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Instrumental & Bio-analysis of Extracts of *Aeromonas sp.* (DKDC1) Found in Puffer Fish and Determined as TTX

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Abstract: *A novel bacterial strain (DKDC1) isolated from Tetrodon cututia (puffer fish) was confirmed by NCBI after 16S rRNA sequencing and by instrumental analysis. The strain was cultured in TCBS media and further analyzed for biochemical characterization. Presence of TTX was examined through different analytical techniques and its action was observed by mouse assay.*

Keywords: *Isolation, Identification,16S rRNA sequencing, TTX, Puffer Fish, extraction, purification, instrumental analysis.*

1. INTRODUCTION

Tetrodon cutcutia (Puffer fish) ,which belongs to the order Tetrodontiformes, is found to be poisonous, if consumed(1). Tetrodotoxin (TTX) a neurotoxin, present in the liver, ovary, skin and other viscera including the intestine of puffer fish is the main cause of poisoning. In human, this neurotoxin blocks the sodium channel in the excitable cell membrane and causes deadening of the tongue and lips, dizziness, and vomiting followed by numbness and pricking over the body, rapid heart rate, decreased blood pressure, and muscle paralysis (3). The level of TTX in different species as well as different organs varies in different seasons.

Earlier in an experiment, where the toxin extracted from the liver, ovary and skin of *Tetrodon cutcutia* and found the presence of some derivatives of tetrodotoxin i.e Saxitoxin. Further, using the above extracted toxin, bioassay was conducted in albino mice, which exhibited symptoms similar to that of TTX poisoning. Thus indicates the presence of toxin in the organs of fish.(4).

According to Lee (2007), bacteria inside TTX bearing organisms make the toxin (5). In further experiments intestinal bacteria of *Tetrodon cutcutia* was cultured in TCBS Agar media. From the isolated bacterial strains different biochemical analysis were performed followed by Bergey's manual. DNA was extracted from isolates and 16s rRNA sequencing was done. NBCI Genbank database confirmed the presence of novel strain of bacteria of genus *Aeromonas* as DKDC1.

This analysis aimed to the molecular study of *Aeromonas sp*.DKDC1 using various analytical techniques for identifying the presence of toxin which can be used as biopesticides.

2. MATERIALS AND METHODS

2.1. Collection and Isolation of Bacterial Strains

Freshwater puffer fishes *Tetrodon cutcutia,* are collected from the tributaries of the river Brahmaputra river and are aseptically dissected to obtain the intestine. The intestine is cut into small pieces and inoculated in alkaline peptone water broth and incubated at 37°C. After 24 hours of incubation the peptone water broth turned turbid indicating the growth of bacteria. Fresh inoculum of bacteria was streaked on TCBS (Thiosulfate Citrate Bile Salts Sucrose) culture media and incubated at 37°C. After 24-36 hours yellow bacterial colonies appeared on the culture plate. Single yellow colonies were picked and streaked on fresh TCBS media. Continuous sub-culturing by streak-plate method resulted in pure isolates of bacteria. A total of three bacterial isolates were found and they are termed as A, B & C.

2.2. Identification of Bacterial Isolates using Biochemical Analysis and Sequencing Method

2.2.1. Biochemical Analysis

The bacterial isolation experiments were conducted on the basis of various staining techniques and their biochemical characteristics prescribed by Bergey's Manual of Systematic Bacteriology. The biochemical methods include Gram's staining, Lactose fermentation, Peroxidase test, Oxidase test, Indole test, Citrate test and MR-VP test.

2.2.2. Sequencing Method

After separation 16S rRNA Sequencing using Sanger's sequencing method:

The most potent bacterial isolate was identified by 16S rRNA Sequencing by Sanger's Sequencing method using 704F forward primer (5'-GTAGCGGTGAAATGCGTAGA-3') and 907R reverse primer (5'-CCGTCAATTCMTTTGAGTTT-3'). Fragment of the 16S rDNA obtained from the bacterial strain was amplified using PCR. Consensus sequence was obtained from forward and reverse sequence data using aligner software and was used to carry out BLAST with the 16S rRNA database of NCBI GenBank Database. Phylogenetic tree was constructed using MEGA 6 software. The evolutionary history was inferred using the Maximum Likely hood method and evolutionary distances were computed using the Kimura-2 parameter method. The 16S rDNA sequence obtained was submitted to GenBank.

2.3. Extraction and Purification of the Toxin from the Isolates(sample A)

0.4% acetic acid (1.5 times the volume of the culture media) was added to 72 hours old bacterial culture in TCBS media and the media contents were homogenized and centrifuged at 17000rpm for 20 minutes. The supernatants were collected, combined and the extract was maintained at slightly alkaline *p*H at around 7.2 using 0.5% NH_4OH and concentrated by heating at 95°C in hot water bath. The aqueous layer was passed through cation exchange resin and subjected to an activated charcoal column. The column was first eluted using double distilled deionised water. The elute or extract is collected and stored for future use. At the end the column was eluted using (0.4%) acetic acid.

The toxic fractions were monitored for TTX using mouse bioassay and other instrumental analysis which includes TLC, HPLC, IR-spectra and NMR.

2.4. Mouse Bioassay

The purified extract was collected and tested for toxicity by the standard mouse bioassay. Toxicity of each sample was expressed as mouse lethal units (MU). One MU is the amount of the toxin that causes death of a 15–25 gram male mouse in 30 minutes after intra-peritoneal administration. 0.8ml of the purified extract was administered and the mouse was kept under observation.

2.5. Instrumental Analysis

Thin Layer Chromatography (TLC): TLC is a very common technique for identifying compounds. TLC plates are prepared and extract containing the lead compound is spotted onto the silica gel plates. The solvent rises by capillary action and a chromatographic separation is obtained. The plate is then sprayed with 10% KOH followed by heating at 100°C for 10 minutes. The toxin is visualized as a yellow fluorescent spot under UV light (365nm).

High Performance Liquid Chromatography (HPLC)

The identification (ID) of individual compounds in the sample;

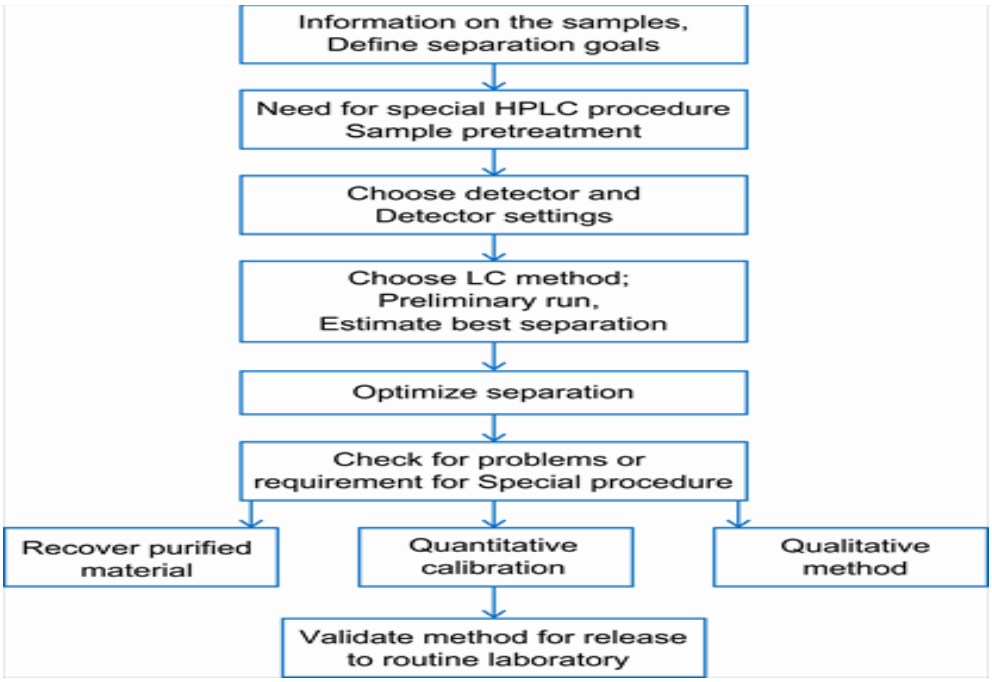
•the most common parameter for compound ID is its retention time (the time it takes for that specific compound to elute from the column after injection);

•depending on the detector used, compound ID is also based on the chemical structure, molecular weight or some other molecular parameter.

We have decided to take Gradient HPLC. This is only a requirement for complex samples with a large number of components or derivatives and traces of components because the maximum number of peaks that can be resolved with a given resolution is much higher than in isocratic HPLC. This is a result of the constant peak width that is observed in gradient HPLC (in isocratic HPLC peak width increases in proportion to retention time).

Column dimension. For most samples (unless they are very complex), short columns (10–15 cm) are recommended to reduce method development time. Such columns afford shorter retention and equilibration times. A flow rate of 1-1.5 mL/min should be used initially. Packing particle size should be 3 or 5 µm.

HPLC instrumentation: The HPLC systems used for the validation studies consisted of Series 200 UV/Visible Detector, Series 200 LC Pump, Series 200 Auto sampler and Series 200 Peltier LC Column Oven (all Perkin Elmer, Boston, Massachusetts, USA). The data were acquired via Total Chrom Workstation (Version 6.2.0) data acquisition software (Perkin Elmer), using Nelson Series 600 LINK interfaces (Perkin Elmer).



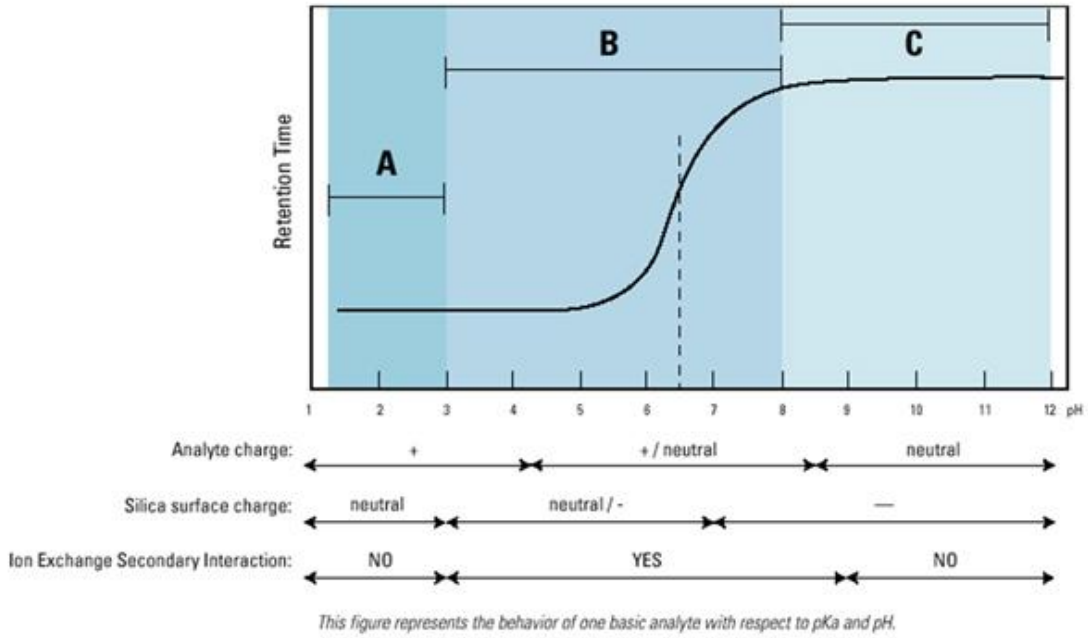
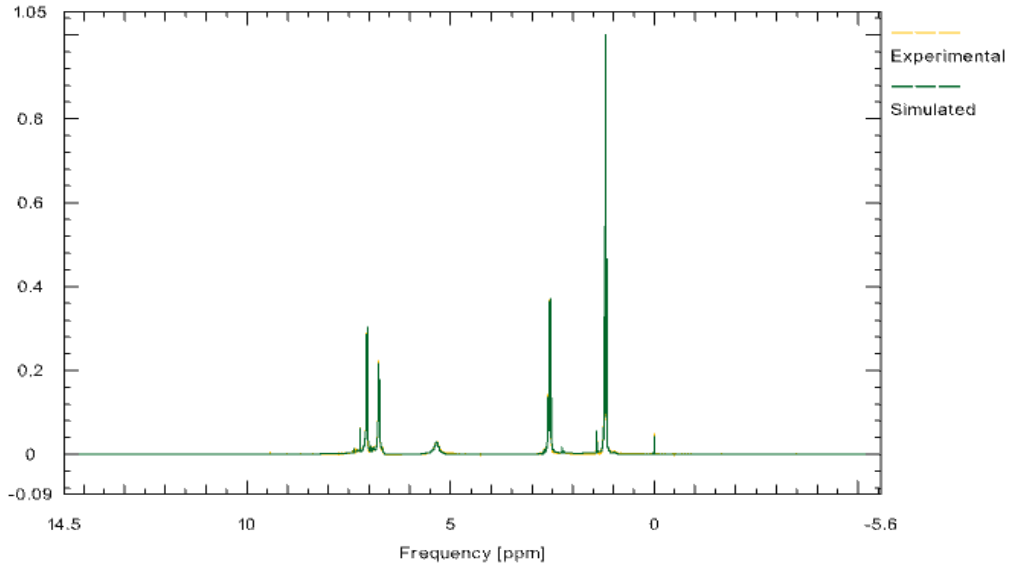
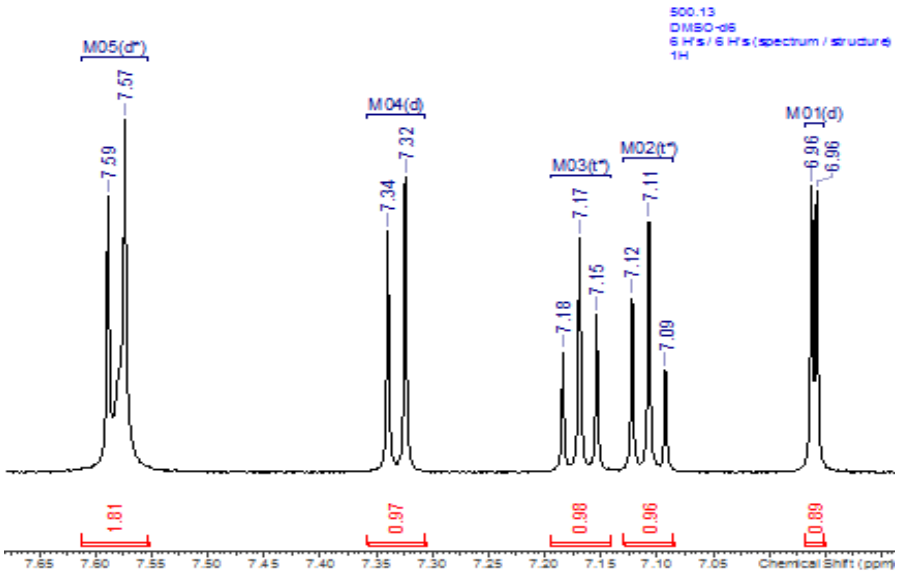


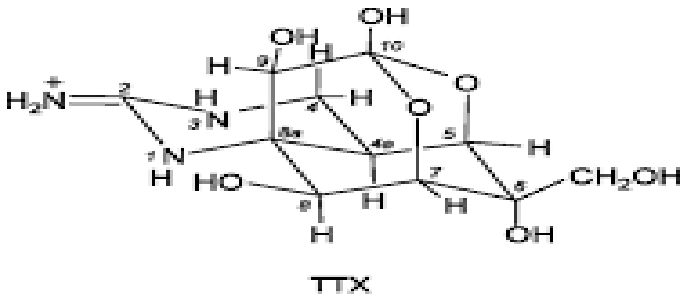
Fig. *Behavior of one basic analyte with respect to pKa*

Nuclear Magnetic Resonance (NMR): Nuclear magnetic resonance spectroscopy is a vital analysis technique. Its principle is based on the spins of atomic nuclei. The spin of protons and neutrons have intrinsic angular momenta hence act as elementary magnet. Such nuclei placed in a magnetic field shows the phenomenon of space quantization and for each allowed direction there is a slightly different energy level.

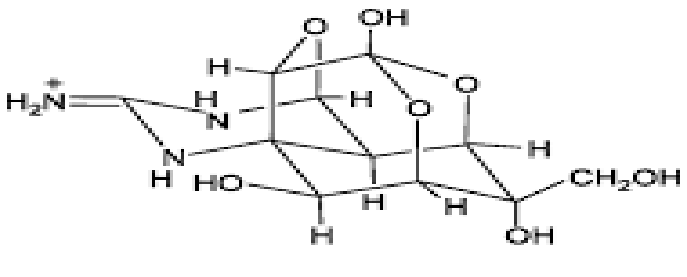


Fig





TTX



4,9-anhydro-TTX

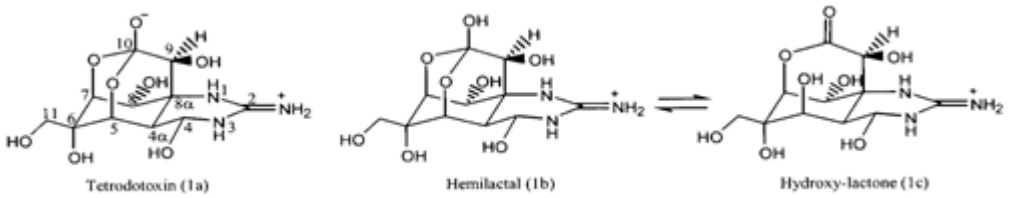


Fig1. *Tetrodotoxin (1a) and its tautomers in dilute acid solution*

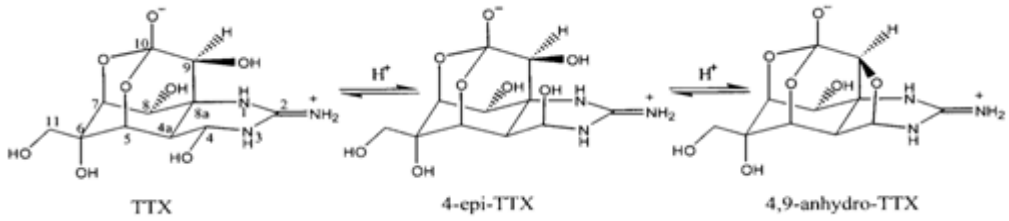


Fig2. *Inter-conversion between TTX and 4. 9-anhydro-TTX*

Mass Spectrometry: Proton transfer reaction mass spectrometry (PTR-MS) provides on-line monitoring of volatile or gaseous organic compounds with a low detection threshold and a fast response time. This is resorted as the compound may contain some volatile traces as evident from initial response. Exposing the sample to heat resulted in vapours which could be trapped in a low vaccum chamber to ascertain the proton emission frequency.

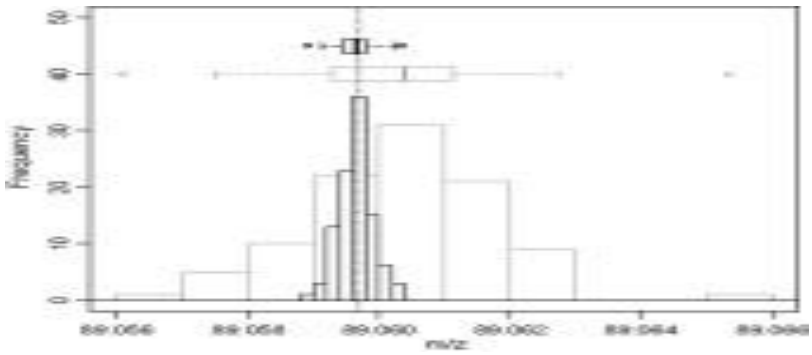


Fig. *Proton transfer reaction mass spectrometry (PTR-MS)*

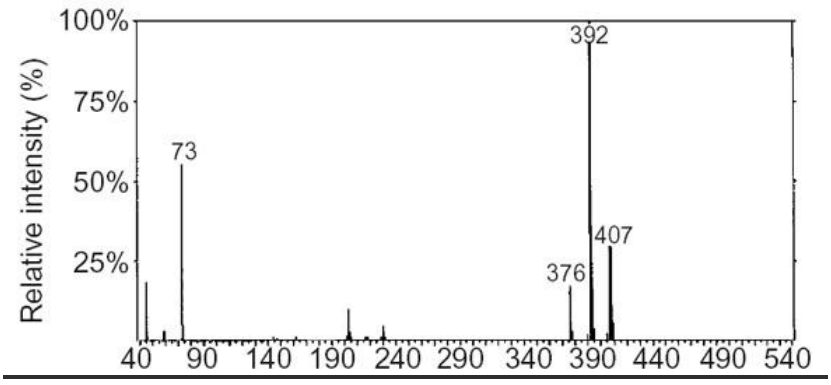


Fig. *Mass spectroscopy*

3. Observation and Result

3.1. Biochemical Analysis of Bacterial Strain A

Biochemical test such as Gram's staining, lactose fermentation test (fig b.i), Peroxidase test, Indole test(fig b.ii), citrate test(fig c.iii) & MR-VP test inference are given the following table-

Table1. *Biochemical test*

Sl. No.	Test	Strain A
1	Gram's Staining	Gram negative bacilli
2	Lactose fermentation	Lactose fermenting
3	Peroxidase test	Positive
4	Oxidase test	Negative
5	Indole test	Positive
6	Citrate test	Positive
7	MR-VP test	------

3.2. Identification of Novel Bacterial Strain by Sanger's Sequencing Method

The work was conducted for the identification of novel tetrodotoxin producing bacterial strain from freshwater *Tetrodon cutcutia*. The most promising isolates were sent for 16S rRNA sequencing. The potential isolate was identified as novel Aeromonas sp. DKDC1 (NCBI GenBank accession no. KT304815).

3.3. Extraction of Toxin

TLC of the bacterial strain A indicates the presence of TTX & Rf value was 58. (fig-c)

3.4. Mouse Bioassay

The treated mouse of 23grams weight behaves like TTX poisoning & deid after 35minutes.

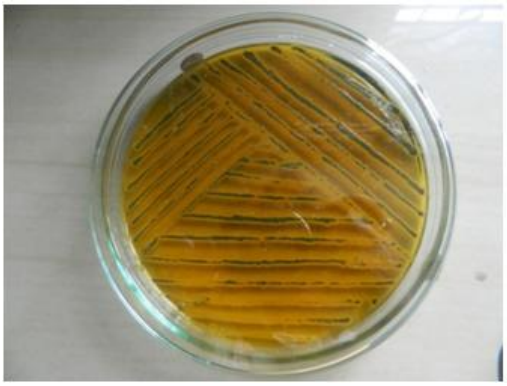
3.5. Instrumental Analysis

The frequency calibration showed little pin-up and further synchronisation yielded no so much quick or dense vibration mode. Small traces of TTX may be present, mostly derivatives with weak bonds. Slight concentration of acid is needed to break the structure and its impulses.

4. CONCLUSION

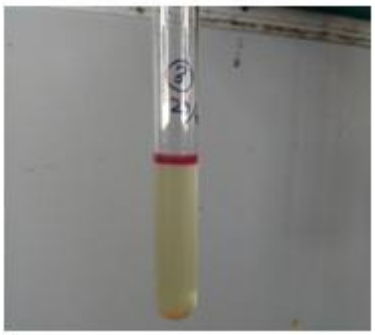
From the observations and results found in the experiments, it confirms that extraction from the bacterial strain A i.e Aeromonas sp. DKDC1 extract contains Tetrodotoxin as on addition of dilute acid most derivatives with weak bonds were formed. There are inter conversion from TTX $\Longleftrightarrow$ 4Epi-TTX $\Longleftrightarrow$ 4,9-Anhydro-TTX. During transformations various intermediate forms are formed and ultimate compound formed is TTX. The symptoms expressed during mouse bioassay indicate the behavior shown when treated with TTX.

5. PHOTO GALLERY

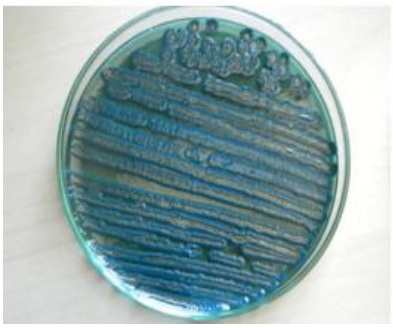


Fig(a). *Bacterial strains in TCBS media*

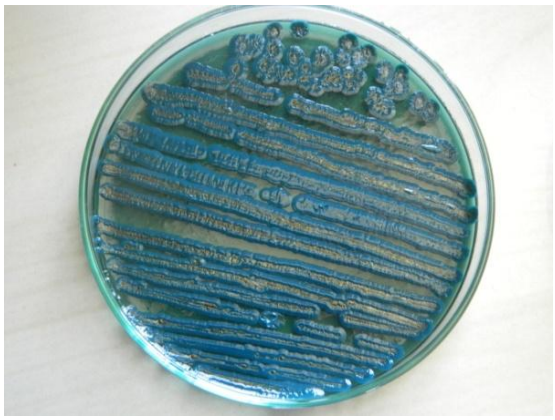
(i) *Lactose Fermentation*



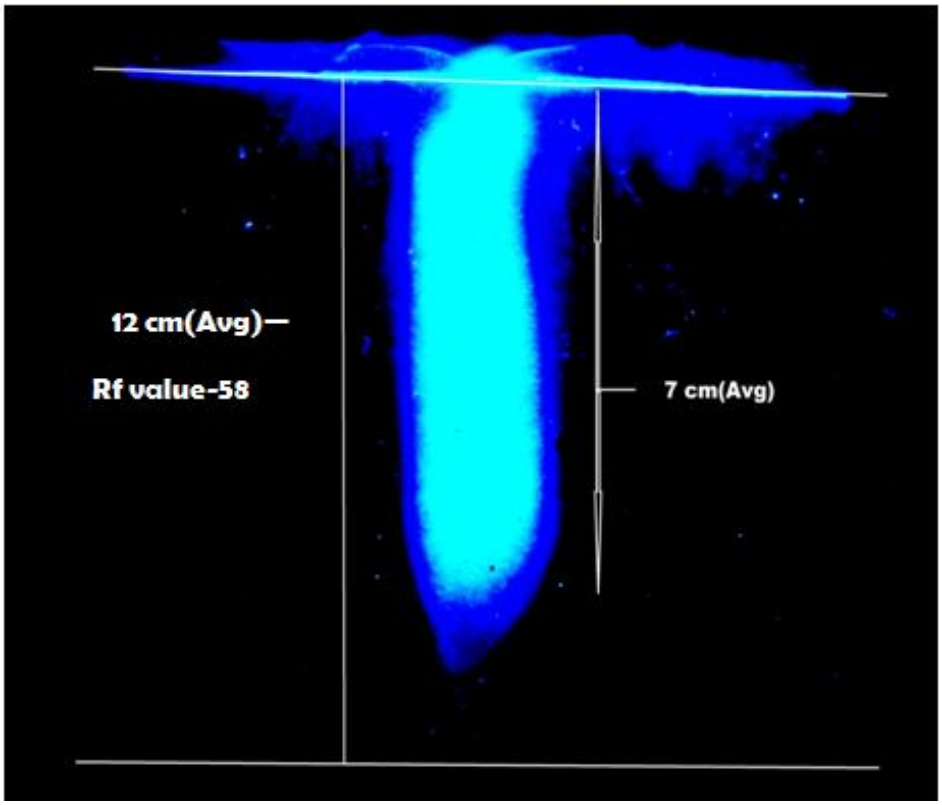
(ii) *Indole test*



(iii) *Citrate test*



Fig(b). *Biochemical test Images*



Fig(c). *TLC of TTX extract from Tetrodon*

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