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In Vitro and In Silico Analysis of the Gut Microbiome of Zebrafish for Bioremediation Approach of Zinc-Contaminated Aquatic Environments

Sugitha S[1], Abirami G[2*]

[1]Department of Biotechnology, School of Life Sciences, Research scholar, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai, India.

[2]Department of Biotechnology, School of Life Sciences, Assistant professor, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai, India.

***Corresponding author:** Abirami G, Department of Biotechnology, School of Life Sciences, Assistant professor, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai, India, Email: drabirami.cas@gmail.com

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ABSTRACT

The discovery of bacterial organisms that aid in heavy metal bioremediation opens up new possibilities for removing radioactive compounds and heavy metals from polluted water sources. Accordingly, this study aims to the identification of Zinc resistant bacteria from the gut microbiome of zebrafish and to evaluate its bioremediation capability. The isolates, namely FG01 (Enterobacter cloacae), FG02 (Citrobacter freundii), and FG03 (Aeromonas hydrophila) identified based on 16S rRNA gene sequencing resistant to Zinc and antibiotics like Ampicillin and Amoxicillin and also selected for bioaccumulation studies. The MTC values of the three zinc-resistant bacteria from the Zebrafish gut were evaluated, and the results revealed that the growth of the isolate FG02 was better than the others, while the growth of FGO3 and FG01 decreased at the concentrations of 10 ppm and 15 ppm. By docking studies, the Zinc binding proteins (ZBPs) were discovered and beta-lactamase showed the best binding affinity compared to the other protein. In the process of treating wastewater, zinc-resistant bacteria from the gut microbiome of zebrafish are normally present and can demonstrate their capacity to adsorb heavy metals. The ZBP can be used later as a novel absorbent for heavy metal removal technology.

Keywords: *Bioremediation, Bioaccumulation, Gut microbiome, zinc-resistant bacteria, Zinc binding protein (ZBP)*

INTRODUCTION

Water contamination is a huge environmental problem because water is so important and plays such a major role in the food chain [1]. The concentration of heavy metals in marine animals or humans that depend on such water bodies can cause severe health problems [2]. The main source of heavy metals is industrial effluents and sewage water.

Heavy metal concentration in humans can affect the liver, kidneys, and even cause cancer [3], [4].

Zinc is essential for life since it is involved in a variety of biological functions in all living things, including people, animals, and plants, such as protein synthesis, immunological function, and development.

But the industries use excess zinc and also other heavy metals for quality of production and these heavy metals finally contaminate the water bodies and pollute the surroundings [5]. Zinc is rapidly being identified as a water quality problem in Canada, Japan, the United Kingdom, China, and Taiwan [6], [7]. An assessment of zinc toxicity and vulnerability in the aquatic ecosystem was carried out using statistical data and equations for calculating zinc exposure to surface waters [8]. Due to high biologically available fractions of metals, site-specific risk assessment is especially needed for rivers heavily contaminated with metals, particularly rivers impacted by metal industries and mining [9], [10].

The zebrafish living in this heavy metal-contaminated water show resistance to some heavy metals like cadmium, zinc, and copper. MRP proteins can act as mediators of heavy metal resistance in Zebrafish cells. Increased expression of several multidrug resistance-associated proteins (MRP) is linked to heavy metal resistance in mammalian cells [11– [13]. After 6 months of selection and chronic cadmium toxicity, toxic heavy metal tolerance is shown by fibroblast-like ZF4 cells from zebrafish (Danio rerio). [14].

The work exhibits newness to the study of novel bacterial species present in the zebrafish gut microbiome that shows resistance to heavy metals like zinc and correlated antibiotics resistance [15], [16]. Identification of new isolates is becoming increasingly important because they contain novel genes and metabolites that help in bioremediation. It remains unclear whether the zebrafish gut bacterial strains are zinc-resistant bacteria. This study gives clear-cut ideas about the zebrafish gut microbiome and its resistance to zinc and antibiotics. In addition to the study, the metal-binding protein can be identified by molecular docking on the proteins of zinc-resistant microbes. Later, a unique adsorbent for heavy metal removal technology may be created using this metal-binding protein.

MATERIALS AND METHODS

Sample collection and isolation of bacterial strains

Zebrafish were collected from Padmanabha Labs/HiBreeds Aquatics, Chennai, and transferred to Medical Research Laboratory. Two individuals were selected according to their weight and height. The Zebrafish skin was washed with 70% ethanol and the bacterial strains were isolated from the Zebrafish gut by dissection method for analysis of zinc-resistant bacteria, this was assessed based on the protocol of C.I Ayo-Olalusi et al (2014) [15], [16].

FIGURE 1: zebrafish dissection

Primary screening of zinc-resistant bacteria

This assay was performed by the standard method of Kais Kassim Ghaima et al. (2017). To determine if the samples contain heavy metal-resistant bacteria, Zinc was added to nutrient agar plates at a concentration of 10 mg/L, and after 24-48 hours, it was discovered that the bacteria could thrive in its presence. The zinc concentration was

increased to 100 mg/L and incubated for 48 hours and only the strains able to grow on the plate were chosen as zinc-tolerant bacteria. Rapid developing heavy metal-resistant bacteria were selected from colonies with different morphological and growth potentials for further study [17], [18].

16s rRNA sequencing

For the purpose of identifying, classifying, and quantifying microorganisms in complicated biological mixtures such as ambient samples and gut samples, the chosen bacterial strains were produced to 16s rRNA sequencing. [19].

Determination of Maximum Tolerance Concentration (MTC) of zinc

Three isolates that showed growth at 100 mg/L concentration were selected for MTC determination of Zinc by broth dilution method. The three bacterial strains were inoculated to the nutrient broth in individual test tubes and one control was placed for each bacterial strain [20]. The zinc salt is added at a concentration of 5 ppm, 10 ppm, and 15 ppm for each bacterial strain except the controls. Optimal density (OD) is checked from the 0th Day of inoculation to the 3rd Day using UV spectrometry. And the values are noted for each day and the results were analyzed according to the protocol of Kais Kassim Ghaima et al. (2017) [17], [18].

Determination of antibiotic susceptibility

The following antibiotics were tested for well diffusion susceptibility method based on Chellaiah et al. (2009) [21], with their concentrations given in parenthesis: Ampicillin (200 mg/l), Amoxicillin (200 mg/l), Ciprofloxacin (200 mg/l), Erythromycin (200 mg/l), and Tetracycline (200 mg/l). The bacterial strain was transferred to Mueller-Hinton agar medium and spread using an L rod and allowed to sit for 10 minutes. Following that, the antibiotic wells were mounted on the agar with sterile forceps pressing tightly against the agar to ensure contact. And the antibiotics suspension (0.3 ml) was transferred to the well. The plates were then kept at 37°C for a further 24 hours [22], [23].

Molecular Docking

Preparation of protein structure

The Protein Data Bank is a resource for all three-dimensional structures of proteins. Using the Protein Data Bank, the 3D structure of the target proteins was obtained, and prepared by PyMOL (PyMOL is a powerful and feature-rich molecular visualization tool for rendering and animating 3D molecular structures) [24], [25].

TABLE 1: Target Proteins of resistant bacterial strains

Organism	Protein name	PDB ID	Exp. Method	Resolution (Å)
Enterobacter cloacae	Contact-dependent inhibitor A	4NTQ	X-ray diffraction	2.40 Å
	Putative cytoplasmic protein	4HFK	X-ray diffraction	2.10 Å
	Competence damage-inducible protein A	5VU3	X-ray diffraction	1.87 Å
	Formate C-acetyltransferase	6XS4	X-ray diffraction	2.33 Å
	sugar-binding protein	7V09	X-ray diffraction	2.00 Å
	Galactose-binding lectin	6YF6	X-ray diffraction	2.00 Å
	Beta-lactamase	1XX2	X-ray diffraction	1.88 Å
	DNA polymerase III subunit beta	6AMQ	X-ray diffraction	2.67 Å
	Metallo-beta-lactamase VIM-31	4FSB	X-ray diffraction	1.88 Å
Citrobacter freundii	Restriction endonuclease	1CFR	X-ray diffraction	2.15 Å
	AmpD protein	1J3G	X-ray diffraction	1.88 Å
	Dihydroxyacetone kinase	1UN9	X-ray diffraction	3.10 Å
	Beta-lactamase	1FR1	X-ray diffraction	2.00 Å

	1,6-anhydro-n-acetylmuramyl-l-alanine amidase AmpD	2Y2D	X-ray diffraction	2.00 Å
Aeromonas hydrophila	(R)-specific enoyl-coa hydratase	1IQ6	X-ray diffraction	1.50 Å
	Prolyl endopeptidase	3IUJ	X-ray diffraction	1.80 Å
	AhlC	6H2D	X-ray diffraction	2.62 Å
	AscE	3PH0	X-ray diffraction	2.40 Å
	Proaerolysin	1PRE	X-ray diffraction	2.80 Å
	Aerolysin	1Z52	X-ray diffraction	2.38 Å

Ligand structure retrieval

PubChem is a large database of freely available chemical compounds prepared specifically for virtual screening. The Zinc compound (Zinc diethyldithiocarbamate: It is used in the rubber industry for prosthetic sleeves and it causes dermatitis and ulceration PubChem CID-26633) is retrieved from the PubChem database [26].

Virtual screening tool

Argus Lab is an open-source software that provides a graphical user interface and a drug design program for Windows operating systems. The zinc compound was selected and docked against each target protein individually on ArgusLab (Windows 10) [27]. The downloaded files were visualized using PyMOL for polar contacts and interactions [28].

J. Phylogenetic analysis

MEGA is a collection of resources that allow researchers to work on phylogenomics and phylomedicine [29]. ClustalW uses progressive alignment methods for multi-sequence alignments. Comparative analysis of the Zinc binding protein (zinc resistant bacterial strains) using a Phylogenetic tree.

RESULT AND DISCUSSION

Screening and selection of zinc-tolerant bacterial strains

In the study of 20 bacterial strains from the zebrafish gut microbiome, only 3 strains were able to grow in the presence of zinc at a concentration of 100 mg/L (FG01, FGO2, and FG03) and were selected for the later experiment. Using common morphological, and physiological, the chosen bacterial strains were selected and described based on similarities to those listed in Bergey‘s Manual of Determinative Bacteriology [30], [31].

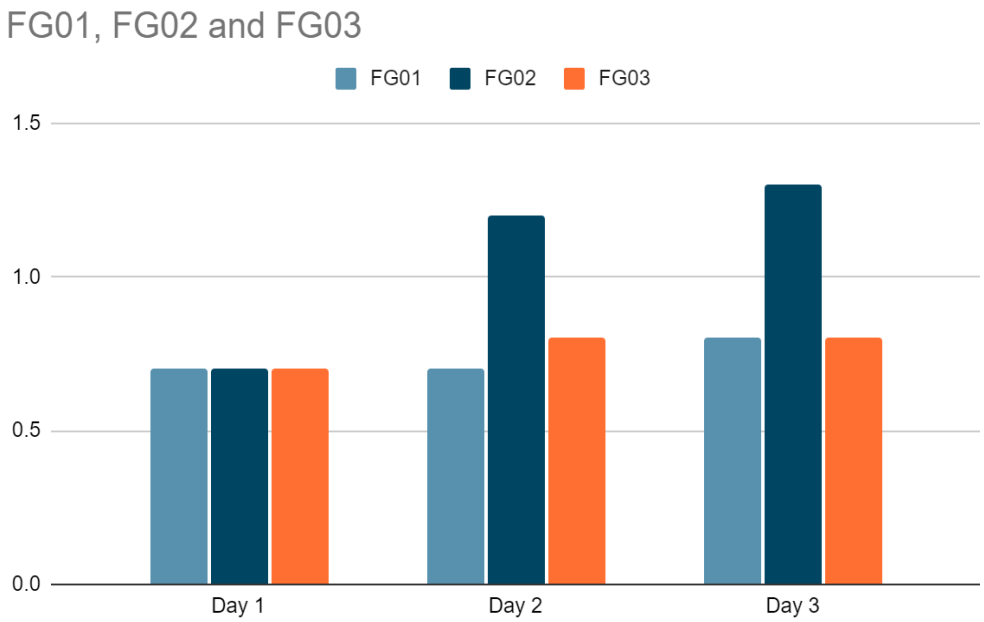
Maximum Tolerance Concentration (MTC) of zinc

To determine the MTC of zinc using the broth dilution process, three isolates were chosen that showed growth at 100 mg/L concentration. The cell density of the broth cultures was measured at 600 nm after incubation. Growth of the isolates decreased at high zinc concentrations compared to the lower concentration [32], [33]. By comparing the growth of the three isolates, the results revealed that the growth of FG02 was better than the others, and the recorded MTC was 15 ppm with an OD equal to 2.16, while the growth of the isolates FG03 and FG01 decreased at concentrations of 10 ppm and 15 ppm, respectively [34], [35].

TABLE 2: Maximum Tolerance Concentration (MTC) of Zinc resistant bacterial strains OD Value at 600 nm

Day	Bacterial Strain	Control	5 ppm	10 ppm	15 ppm
	FG01	0.67	0.66	0.72	0.70
Day 1	FG02	0.52	0.64	0.56	0.71
	FG03	0.75	0.74	0.73	0.75
	FG01	0.87	0.79	0.84	0.72

Day 2	FG02	1.27	1.57	1.24	1.35
	FG03	0.84	0.89	0.81	1.33
	FG01	1.79	1.21	0.81	0.85
Day 3	FG02	1.35	1.34	1.25	2.16
	FG03	1.87	0.95	0.94	1.28



GRAPH 1: Maximum Tolerance Concentration (MTC)

The optical density decreased as the zinc content in the medium increased, indicating that Zinc has a toxic impact on cell formation. A diverse range of microorganisms has evolved pathways to shield themselves from heavy metal exposure, including uptake, oxidation, adsorption, reduction, and methylation [36]. Some bacteria have also been confirmed to be able to use heavy metal tolerance and detoxification mechanisms by producing chelating agents that bind metals and reduce their toxicity. Zinc inhibitory effect on bacteria may be attributed to the metal's high affinity for binding organic matter, suggesting a reduction in their bioavailability [37].

Antibiotic susceptibility

Five antibiotics (1 ampicillin, 2 amoxicillin, 3 ciprofloxacin, 4 erythromycin, and 5 tetracycline) were used in this study, and the results showed that the three isolates were resistant to ampicillin and amoxicillin. And these isolates were sensitive to ciprofloxacin, erythromycin, and tetracycline [38], [39]. The synthesis of enzymes that can inactivate or alter specific antibiotics, as well as changes in the bacterial cell membrane, alteration of the target site, and the evolution of metabolic pathways by bacteria, could explain the tolerance of these strains. Bacterial resistance to antibiotics and zinc appears to be the result of exposure to zinc-contaminated conditions, which causes a coincidental selection of antibiotic and zinc resistance factors [35] .

FIGURE 2: FGO1 Antibiotic test

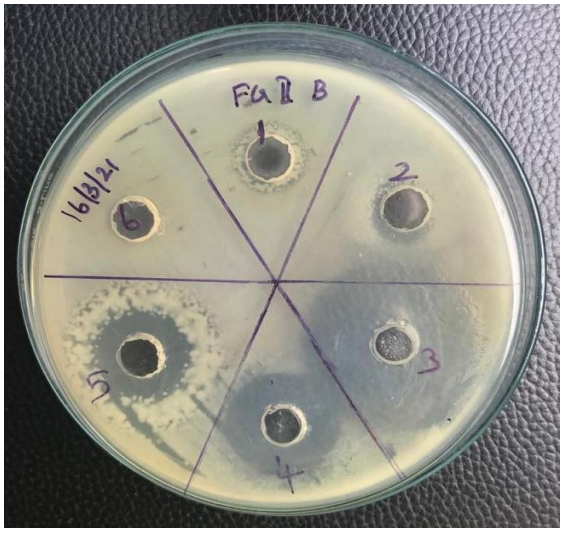


FIGURE 3: FGO2 Antibiotic test

FIGURE 4: FGO3 Antibiotic test

TABLE 3: Antibiotic-resistant patterns of Zinc resistant isolates

Antibiotics	**Fg01**	**Fg02**	**Fg03**
Ampicillin	R	R	R
Amoxicillin	R	R	R
Ciprofloxacin	S	S	S
Erythromycin	S	S	S
Tetracycline	S	S	S

R: Resistant, S: Sensitive

Zinc uptake capacity

The toxic effects of the pollutant cause cell damage when a microbe is added to a fresh culture of hazardous substances [37]. The bacterium in this instance uses energy to repair cell damage and conform its enzymatic route to a new environment. Around 85% of the maximal quantity of zinc was extracted by the strain FG02. This method causes bacteria to multiply quickly over time, which led to an increase in zinc bioaccumulation. The amount of zinc bioaccumulation in strain FG02 rose from the start of the experiment to the conclusion. There was a little decline in zinc elimination in the

medium after 32 hours. Some researchers theorize that growth would slow and the number of viable cells in the culture would decline when metal bioaccumulation peaked [37], [40]. Since it has a number of transition metal efflux mechanisms, the bacterium can live in metal-contaminated conditions [41]. However, two general efflux mechanisms used by a P-type ATPase efflux system and an RND-driven transporter system enable bacteria to resist zinc [42], [43]. Hou et al (2015).'S [44] experiments revealed that the biosorption process may be described as a chemical interaction between ions and chemical groups on the surface of biomass.

Analysis of sequencing results

The sequences of the three bacterial strains obtained from 16S rRNA sequencing were analyzed with the NCBI database (Nucleotide BLAST) that showed these strains were related to the members of the genus Enterobacter, Citrobacter, and Aeromonas. The highest sequence similarities of bacteria strains are as follows: FG01, Enterobacter cloacae (100% similarity to Accession number: MT074035.1), FG02, Citrobacter freundii (99% similarity to Accession number: MT258989.1), and FG03, Aeromonas hydrophila (99% similarity to Accession number: OP221548.1) [19].

Enterobacter cloacae

Scientific classification

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Enterobacterales

Family: enterobacteriaceae

Genus: *Enterobacter*

Species: *E. cloacae*

16s rRNA sequence:

>SR2154-FG01-RSR1_H04.ab1

TAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTC
TCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGAC
GTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATA
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCA
GAGTTCCCGAAGGCACCAAACCATCTCTGCTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT

FIGURE 5: Taxonomic classification of Enterobacter cloacae [45].

Citrobacter freundii

Scientific classification

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Citrobacter*

Species: *C. freundii*

16s rRNA Sequence:

>SR2154-FG02-RSR1_G04.ab1

ACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAG
GTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGG
CCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGACCGAACC
GCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGAC
AGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAGAG
TAGGTAAGGTTCTTCGCGTTGC

FIGURE 6: Taxonomic classification of Citrobacter freundii [46].

Aeromonas hydrophila

Scientific classification

Domain: Bacteria

Phylum: Pseudomonadota

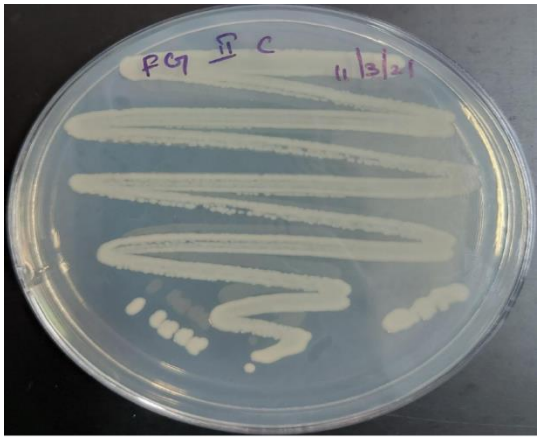
Class: Gammaproteobacteria

Order: Aeromonadales

Family: Aeromonadaceae

Genus: *Aeromonas*

Species: *A. hydrophila*



16s rRNA Sequence:

>SR2154-FG03-RSR1_A06.ab1

GAGGATTCGCTCACTATCGCTAGCTTGCAGCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCA
TGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTTGAGTTCCCACCATTACGTGCTGGCAACA
AAGGACAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTG
TTCTGATTCCCGAAGGCACTCCCGTATCTCTACAGGATTCCAGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCAA
ATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTT

FIGURE 7: Taxonomic classification of Aeromonas hydrophila [47].

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Docking Result

TABLE 4: Docking result

Organism	Protein	Binding affinity (Zinc) Kcal/Mol
Enterobacter cloacae	Contact-dependent inhibitor A	-6.6
	Putative cytoplasmic protein	-7.9
	Competence damage-inducible protein A	-7.6
	Formate C-acetyltransferase	-8.9
	sugar-binding protein	-7.8
	Galactose-binding lectin	-8.5
	Beta-lactamase	-9.4
	DNA polymerase III subunit beta	-7.2
	Metallo-beta-lactamase VIM-31	-8.7
Citrobacter freundii	Restriction endonuclease	-6.8
	AmpD protein	-7.3
	Dihydroxyacetone kinase	-8.5
	Beta-lactamase	-9.1
	1,6-anhydro-n-acetylmuramyl-l-alanine amidase AmpD	-7.3
Aeromonas hydrophila	(R)-specific enoyl-coa hydratase	-8.8
	Prolyl endopeptidase	-7.7
	AhlC	7.5
	AscE	-7.4
	Proaerolysin	-7.8
	Aerolysin	-7.9

TABLE 5: Interacting Residues and H-Bond Distance

Organism	**Protein Name**	**Interacting residues**	**H- bond Distance (Å)**
Enterobacter cloacae	Formate C-acetyltransferase	GLY ARG HIS SER TYR ILE	3.1 2.7 2.7 2.1 2.8 3.2
	Beta-lactamase	MET SER VAL LEU ILE	2.8 2.6 3.2 3.8 2.3
Citrobacter freundii	Beta-lactamase	TYR SER ARG GLN	2.7 2.3 2.2 2.5

Aeromonas hydrophila	(R)-specific enoyl-coa hydratase	GLY HIS SER TYR ILE VAL	2.0 2.1 1.6 3.2 2.9 2.8

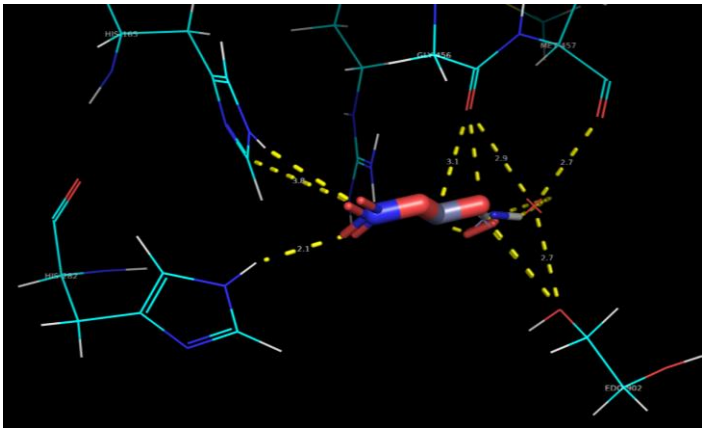

FIGURE 8: Formate C-acetyltransferase interaction with Zinc compound

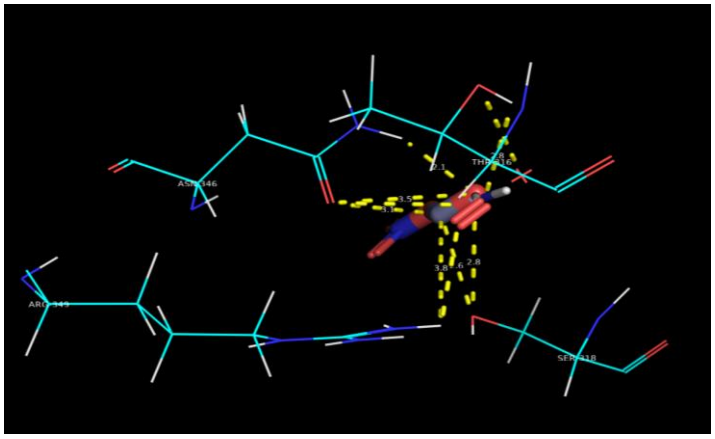

FIGURE 9: Beta-lactamase (Enterobacter cloacae) interaction with Zinc compound

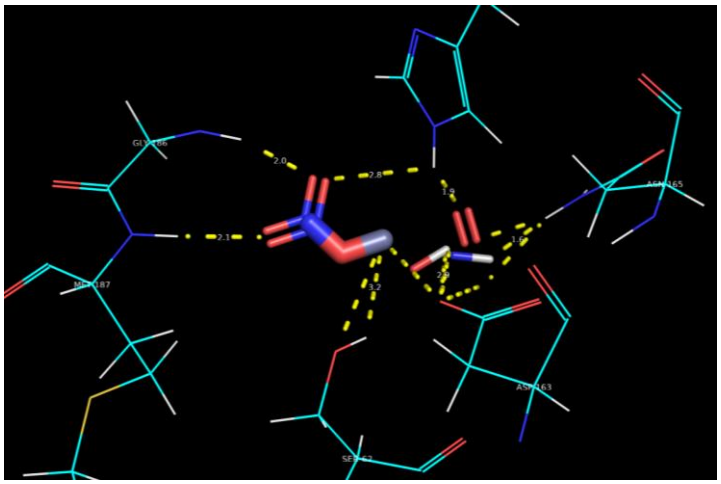

FIGURE 10: Beta-lactamase (Citrobacter freundii) interaction with Zinc compound

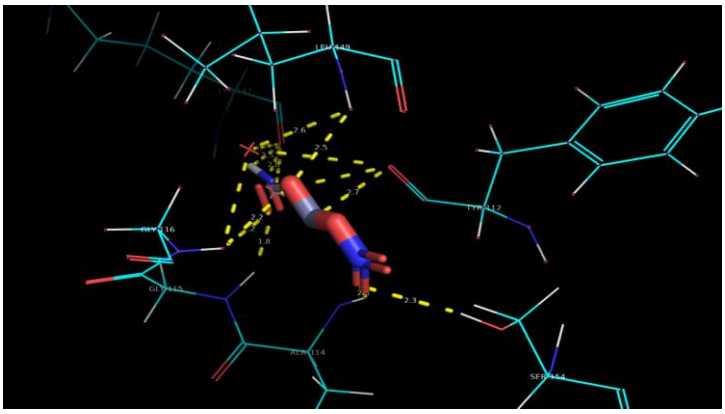

FIGURE 11: (R)-specific enoyl-coa hydratase interaction with a Zinc compound

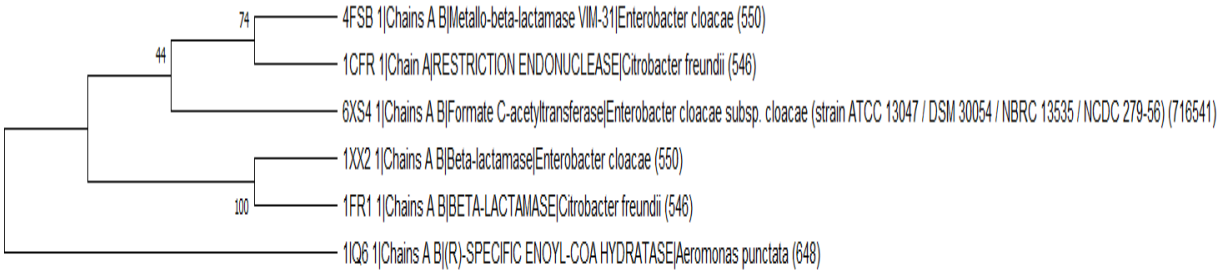

FIGURE 12: The proteins from the resistant bacteria that showed the best binding result with zinc are checked for phylogenetic analysis based on the neighbor-joining method [48].

By docking the proteins with a zinc compound using Argus Lab, the binding affinity is obtained. The proteins that show the least binding affinity are Formate C-acetyltransferase (-8.9), Beta-lactamase (-9.4, Enterobacter cloacae), Beta-lactamase (-9.1, Citrobacter freundii) and (R)-specific enoyl-CoA hydratase (-8.8). These protein interaction residues and H-bond distances are given in the above table and also checked for phylogenetic analysis based on the neighbor-joining method. The zinc compound with beta-lactamase (Enterobacter cloacae) shows the best binding affinity of all the above and has five hydrogen bond interactions where 265MET with a distance of 2.8 Å, 264SER with a distance of 2.6 Å, 291VAL with a distance of 3.2 Å, 296LEU with a distance of 3.8 Å, and 262ILE with a distance of 2.3 Å [49], [50]. Beta-lactamase of both bacteria (Enterobacter cloacae and Citrobacter freundii) shows the least binding affinity, and beta-lactam antibiotics are rendered inactive by this enzyme by hydrolyzing the peptide link of the distinctive four-membered beta-lactam ring. The beta-lactamase resistance towards antibiotics is clearly shown in the below given pathway from the (PATH: map01501) KEGG pathway database [51].

According to Worthington, R. J et.al (2013), a majority of beta-lactam antibiotic resistance results from one of two mechanisms, either the creation of beta-lactamases, the most frequent resistance mechanism in Gram-negative bacteria, and the formation of an altered PBP (Penicillin-binding proteins) with a decreased affinity for the majority of beta-lactam antibiotics [52], [53]. Antibiotic resistance is created for the bacterium by the antibiotic's inactivation, and this proves beta-lactamase is resistant to heavy metals like zinc and correlates with antibiotic resistance. Since ZBPs from zinc-restricted bacteria are typically identified and demonstrate their potential to absorb heavy metals in the wastewater treatment process, these ZBPs may be available as heavy metal adsorbents in water and wastewater treatments.

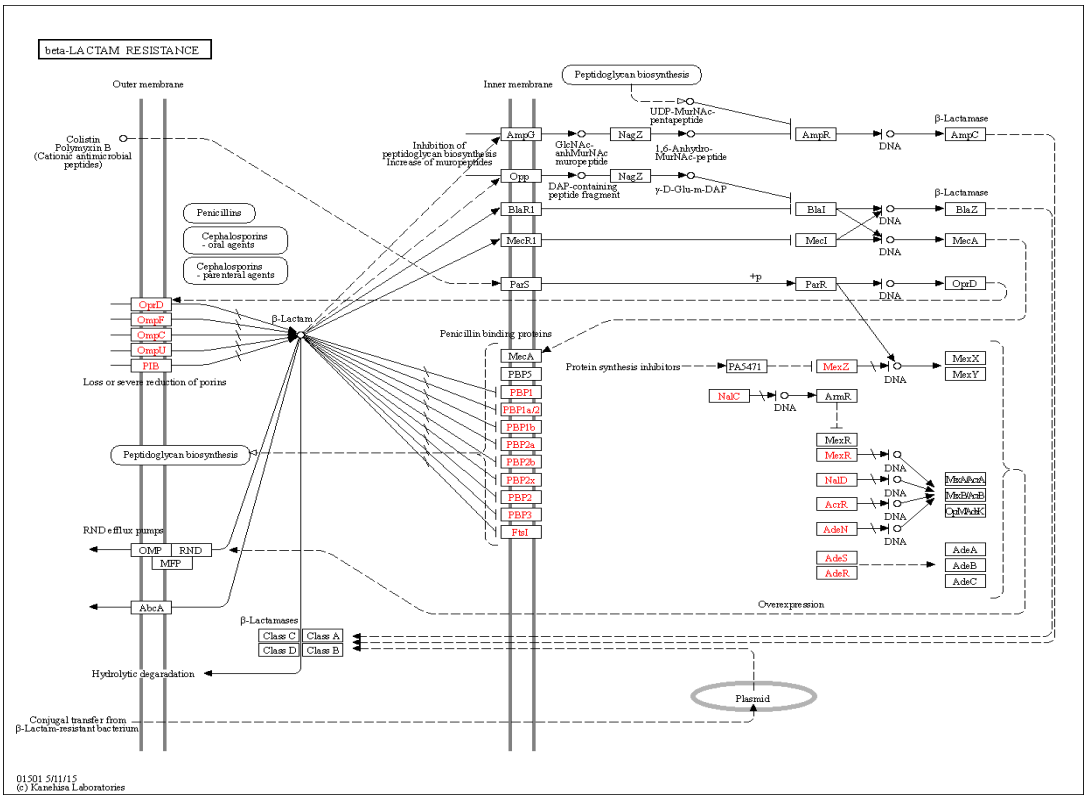

FIGURE 13: Beta-lactamase resistance to antibiotics

CONCLUSION

As evidence grows to demonstrate the critical role of microbiota in animal growth, metabolism, behavior, and the environment, the field of microbiome study broadens. We believe that the zebrafish model is an important player in this expansion, showing resistance to heavy metals and antibiotics, and enabling researchers to research more about bioremediation. The findings of this study provide credence to the idea that three bacteria have high bioremediation potential and may be exploited to create bioremediation agents that may detoxify zinc effluents from industrial sites in natural surroundings. The final result of docking proves that beta-lactamase is resistant to heavy metals like zinc and correlates with antibiotic resistance. Thus, the present study demonstrated that the use of ZBP from the zinc-resistant bacteria in the zebrafish gut microbiome is helpful in the removal of zinc from contaminated sources, and it depends on the conditional elements to increase the removal. As an alternative to the conventional process of heavy metal biosorption, the method may be cost-effective, environmentally safe, and multipurpose.

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