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Research Article

MOLECULAR ANALYSIS OF BACTERIAL COMMUNITIES AND DETECTION OF POTENTIAL PATHOGENS IN A RECIRCULATING AQUACULTURE SYSTEM FOR YELLOWTAIL CATFISH, *Pangasius pangasius* (Hamilton, 1822)

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Abstract- Raising interest for consumable marine items like fish, mollusks, algae *etc.* have scientists to discover progressively sustainable environmentally friendly, economically viable, and socially acceptable strategies for aquaculture production. Among all Recirculatory Aquaculture System best in any case, however RAS has its own disadvantages high chances of contamination with diseases which are microbial in origin, formation of super bugs, high cost intensive, continues monitoring *etc.* The motivation behind the investigation is to break down the complete microbial populace in a freshwater RAS and to discover any possible pathogens in RAS for Yellowtail catfish pangasius (Hamilton, 1822), for knowing the away from of the bacterial communities in the RAS framework traditional strategies are not sufficient because, there are no standard protocols to isolate all the microscopic organisms from sample so for community 16srRna examination is done by separating DNA legitimately from the sample. However, we were unable to discover expected pathogens in the drawn samples as the water used in RAS was not drawn from any water bodies, rather than drawing water from water table (ground water) and RAS established recently, maintained scientifically.

Keywords- *Quantitative research, Applied Research, Problem-solving research, exploratory, and descriptive*

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Introduction

All the fishes carry pathogens and parasites. Normally, this is at some expense to the fish. On the off chance that the expense is adequately high, at that point the effects can be portrayed as a malady. In any case, the illness in fishes isn't studies well [1]. The malady is a prime operator influencing fish mortality, particularly when fish are young. Fish can restrict the effects of pathogens and parasites with behavioral or biochemical methods, and such fish have regenerative advantages. Interacting factors bring about poor quality contamination turning out to be lethal illnesses. Specifically, things that cause pressure, for example locality of growing, technique of growing number of fishes/ stocking density, natural droughts or pollutants or predators, can hasten the outbreak of disease [2]. What is thought/ known about the fish illness frequently refers with aquaria fish/ all the cultivated fish. Major among those diseases are caused by microscopic organisms like viral [3, 17], bacterial, fungal, water mould, metazoan, archaebacteria *etc.* The present-day studies say that, traditional aquaculture production is not sustainable and causes numerous numbers of issues, to solve these problems researchers have invented new-generation production techniques like RAS, Raceways *etc.* This paper deals with, analyzing water samples are from every component of the RAS system for Yellowtail catfish *Pangasius pangasius* (Hamilton, 1822), and samples are: water and gel from the fish gills. A variety of microbial species can be identified in RAS by utilizing advanced molecular-biology techniques like, 16srRna analysis can be done for getting a clear picture, on the bacterial community in the system we can easily restrict the outburst of diseases in the RAS system

Materials and Methods

Sample Collection: Sample was collected from the one of the few facilities' where RAS technology is present in India which is Kravis Aqua, Gunded, Telangana with GPS coordinates of 17°7' 23.4624" N and 79°12' 31.7664" E, and sample was collected by keeping the all parameters at constant for that I have to understand the functionality of RAS clearly so that I can collect samples perfectly without any contamination and the collected samples are then sipped by using an ice box to arrest the overgrowth and contamination of sample [11, 12, 13, 26].

Fig-1 GPS location of Kravis Aqua company

Water Quality Testing

Water is the medium in which the fishes are grown so the water quality is maintained at a level where the fish can grow without stress, as we are growing the fish in ultra-high density technique there is need to check water quality continuously because change in pH is directly proportional to the buildup of the toxic ammonia in the system. To archive our goal so many bio sensors and so many water quality testing kits are available in market among them, Libelium water quality smart sensor is much more advanced than other and aureate.

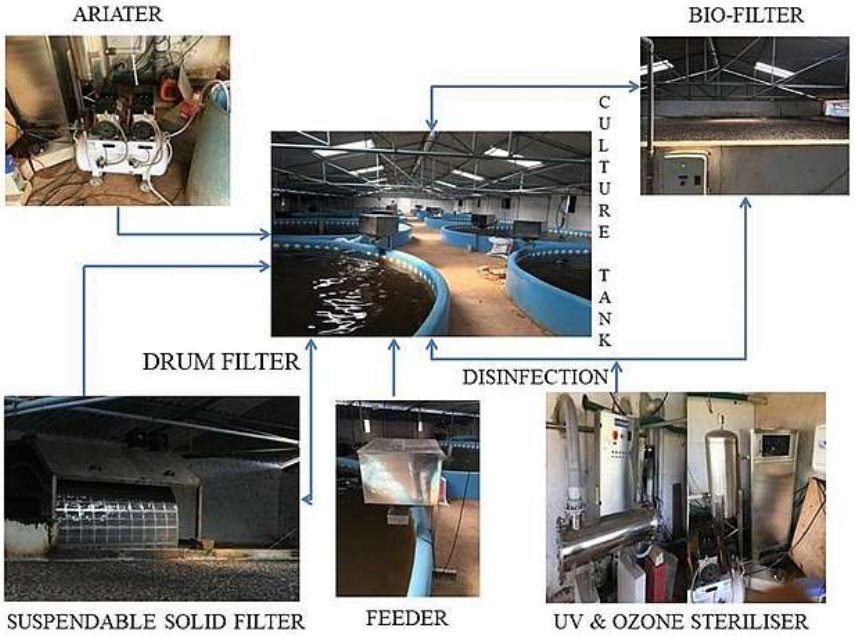

Fig-2 Components of RAS

Libelium water quality smart sensor is used to check the water quality in this RAS system which automatically tests the water more than a dozen parameters are checked using this smart sensor but as the RAS is controlled system it is set to measure the pH, ORP, ammonia (NH_4), nitrate(NO_3-), nitrite(NO_2-). For every 10 days the device will be giving average mean data will be given which have collected from Mr. Vishwanatharaju owner of Kravis Aqua for 2 months. The date obtained is used to construct a relationship graph between ammonia level in water and bacterial abundance.

Formula for conversion of ammonia in to nitrate, nitrite is calculated

NH_4 (ammonium) + 1.5 O_2 → NO_2 (nitrite) + H_2O + 2H+ + 2e NO_2 (nitrite) + 0.5 O_2 → NO_3 (nitrate)+ e

$NH_4 + 2\ O_2 \leftrightarrow NO_3 + H_2O + 2H+$

Table-1 *Collected samples and their quantity*

SN	Component	Sample Collected
1	Culture tank	Water (50 ml)
2	Sedimentation tank	Water (50 ml)
3	Biofilter tank	Water (50 ml)
4	Ozone tank (Ozone sterilizer).	Water (50 ml)
5	UV sterilizer	Water (50 ml)
6	Fish gills gel.	Gel (15 ml) from 10 fish 1 fish from each tank
7	Final filterate	Water (50 ml)

Table-2 *Mean values of ammonia, nitrate, and nitrate in the span of 60 days*

Days	Mean values (+/- 0.5) of Nitrate N Mg/l	Mean values (+/- 0.5) of Nitrite n Mg/l	Mean values (+/- 5) of Ammonia N Mg/l
0	0.7	0.019	138
10	0.46	0.05	97
20	0.5	0.1	77
30	0.45	0.21	64
40	2.1	0.78	53
50	6.5	0.98	47
60	8.1	1.07	42

Molecular Characterization

Bacterial classification, identification of microbes is progressively utilizing molecular techniques. Diagnostics utilizing DNA-based instruments, for example, polymerase chain response, are progressively increasing because of their particularity and speed, when compared to other culture-based methods [4, 18]. These strategies additionally permit the recognition and identification of "live yet nonculturable" cells that are metabolically dynamic yet non-dividing [5,19]. However, in any event, utilizing these improved techniques, the complete number of bacterial species cannot be known and can't be assessed with precision. Following current classification, there are somewhat less than 9,300 known types of prokaryotes, which incorporates bacteria and archaea [6,20]; however, endeavors to assess the genuine number of bacterial decent variety have not been successful [7,8,21,22]. This step confirmed the species or Genus of organisms by the PCR amplification of the genomic DNA with 16s rDNA marker.

Isolation of Bacterial Genomic DNA

DNA, deoxyribonucleic acid, is the particle of life. Each living being has DNA in every cell of the living beings and every atom of DNA conveys the rough diagram for that living being. The DNA atom is additionally liable for heredity, passing on hereditary data from one generation to other. DNA particles are huge strands or chains of little atoms known as nucleic acids, which are receded in the core of a cell. DNA from sample is isolated by pre-existing traditional techniques.

PCR Amplification of The Genomic DNA using 16s-rDNA Marker

To over-power the time-consuming and difficult procedure of test groundwork for DNA sequencing, we attempted to enhance the 16S rRNA quality straightforwardly from samples. We utilized enhanced primers from department of molecular biology IIT madras, for 16S rRNA amplicon sequencing. The primers were intended to enhance the near-full-length 16S rRNA quality for bacterial recognizable proof [9, 10, 23].

Reaction Mixture Composition

1. 1 X PCR buffer
2. 1.5 mM $MgCl_2$
3. 1.0 mMdNTPs
4. 1.5 U Taq Pol
5. 0.5 pmol/µl Primer 1
6. 0.5 pmol/µl Primer 2
7. 0.4 ng/µl Bacterial DNA

Total volume was 25 µl

PCR profile

Initial hot start - 95°C for 5 minutes.
Denaturation - 95°C for 30 seconds.
Annealing - 55°C for 45 seconds.
Extension - 72°C for 2 minutes.
Final Extension - 72°C for 10 minutes.
Storage - 4°C for no time limits.
Total 35 cycles

Molecular Identification

Microbes are omnipresent, ubiquitous in nature; all most all ecosystems in soil, water, air are inhibited by microbes. Even after the huge advancements in science and technology we don't have the adequate technologies to isolate all the microbes from the given sample, according to scientific studies only 5% of microbial load in a sample can be cultured, in that case the isolated bacteria will be a small fraction from the sample. However, my work title is Molecular Analysis of Bacterial Communities and Detection of Potential Pathogens in a Recirculating Aquaculture System (RAS) for yellowtail catfish, *Pangasius pangasius* (Hamilton, 1822) that it self say I have to know all the bacterial communities in that system, for that reason traditional methods are not enough, so I have went for a very advanced molecular identification technique which involves the 16S rRNA gene sequence analysis

The bacteria present in sample will be identified on the basis of16S rRNA gene analysis. The purified gel-eluted rDNA fragments will be outsourced for sequencing from Molecular and Biotechnology Laboratory, IIT Chennai, with selected primers. Resulted nucleotide sequences will be compared to publish sequences in the NCBI GenBank using the nucleotide BLAST for identification.

Phylogenetic Analysis

A phylogenetic tree is a diagram that show the relationship between different species based on the molecular characteristics, for phylogenetic analysis, the bacterial partial 16S rDNA gene sequences from the samples collected are aligned using the multiple sequence alignment programme 'Clustal Omega' [16]. The phylogenetic analysis was performed with the software MEGA 6 using the neighbor-joining method. The reliability of the tree nodes was analyzed by using the bootstrap resampling technique with 1000 replication.

Results

Water Quality Testing

Water quality plays a major role in growth and development of fish high levels of the ammonic ions in the water may cause lethal effects on the fish and for reduction of available nitrogen in the water we use the bio filters beds with duck weed *etc* hear the farm have used the bio-filter and the following test conforms the presence of the bacteria in the system, by seeing graph we can infer that bye coerce of time ammonia level are reducing and increasing of the level of nitrate and nitrate increasing that indicates that microbial activity is there in the RAS.

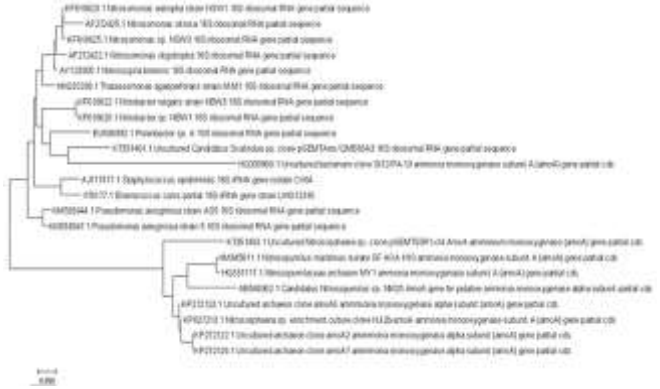

Fig-2 Evolutionary relationships of taxa

Molecular Characterization of the isolates

The sequences were submitted to the NCBI-GenBank and accession no were also received. The thirteen isolates were identified by the similarity of their partial 16S rDNA sequences to sequences in NCBI GenBank Database and results are summarized. The phylogenetic tree based on neighbor joining method and Kimura 2 parameter model with bootstrap value 1000 among 13 selected bacterial isolates were developed. The percentage similarity by sequence alignment using Clustal Omega was also obtained. It was found that the maximum isolated bacterial strains are *Nitrosomonas* sp ,*Nitrososphaera* sp, *Pseudomonas* sp, *Nitrobacter* sp, *Staphylococcus* sp (1) ,*Thalassomonas* sp (1) and along with this I have isolated some uncultured from the water samples and maximum isolated species are Aerobic, Autotrophs which are AOB bacteria and 2 heterotopic bacteria. The distribution of the isolates in the phylogenetic tree again confirmed the pattern of variation among the isolates in the 16S rDNA sequences.

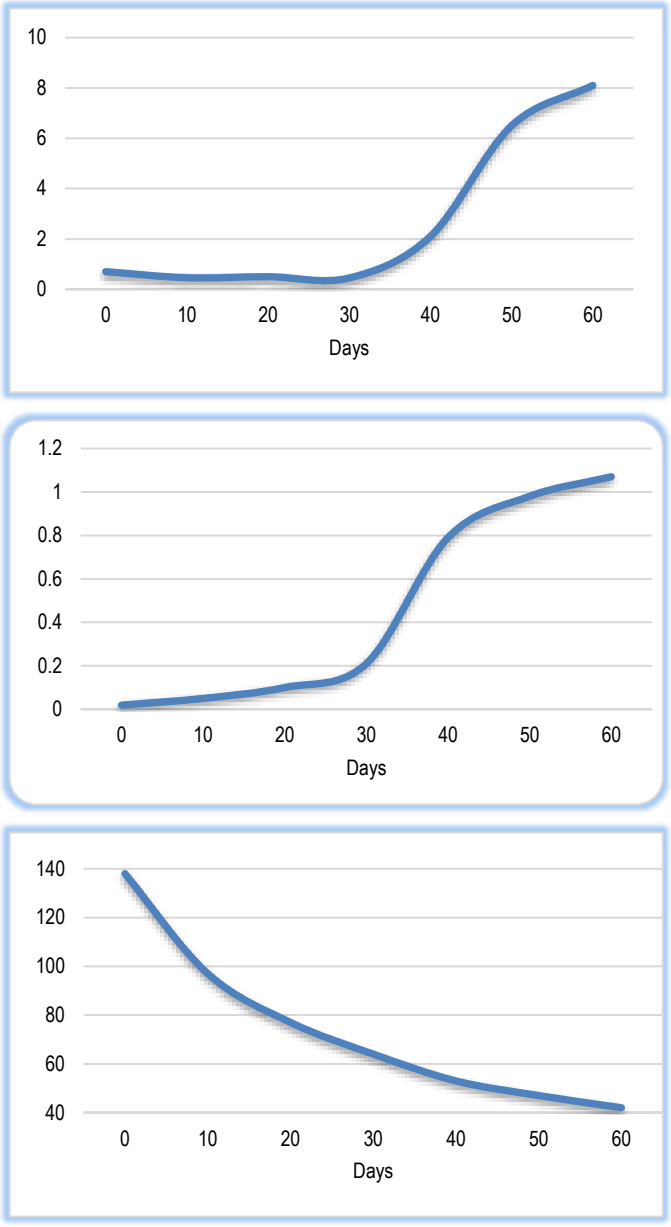

Fig-3 Rate of change of ammonia, nitrate, and nitrate in the span of 60 days

Discussion

The evolutionary history was inferred using the Neighbor-Joining method [14, 27]. The optimal tree with the sum of branch length = 2.33784721 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The developmental separations were figured utilizing the Most extreme Composite Probability strategy [15, 28] and are in the units of the quantity of base replacements per site. The examination included 23 nucleotide arrangements. All positions containing holes and missing information were killed. There was an aggregate of 135 situations in the last dataset. Developmental investigations were directed in MEGA7 [16].

Conclusion

Staphylococcus epidermidis was found in the system so if we use the *Staphylococcus* suppressing probiotics the RAS system than the system will be free of pathogens.

Its new technique with high investments and high profit in Indian aquaculture scenario but maximum Indian farmers who are in Aquaculture cannot afford that huge investments.

Application of research: The presence of pathogenic bacteria in the RAS system which indicates that if system is not maintained properly infection logs in the system, high chances of getting economic loss, but if we maintain the system properly note the water quality parameters and to wash the bio filter frequently will reduce the risk factors in RAS which ultimately give high amount of yield if yield is high then 75-80% chances of getting huge benefits.

Research Category: Aquaculture

Abbreviations: RAS: Recirculatory Aquaculture System.
DNA: Deoxyribose Nucleic Acid.
16S rDNA: 16S ribosomal Deoxyribose Nucleic Acid.
NCBI: National Center for Biotechnology Information.
BLAST: Basic Local Alignment Search Tool.
Aquaphonics: Aquaculture coupled with hydroponics is Aquaphonics.

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Research project name or number: MSc Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Kravis Aqua, Gunded, Telangana

Cultivar / Variety / Breed name: *Pangasius pangasius* (Hamilton, 1822)

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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