimate and left for an hour. In mean, while the oxygen content and ammonia of the water were found by Winkler's method and Ammonia estimation. After an hour, water samples were siphoned out and analyzed for oxygen and ammonia. The difference in oxygen content of the first and second samples revealed the amount of oxygen consumed by the fish in an hour. Similarly, the difference in the ammonia content of the two samples resulted the ammonia excretion in an hour by the fish. The experiment was repeated thrice with appropriate intervals in each system. Mean values were taken and rates of oxygen consumption and ammonia excretion of the fishes were calculated in mg/fish/hr

From the rates of Oxygen consumption and Ammonia excretion, the Ammonia quotient (A.Q) were calculated by the formula

$$\text{Ammonia Quotient (A.Q)} = \frac{\text{Amount of ammonia excreted}}{\text{Amount of oxygen consumed}}$$

2.2.8. Biological Oxygen Demand (BOD):

The Biochemical Oxygen Demand determination is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of waste waters, effluents and polluted waters. The test has its widest application in measuring waste loadings to treatment plants and in evaluating the BOD removal efficiency of such treatment systems. The test measures the oxygen utilized during a specified incubation period for the biochemical degradation of organic material and the Oxygen used to oxidize inorganic material such as sulphides and Ferrous ion. It also may measure the oxygen used to oxidize reduced forms of nitrogen unless their oxidation is prevented by an inhibitor. The seeding and dilution procedures provide an estimate of the BOD at pH 6.5 to 7.5.

The BOD concentration in most waste waters exceeds the concentration of dissolved Oxygen (DO) available in an air-saturated sample. Therefore, it is necessary to dilute the sample before incubation to bring the Oxygen demand and supply into appropriate balance. Because bacterial growth requires nutrients such as Nitrogen, Phosphorus and trace metals, these are added to the dilution water which is buffered to ensure that pH of the incubated sample may require a period of incubation too long for practical purposes. Therefore 5 days have been accepted as the standard incubation period.

Principle: The sample is placed in a full, air tight bottle and is incubated in the bottle under specified conditions for a specific time. Dissolved Oxygen is measured initially and after incubation, the BOD is computed from the difference between the initial DO and final DO

Reagents required:

a. Phosphate buffer solution

8.5gms KH_2 PO_4, 21.75gms K_2HPO_4, 33.4gms, Na_2 $HPO_4$7H2O and 1.7 NH_4 C1 were dissolved in about 500 ml distilled water and diluted to one litre.

b. Magnesium Sulphate Solution

22.5gms. $MgSO_4$. $7H_2O$ was dissolved in distilled water and diluted to 1 litre.

c. Calcium Chloride Solution

27.5gms. $CaC1_2$ was dissolved in distilled water and diluted to 1 litre.

d. Ferric Chloride Solution

0.25gms. $FeC1_3$. 6 $H_2O$was dissolved in distilled water and diluted to 1 litre.

e. Acid and alkali Solutions

1N acid and alkali solutions for neutralization of alkaline or acidic waste samples.

f. Sodium Sulphite Solution

1.575 gms. Sodium Sulphite was dissolved in distilled water and diluted to 1 litre.

Procedure:

A desired volume of distilled water with 1 ml per litre of phosphate buffer, $MgSO_4$, $CaC1_2$ and $FeC1_3$ were mixed and aerated for 30 mins. This is known as as Dilution water. The samples were diluted suitably using standard dilution tables and dilution water. 2 sets of BOD bottles were filled with the respective solutions of dilution range. One bottle was incubated with label at 20 degree Celsius in a BOD incubator for 5 days. Initial DO level was estimated in the other set of bottle and noted down. After 5 days the final DO was estimated and the BOD was calculated using the following formula

BOD mg/1 = (Initial DO-Final DO) x Dilution factor(100 to 1000 times)

2.2.9. Chemical Oxygen Demand(COD) :

Principle:

Chemical Oxygen Demand is the measure of oxygen consumed during the oxidation of the oxidisable organic matter by a strong oxidizing agent. Potassium Dichromate in the presence of Sulphuric acid is generally used as an oxidizing agent in the determination of COD.

The samples and refluxed with Potassium Dichromate and Sulphuric acid in the presence of mercuric sulphate to neutralize the effects of chlorides and Silver Sulphate as catalyst. The organic matter gets oxidized completely to Carbon dioxide and Water. The excess of Potassium dichromate remaining is

titrated against Ferrous Ammonium Sulphate using Ferroin as indicator. The amount of Potassium dichromate consumed is proportional to the oxidisable matter present in the sample.

Reagents required:

Potassium dichromate solution

12.25g of dried A.R grade potassium dichromate was dissolved in double distilled water and was made upto 1000ml.

Ferrous Amonium Sulphate (0.1N)

39.2g of FAS was dissolved in distilled water along with the addition of 20ml of concentrated Sulphuric acid. It was made upto 1000ml in a standard flask.

Silver Sulphate Concentrated Sulphuric Acid mixture:

10g of Silver Sulphate was dissolved in 1000ml of conc. Suphuric acid.

Ferroin indicator:

1.485g of 1,10 phenonthroline and 0.695g of ferrous sulphate was dissolved in distilled water and made upto 100ml.

Mercuric Sulphate

Procedure:

20 ml of the sample was taken in a round bottomed flask and a pinch of mercuric sulphate was added. Antibumping granules were added. 5ml of Sulphuric acid-silver sulphate mixture was added to the solution in the flask and mixed well to facilitate the dissolution of mercuric sulphate. 10ml of potassium dichromate was added and 25 ml of sulphuric acid-silver sulphate mixture was added. While adding this mixture, the round-bottomed flash should be kept in ice water bath. This was to prevent the escape of fatty acids due to higher temperature. Then the RB flask was connected to the reflux condenser and refluxed for 2 hours.

After 2 hours the flask was cooled and 80ml of distilled water was added and mixed well. This was titrated against FAS solution using ferroin indicator. The end point was the sharp colour change from blue green to wine red. Simultaneously, the blank was refluxed in the same manner using distilled water instead of sample with the same amount of chemicals.

Calculation:

$$\text{COD (mg/1)} = (B - A) \times \frac{N_{FAS} \times 8 \times 1000}{\text{Volume of sample}}$$

Where,

A = Volume of FAS for Sample ----- ml.

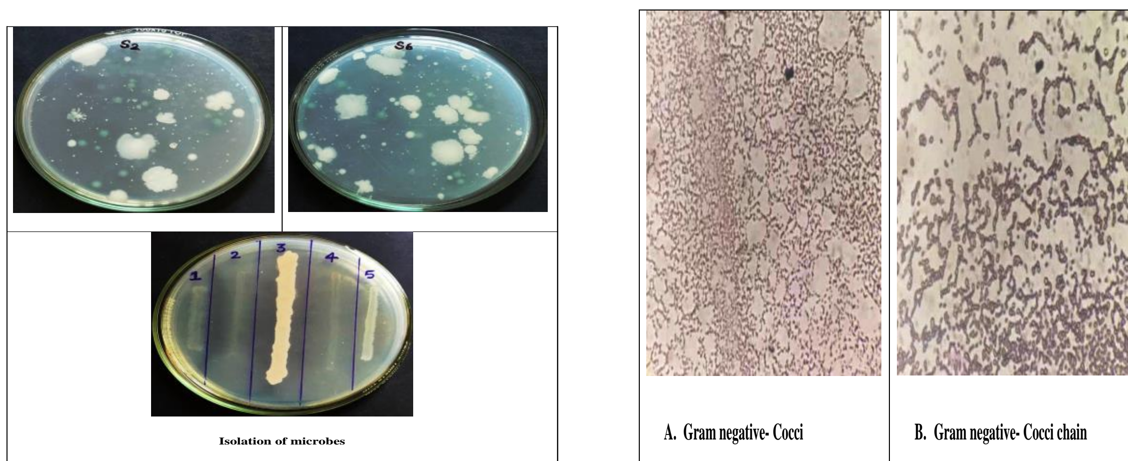
B = Volume of FAS for Blank ------ ml.

2.2.10. Growth and Mortality rate:

The growth and weight gain in percentage were recorded. The mortality rate was also observed and recorded during the course of the experiment in fishes after probiotic treatment

fig.1: Images for enumeration of microbes

Results



S. No.	Name of the test sample concentration	Name of the Biochemical characterization						Identified bacteria
		Gram staining	Motility	Indole test	Methyl red test	Voges Proskauer	Simmons' test	
1.	A	Gram negative–Cocci	Motile	–	+	–	+	Nitrosococcus
2.	B	Gram negative–Cocci chain	Motile	–	+	–	+	Neisseria
3.	C	Gram positive Bacillus sp.	Motile	+	+	+	–	Lactobacillus acidophilus

Table 1 : Biochemical characterization and Indentification of Bacteria

2.2: APPLICATION IN AQUACULTURE:

Fig 2 : Acclimatization of the Tilapia fish and application of the probiotic supplementary food

PHYSICO – CHEMICAL PARAMETERS OBSERVED IN THE PROBIOTIC TREATED WATER:

Tab. 2. CHLORIDE CONTENT:

DAY	CONTROL			FISH TREATED WITH PROBIOTICS (10mg/l)		
	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
0	67.4 ± 2.80	67.4 ± 2.80	67.4 ± 2.80	67.4 ± 2.80	67.4 ± 2.80	67.4 ± 2.80
7	75.7 ± 1.60	80.4 ± 1.60	76.9 ± 1.60	82.8 ± 1.60	79.2 ± 1.60	79.2 ± 1.60
14	76.9 ± 1.60	73.3 ± 1.60	84.0 ± 1.60	80.4 ± 1.60	76.9 ± 1.60	80.4 ± 1.60
21	76.3 ± 1.60	79.8 ± 1.60	86.9 ± 1.60	83.4 ± 1.70	83.4 ± 1.70	79.8 ± 1.70

Tab. 3. NITRITE CONTENT RECORDED:

DAY	CONTROL			FISH TREATED WITH PROBIOTICS (10mg/l)		
	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
0	0.073 ± 0.002	0.073 ± 0.002	0.073 ± 0.002	0.073 ± 0.002	0.073 ± 0.002	0.073 ± 0.002
7	0.042 ± 0.002	0.40 ± 0.004	0.042 ± 0.002	0.030 ± 0.007	0.030 ± 0.001	0.032 ± 0.002
14	0.015 ± 0.001	0.019 ± 0.002	0.010 ± 0	0.005 ± 0.001	0.012 ± 0.002	0.010 ± 0.003
21	0.015 ± 0.002	0.018 ± 0.001	0.0015 ± 0.002	0.013 ± 0.003	0.015 ± 0.002	0.014 ± 0.001

Tab. 4. NITRATE CONTENT RECORDED: _

DAY	CONTROL			FISH TREATED WITH PROBIOTICS (10mg/l)		
	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
0	2.2 ± 0.78	2.2 ± 0.78	2.2 ± 0.78	2.2 ± 0.78	2.2 ± 0.78	2.2 ± 0.78
7	1.6 ± 0	3.3 ± 0	5.8 ± 0.83	5.8 ± 0.83	5.8 ± 0.83	5.0 ± 0
14	5.5 ± 0.50	4.5 ± 0.50	5.5 ± 0.50	6.5 ± 0.50	7.0 ± 0.10	7.5 ± 0.50
21	6.8 ± 0.62	5.6 ± 0.62	6.2 ± 0	7.5 ± 0	8.1 ± 0.62	8.1 ± 0.62

Tab.5. DISSOLVED OXYGEN RECORDED:

DAY	CONTROL			FISH TREATED WITH PROBIOTICS (10mg/l)		
	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
0	6.3 ± 0.30	6.3 ± 0.30	6.3 ± 0.30	6.3 ± 0.30	6.3 ± 0.30	6.3 ± 0.30
7	2.8 ± 0.19	0.9 ± 0.07	0.7 ± 0.07	2.5 ± 0.07	2.3 ± 0.15	1.7 ± 0.07
14	3.3 ± 0.13	1.2 ± 0.52	0.02 ± 0.01	4.64 ± 0.12	1.1 ± 0.72	0.02 ± 0.007
21	1.8 ± 0.004	1.0 ± 0.009	2.6 ± 0.09	2.1 ± 0.004	1.0 ± 0.004	2.0 ± 0.11

Tab. . OXYGEN CONSUMPTION RATES:

DAY	CONTROL			FISH TREATED WITH PROBIOTICS (10mg/l)		
	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
0	2.2 ± 0.22	2.2 ± 0.22	2.2 ± 0.22	2.2 ± 0.22	2.2 ± 0.22	2.2 ± 0.22
7	3.6 ± 0.07	3.3 ± 0.13	3.6 ± 0.06	2.6 ± 0.07	2.7 ± 0.07	2.1 ± 0.07
14	1.2 ± 0.17	0.4 ± 0.20	0.4 ± 0.07	0.8 ± 0.26	0.4 ± 0.26	0.3 ± 0.16
21	1.3 ± 0.15	1.2 ± 0.17	0.2 ± 0.09	1.6 ± 0.09	0.7 ± 0.13	0.4 ± 0.06

table . AMMONIA EXCRETION RATES:

DAY	CONTROL			FISH TREATED WITH PROBIOTICS (10mg/l)		
	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
0	0.008 ± 0	0.008 ± 0	0.008 ± 0	0.008 ± 0	0.008 ± 0	0.008 ± 0
7	0.055 ± 0.003	0.033 ± 0.004	0.011 ± 0.004	0.025 ± 0.003	0.029 ± 0	0.040 ± 0.003
14	0.085 ± 0.002	0.051 ± 0.001	0.029 ± 0.004	0.010 ± 0.002	0.020 ± 0.001	0.018 ± 0.002
21	0.039 ± 0.005	0.017 ± 0.003	0.010 ± 0.003	0.007 ± 0	0.014 ± 0	0.007 ± 0

4. DISCUSSION:

As new findings emerged, several definitions of probiotics have been proposed. Few researchers gave a precise definition of probiotics which is still widely referred to, i.e., a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance. It was stated above that the interaction between the microbiota, including probiotics, and the host is not limited to the intestinal tract. Probiotic bacteria could also be active on the gills or the skin of the host but also in its ambient environment. The intensive interaction between the culture environment and the host in aquaculture implies that a lot of probiotics are obtained from the culture environment. Knowledge of probiotics has increased, currently it is known that these microorganisms have an antimicrobial effect through modifying the intestinal microbiota, secreting antibacterial substances (bacteriocins and organic acids), competing with pathogens to prevent their adhesion to the intestine, competing for nutrients necessary for pathogen survival, and producing an antitoxin effect. Probiotics are also capable of modulating the immune system, regulating allergic response of the body, and reducing proliferation of cancer in mammals. Because of this, when provided at certain concentration and viability, probiotics favorably affect host health. In fact, terms such as “friendly bacteria,” “friendly,” or “healthy” are commonly used to describe probiotics . For many years, studies focused on microorganisms characteristic from intestinal microbiota, and the term “probiotic” was mainly restricted to gram-positive lactic acid bacteria, particularly representative of the genera Bifido bacterium, Lactobacillus, and Streptococcus . In contrast to terrestrial animals, gastrointestinal micro biota of aquatic species is particularly dependent on the external

environment due to the flow of water passing through the digestive tract. Thus, the majority of bacteria are transient in the intestine, due to constant intake of water and food, together with microorganisms present in them. Although in the gastrointestinal tract (GIT) of aquatic animals have been reported potentially pathogenic bacteria such as Salmonella, Listeria, and Escherichia coli, probiotic bacteria and other microorganisms have also been identified. These include gram-positive bacteria such as Bacillus, Carno bacterium, Enterococcus, and several species of Lactobacillus; gram negative, facultative anaerobic such as Vibrio and Pseudomonas, as well as certain fungi, yeasts, and algae of the genera Debaryomyces, Saccharomyces, and Tetraselmis, respectively. Due to the increasing interest of probiotics in aquaculture, Moriarty proposed extending the definition of these to "living microbial additives that benefit the health of hydrobionts and therefore increase productivity. From the review of the articles published earlier, too many works concentrated much about the GIT bacteria or probiotic bacteria from the GIT(Gastro Intestinal Tract) also known as gut bacteria to show enormous benefits in aquatic organisms. Hence, this study was done to test the unique role of the soil probiotic bacteria and their effect in the Oreochromis(Tilapia)fish sp. With the present findings, this study promotes the following:

Enhanced growth and survival rate in fish, Improved quality of the probiotic fed fishes – like, enhanced digestion , high immune response to pathogenic microbes and tolerance to stress, Reduced accumulation of dissolved and particulate organic carbon, ammonia in fish ponds, Reduced level of nitrites in fish ponds and played vital role in the conversion of nitrites to nitrates, Reduced pollutant level in the fish ponds, Increased fish biomass in the edible tilapia fish and is possible in good marketing of the probiotic fed tilapia fish , Thus, this study hence proved the enhanced effect of the soil probiotic bacteria in the improvement of the metabolic activities of the fish thereby increasing the bio mass, disease susceptibility, enhanced digestive process in fish and also in improving the water quality.

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