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Research Article

Isolation of Metal scavenging Microorganism in an Industrial Backdrop

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ABSTRACT

The metal electroplating and finishing industries claim a major proportion of heavy metal pollution of the water bodies. In such an environment few microbes who are able to utilize or scavenge those heavy metals, manage to survive. Effluents from a Metal plating industry located in Howrah were collected for the present investigation that had significant concentrations of Zinc (4.76 mg/ml) and Iron (2.56 mg/ml). The bacterium was further identified to be *Shewanella xiamenensis* by 16S rDNA sequencing technique. *Shewanella sp.* is a Gram negative motile marine organism. The microbe was then subjected to a series of different concentrations of metal (in ppm) and this revealed that the growth was maximum at 150 ppm (0.67), minimum at 500 ppm (0.07),no growth beyond 500 ppm of Iron and it was 0.41 in the control setup. This was followed up with an experiment to analyze the potential of the organism to remove iron salt from the aqueous solution and this showed *Shewanella xiamenensis* exhibited 97.09% (725.2623 mg) iron removal in the 150 ppm setup and around 89.71% (1787.0232 mg) of the available iron in the 400 ppm setup. Thus the overall results clearly indicate that the bacterium *Shewanella xiamenensis*scavenges heavy metal after achieving osmotic tolerance in the sweet water environment, which being a marine bacteria perhaps entered the Ganges through the reverse current created due to unequal heating of the Earth's surface.

Keywords: *Shewanella xiamenensis,* 16S rRNA sequencing, Iron removal.

INTRODUCTION

Human beings have utilized the resources of nature and in turn inflicted damage to it and this has been its constant reputation since the age of the caveman. Industries exploit the resources of nature for energy and other essential steps in the manufacture process and in turn return the favor by polluting it. The industries in the coastal areas contribute to the discharge of their effluents containing heavy metals and chemicals into the coastal bodies. The living organisms in a food chain get affected by the toxic substances released in the environment (Dembitsky et al, 2003) and this mainly occurs through bioaccumulation and bio-magnification (Manohar et al, 2006).In the scenario attenuating heavy metal pollution several Sulfate reducing bacteria (SRB) are employed generating sulfide in the process (Alexandria et al, 2014).The current investigation involves the collection of the effluent of a factory in the Salkia, Howrah region that manufactures metal plates (mainly Iron plates with the provision of galvanization). The Metal plate Industry is located very close to Badaghat in Salkia. This location thus ensures quick and easy disposal of the effluents into the Hooghly, hence the sample was collected from the effluent drain near the Badaghat region. The Industrial wastewater naturally contains high level heavy metals which are potential areas for organisms with the ability to remove heavy metals from the environment. Several experiments have established that previously. In the Damodar River a thermophilic bacteria *Geobacillus thermodenitrificans* have showed considerable biosorption ability towards heavy metals reducing the concentration of Fe (+3) by almost 91.31% at optimum pH (Chatterjee et al, 2010). Certain species of *Cyanobacteria Synvhocystis* sp. EP35 in the Kucukcekmece Lake, Turkey have

been reported as a potent adsorber of Fe (iron) on its cell surface as well as intracellular accumulation (Demrel et al, 2009). Another investigation brought forward the ability of a spore-forming bacteria *Lysinibacillus sphaericus* as a potent bioremediation agent. It was found to be tolerant towards heavy metals at concentrations above the permissible limit for industrial waste water. The objective was to isolate such organisms. The effluent waters are expected to maintain certain high levels of different metals and consequently it was assumed that such conditions would usually favor and select organisms with the ability to tolerate the metal concentrations and few of those who might manage to utilize it to its benefit thereby biologically remove the metals from the environment. Thus the objectives were:

To isolate an organism that could tolerate considerable levels of metal concentrations (in ppm)

To determine the tolerance limit of such an organism.

To estimate the metal scavenging potential of the organism.

MATERIALS AND METHODS

1) Sample collection:
   The water sample used in this investigation was collected from the effluents of a Metal plate manufacturing (mainly iron plates and some are also galvanized) industry in Salkia, Howrah.
2) Physical characterization of the sample:
   Around 50 ml water sample was taken in a beaker; the electrical conductivity meter (rinsed thrice with de-ionised water) was used to measure the electrical conductivity and the pH meter to measure the pH of the sample. The concentration of the metals (iron and zinc) in the sample water was calculated after acidulation of the same followed by Atomic Absorption Spectroscopy.
3) Isolation and staining of micro-organism:
   The objective was to isolate and then identify certain metal tolerant microbes in the sample. Thereby in order to properly characterize and identify the different kinds of organism present n the given sample, around 100 µl of the water sample was spread on nutrient agar media under aseptic conditions. The plate was then incubated at 30˚C for 48 hours. Pure colonies were isolated plated and then Gram stained.
4) Identification of micro-organisms:
   Bacterial 16S rDNA sequences are attractive targets for developing identification methods because they represent conserved regions in all bacteria and species having 70% or greater DNA similarity usually have more than 97% sequence identity (Stackebrandt and Goebel, 1994).Bacterial identification based on % similarity of 16S rDNA has been using PCR technique, DNA sequencing and similarity analysis of rRNA genes. A direct comparison of 16S rDNA sequence is probably the most powerful tool for the identification of many bacteria (Stackebrandt and Goodfellow, 1991). 16S rDNA was amplified and sequenced using oligonucleotide primers complementary to highly conserved regions of bacterial rRNA gene. For identification, DNA was isolated from the slant culture of Sample B. Its quality was evaluated on 1.2% Agarose Gel. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. The PCR amplicon was purified to remove contaminants.Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and consensus sequence was generated by Aligner software.The 16S rDNA gene sequence was used to carry out BLAST alignment search tool ofNCBI gene bank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.
5) Biochemical Tests:
   The isolated organism was used to perform the following biochemical tests-
   Test for Indole production; Methyl Red Test; Voges-Proskauer Test; Citrate utilization Test; Urease Test; Catalase Test; Oxidase Test.
6) Metal Tolerance:
   The water sample was collected from the effluents of a metal plating industry in Salkia, Howrah. The aim was to determine the tolerance of the isolated organism towards the heavy metals as they are accustomed to an environment containing considerable levels of both Zinc and Iron. In order to do so, pure culture of the organism was inoculated in 15 ml Nutrient broth containing increasing concentrations of Zinc and Iron which was measured in terms of parts per million (ppm) . The culture was then incubated at 37˚C for 48 hours and the absorbance was measured at 600 nm.
7) Metal Scavenging:
   The determination of the metal removal capacity of the organism was the central objective of the investigation. In this regard, equal amounts of the pure culture were inoculated in equal quantities of Nutrient broth in separate setups, each differing in their parts per million-concentration value of the metals. The setups for metal removal were incubated at 37˚C and after 48 hours distinct

precipitate was observed in each of them. The precipitate was filtered to get the amount of deposit. Consequently, the filter paper was subjected to suitable treatments prior to the estimation of the amount of residual heavy metals for the set ups containing 150 ppm and 400 ppm of iron.

Determination of Iron in Filtered Sediment

a) The sample was weighed and taken in a glass beaker.
b) 10 ml Nitric acid and 2.0 ml Hydrochloric acid was added
c) Heated on a Hot plate for 45 minutes
d) Cooled and 2.0 ml HNO3, 2.0 ml H2O2 was added.
e) It was heated another 30 minutes.
f) Cooled and filteredusing Whatman 42.
g) Volume was made up to 50 ml by distilled water.
h) This is the prepared sample for ICP.

Instrument information

Name: ICP (OES), Model No: ICAP 6300 duo.

Instrument Condition:

RF Power: 1200 watts.
Flash pump rate: 100 rpm.
Analysis pump rate:50 rpm.
Argon pressure: 95 psi.
Auxiliary argon flow rate: 500 ml/min.
Sample uptake rate: 1.5 ml/min

RESULTS

1) Physical characteristics of the sample:
The electrical conductivity of the sample water was found to be 222.8 µS. The total dissolved solvent and pH of the sample were 214.6 ppm (parts per million) and 8 respectively. The Iron and Zinc concentration in the sample estimated through Atomic Absorption (AAS) was found to be 2.56 mg/ml (Fe) and 4.76 mg/ml (Zn). (Table 1)
2) Isolation of the microorganism and staining:
Single colonies from the Nutrient agar were plated to obtain pure culture. The pure colony was gram stained the microscopic features were observed and it showed that the bacteria isolated was Gram Negative rods. (Table 2 and Figure 1)
3) Identification of the isolated organism by 16S rDNA technique
The BLAST report of query consensus sequence (1439 bp) of Sample B showed that nucleotides sequences were similar to marine bacterium *Shewanella xiamenensis*. (Table 3, 4 and Figure 2, 3)
4) Biochemical Tests:
Organism isolated was found to be:
Indole negative, negative for Methyl red test; positive for Voges-Proskauer test; Citrate positive; Urease negative. (Table 6)
5) Metal tolerance:
The metal tolerance experimental set-ups revealed that the organism isolated (*Shewanella xiamenensis*) was tolerant till 3 ppm of zinc and around 400 ppm of Iron. The absorbance of the culture tubes after 48 hours of incubation were measured at 600 nm. This revealed that the organism showed maximum growth rate at the 150 ppm concentration of iron. Moreover the growth rate at 150 ppm concentration of Iron was greater than the control setup. (Table 7)
6) Metal scavenging:
The central objective of the experiment was to determine the potential of the organism in metal removal. Distinct brown precipitate was observed in the experimental setups after 48 hours of incubation in varying amounts. The amount of this precipitate (for 150 ppm and 400 ppm) was determined by filtering the broth through a Whatman filter paper and then measuring its dry weight. Following this, the filter papers (150 ppm and 400 ppm) were appropriately treated and the residual metal content in the filter estimated. It was observed that around 97.09%% of the iron was utilized in the 150 ppm and around 89.71% in case of the 400 ppm set up.(Table 8)

DISCUSSION

Global development is often seen to cause new challenges especially in the field of environmental pollution and conservation (Bennett at al. 2003). The presence of heavy metals in the water and soil thus became a matter of huge concern due to its potential harmful effects on all living organisms including humans. Fishes are sources of methyl mercury exposure whereas the risk of Lead exposure is both in food and air (lead emission from petroleum driven automobiles) (Jarup et al, 2003).The high affinity of mercury towards sulfur results in its toxicity, causing accumulation in parenchymal organs, metallic mercury lipophilicity as well as long biological half-life period (Kiedrowski et al, 2014).

Thus health effects of such exposures are widespread for example cadmium has been established as a Carcinogen on the basis of its ability to cause cancer both in humans and experimental animals (IARC). The urge of maximization of profits has caused Industries to consistently neglect the efforts towards Damage control. Heavy metals from the industrial and urban discharges are therefore one of the common pollutant of the aquatic bodies (Ramos et al, 1999). Few cases have been reported which have shown higher levels of heavy metals in the soil

surrounding the effluent basin compared to the background soil (Gupta et al, 2007). Moreover the use of effluent water in irrigation is a common practice in India (Fazeli et al 1998).In the current investigation, the water sample was collected from the effluent drain of a Metal plates manufacturing industry in Salkia, Howrah. Iron is a common industrial pollutant. The chemical properties of iron have been seen to be exploited by organisms in a variety of biochemical reactions, including respiratory and photosynthetic electron transport and others. Certain small molecules (Siderophores) have been reported which have shown high complex formation with Fe (III) (Raymond et al 1984). Several heterotrophic bacteria from marine habitat have been shown to produce siderophores (Trick et al 1989). Heterotrophic marine bacteria account has been shown to account for almost 50% of the carbon biomass (Fuhrman et al 1989) as well as the equivalent amount of the biogenic iron (Tortell et al 1996). Exhaustive research on iron metabolism has proven the existence of several mechanisms to acquire chelated iron in certain terrestrial pathogenic strains of bacteria. (Byers and Arceneaux 1998). A well characterized hydroxamate siderophore (aerobactin) is reported to be produced by planktonic Vibrio sp. from the coast of West Africa (Haygood et al, 1993) which are further utilized by the Escherichia coli (Winkleman 1990). The feasibility of a safe and healthy future on earth lies within the scope of decontaminating soils, sediments or water. In this regard the bacteria resistant to toxic metals are form an attractive biomass (Francois F. et al. 2012). Therefore bioremediation of contaminated water and soil is an emerging process. Several bacteria isolated from toxic metal contaminated soil and effluents established seven bacteria species which were tolerant to Hg and the presence of a mucoid phenotype (indicative of the products of extracellular polymeric substances) further shows that they are effective biosorption agents (Francois F. et al. 2012). In a certain contaminated soil in Korea a metal resistant bacterium Bacillus sp. strain CP134 (showing high removal potential in the order of preference Pb>Cd>Cu>Ni>Co>Mn>Cr>Zn) was reported and the heavy metals (Cheong et al, 2007) were mainly adsorbed on the cell wall and membrane (Cheory at al, 2007). In the present investigation, the objective was to isolate such metal resistant organisms and for this purpose the sample chosen was an Industry (Metal plate manufacturing Industry) effluent as it is theideal environment with the maximum probability to come across the desired metal resistant species. The organism isolated and cultured was identified using 16S rDNA technique and it was found to be *Shewanella xiamenensis*. *Shewanella sp.* is a motile marine bacterium and hence it is extremely unique as it was found in inland water body. This further confirms the effects of global warming and the unequal heating and cooling of the earth which often results in reverse currents (like El Nino) which are responsible for carrying the bacterium from its marine habitat to Inland water of Hooghly. This organism has shown metal tolerance till 500 ppm of Iron. The growth was observed to be maximum at 150 ppm Iron concentration setup (indicated by the Absorbance of the culture measured at 600nm) and an effective 97.09% of Iron removal in the 150 ppm Iron concentration in Nutrient broth. Thus all these results imply that the isolated organism *Shewanella xiamenensis* is a potent metal tolerant bacterium found in the industrial discharge water. Moreover it has also proven to be an efficient bioremediator with an iron removal efficiency of 97.09% from aqueous solutions. It was also found that in an iron dominated environment that was created invitro (mimicking the discharge water environment by creating set ups with increasing ppm concentrations of iron), the organism grew at a much faster rate as well as to a larger extent compared to a control environment (without the presence of Iron). This further validated its commitment in surviving in an iron enriched environment and also utilizing the iron content in the water enabling greater proliferation and consequently greater removal.

CONCLUSION

This particular investigation revealed the presence of *Shewanella xiamenensis* in the waters of the Hooghly river. The metal tolerance and the metal removal experimental setup results conclusively suggest that not only has *Shewanella xiamenensis* inhabited the inland waters but over and above that it has adapted itself to the predominant heavy metal environment and consequently adapted itself to make the best out of it. This unique ability of a marine bacterium in polluted waters is of great potential in the field of remediation technologies like effluent water treatment, where it might find extensive application to reduce the damage inflicted on the quality of inland water sources by the industrial community.

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Table 1
Physical characters

PARAMETERS	CHARACTERS
Colour	Brownish Yellow
Odour	Odourless
Electrical conductivity	222.8 µS
Total dissolved solvent	214. ppm
pH	8
Metal content (by AAS)	Fe=2.56 mg/ml; Zn=4.76 mg/ml.

Table 2
Colony characters

Colony Parameters	Characters
Colour	Pale white
Border	Regular
Elevation	Slightly raised

Table 3
CONSENSUS SEQUENCE

ACATGCAAGTCGTGCGGCAGCACAAGGGAGTTTACTCCTGAGGTGGCGAGCGGCGGACGGGTGAG
TAATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATAC
GCCCTACGGGGGAAAGAGGGGGACCTTCGGGCCTCTCGCGATTGGATGAACCTAGGTGGGATTAG
CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
GGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAG
TAGGGAGGAAAGGGTGAGTCTTAATACGGCTCATCTGTGACGTTACCTACAGAAGAAGGACCGGC
TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTA
AAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCTGGGCTCAACCTAGGAATAGCATT
TCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCATGCACGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTCGGAG
TTTGGTGTCTTGAACACTGGGCTCTCAAGCTAACGCATTAAGTAGACCGCCTGGGGAGTACGGCC
GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAAGACTGCAGAGATGCGGTTGTGC
CTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCCTATCCTTATTTGCCAGCACGTAATGGTGGGAACTCTAGGGAG
ACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGG
GCTACACACGTGCTACAATGGCGAGTACAGAGGGTTGCAAAGCCGCGAGGTGGAGCTAATCTCAC
AAAGCTCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAAT
CGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG
GAGTGGGCTGCAAAAGAAGTGGGTAGCTTAACCTTC

Table 4
Sequence producing significant alignments (source:http://blast.ncbi.nlm.nih.gov/)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ418493.1	Shewanella xiamenensis strain H3	2566	2566	100%	0.0	99%
HQ418492.1	Shewanella xiamenensis strain H2	2558	2558	100%	0.0	99%
JQ795827.1	Shewanella putrefaciens strain M-T-TSA_26	2549	2549	100%	0.0	99%
NR_116732.1	Shewanella xiamenensis strain S4	2549	2549	100%	0.0	99%
DQ307731.1	Shewanella putrefaciens strain Hac411	2549	2549	100%	0.0	99%
JN555612.1	Shewanella putrefaciens strain SYY-1	2547	2547	100%	0.0	99%
JX119023.1	Shewanella xiamenensis strain BC01	2538	2538	100%	0.0	99%
JQ795830.1	Shewanella putrefaciens strain M-T-TSA	2538	2538	100%	0.0	99%
DQ307730.1	Shewanella putrefaciens strain Hac318	2538	2538	100%	0.0	99%
KJ542802.1	Shewanella xiamenensis strain A2	2534	2534	100%	0.0	99%
KC607521.1	Shewanella putrefaciens strain Pt400	2499	2499	98%	0.0	99%
KC607508.1	Shewanella putrefaciens strain K696	2495	2495	98%	0.0	99%
KC607506.1	Shewanella putrefaciens strain St15	2494	2494	100%	0.0	99%
NR_074817.1	Shewanella putrefaciens CN-32 strain CN-32	2494	2494	100%	0.0	99%
JQ795825.1	Shewanella putrefaciens strain M-T-TSA_10	2494	2494	100%	0.0	99%

Table 5
Distance matrix

SAMPLE	1		0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
HQ418493.1	2	0.001		0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
HQ418492.1	3	0.002	0.002		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
JQ795827.1	4	0.005	0.005	0.006		0.002	0.002	0.002	0.003	0.001	0.003	0.002
NR_116732.1	5	0.005	0.005	0.004	0.009		0.000	0.001	0.001	0.002	0.001	0.001
DQ307731.1	6	0.005	0.005	0.004	0.009	0.000		0.001	0.001	0.002	0.001	0.001
JN555612.1	7	0.005	0.005	0.004	0.009	0.003	0.003		0.001	0.002	0.001	0.002
JX119023.1	8	0.007	0.007	0.005	0.010	0.001	0.001	0.003		0.003	0.000	0.002
JQ795830.1	9	0.006	0.004	0.007	0.001	0.010	0.010	0.010	0.011		0.003	0.002
DQ307730.1	10	0.007	0.007	0.005	0.010	0.001	0.001	0.003	0.000	0.011		0.002
KJ542802.1	11	0.005	0.005	0.004	0.009	0.003	0.003	0.004	0.004	0.010	0.004	

Table 6
Biochemical Tests

Test	Result
Indole	Negative
Methyl-Red	Negative
Voges-Proskauer	Positive
Citrate	Positive
Urease	Negative
Catalase	Positive
Oxidase	Negative

Table 7
METAL TOLERANCE
A.IRON TOLERANCE

Concentration (in ppm)	Absorbance at 600 nm
10	0.22
25	0.25
50	0.35
100	0.40
150	0.67
200	0.46
250	0.40
300	0.31
350	0.25
400	0.28
450	0.12
500	0.07
550	0.00

B. ZINC TOLERANCE

Concentration (in ppm)	Absorbance at 600 nm
5	0.21
10	0.13
15	0.11
20	0.02

Table 8
IRON REMOVAL FROM AQUEOUS SOLUTION BY THE ORGANISM *Shewanella xiamenensis*

Concentration (in ppm)	Amount of precipitate	Residual Iron	Amount of metal removal	Percentage of metal removal
150	155.7141	13.96% of 155.7141 = 21.7377 mg	150 ppm = 747 mg (747 – 21.7377) mg = 725.2623 mg	97.09
400	601.810	34.06% of 601.810 = 258.96 mg	400 ppm = 1992 mg (1992 – 258.96) mg = 1787.0232 mg	89.71

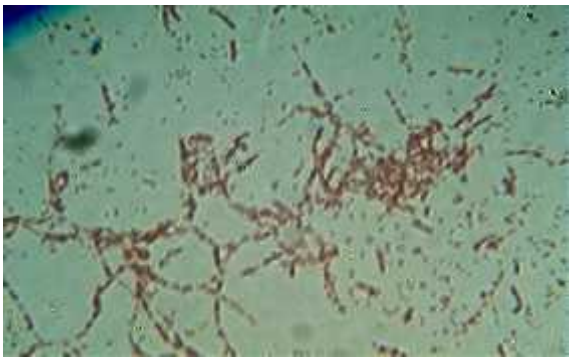

Fig 1
Microscopic view of the sample

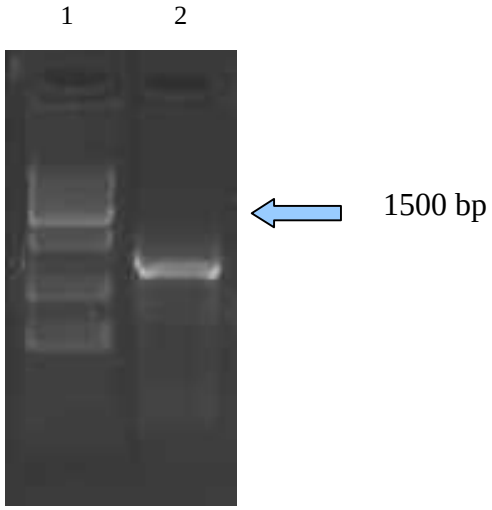

Fig 2
Gel Image Of 16s rDNA Amplicon.
1.2% Agarose gel showing single 1.5 kb and 16S rDNA amplicon

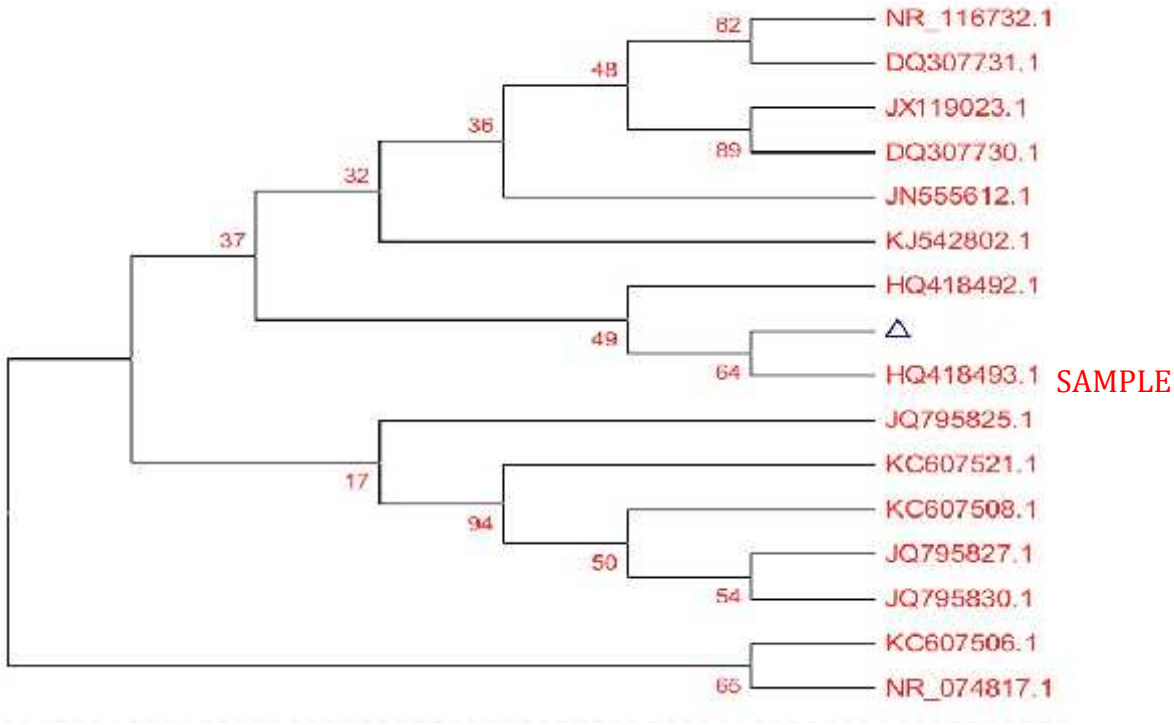

Fig 3
Phylogenetic tree

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