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ISOLATION AND DETERMINATION OF CHROMIUM (VI) AND NICKEL (II) RESISTANT SIDEROPHORE PRODUCING *BACILLUS CEREUS(sp)* AND ANALYSIS OF THE PROTEIN EXPRESSION USING BIOINFORMATICS TOOLS

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ABSTRACT

Siderophores are produced by bacteria, fungi and plants to facilitate uptake of iron, they are low-molecular-weight chelation agents and protect from stress responses. Pyoverdines act as fluorescent siderophores, plays an important role under iron-limiting conditions. During the stress condition a change in the broth culture was observed where it turned green from yellow, indicating some kind of pigment release. According to some reports the pigment release happens due to the presence of Siderophore in microorganisms. This colour change can also be considered as microbial sensor for color change detection. The bacterial sample was tested for the chromium (VI) and Nickel (II) resistant capacity, presence of siderophore, DNA and protein content. The chromium (VI) and nickel (II) resistant isolates were identified as *Bacillus cereus* (BF2). The siderophore was detected using qualitative and quantitative assays. Using various bioinformatics softwares the siderophore produced by *Bacillus cereus* (Rock 3-28) was compared with the isolated culture because both of their molecular weight varied from the range of 48kDa-65kDa. National Center For Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST), Clustal Omega, Expert Protein Analysis System (ExPASy), Self-Optimized Prediction Method with Alignment (SOPMA) and SWISS-MODEL tools were used to study the pyoverdine protein which coded for the enzyme L-ornithine N5-oxygenase. The FASTA sequences of the query sequence were compared with other species using BLAST. The sequences of protein were used for structural and functional analysis and for phylogenetic tree construction.

Keywords: Heavy Metals, Siderophore, NCBI, SWISS-MODEL, East Kolkata Wetland

INTRODUCTION

Microbial biosensors are the devices that uses microorganisms and enables a rapid, accurate and a sensitive detection of target analytes in different fields. For environment monitoring microbial biosensors are mainly used. Biosensors help in monitoring the contaminants present in the environment as they pose a risk to human health as well as to the environment **(Chiadò *et al.,* 2013)**. In the perspective of sustainable development, there has been a considerable momentum in the discharge of unplanned industrial effluents which is causing an undesirable impact on our environment. These industrial effluents have different impacts or effects on the ecosystems. The East Kolkata Wetlands has recently been declared a ramsar site covering around 12,500 ha land area including 286 wastewater-fed fishponds. **(Mukhopadhyay et al., 2007)**. It receives the effluents from 538 tanneries and nearly 5500 various other small-scale industrial establishments like rubber industries, electroplating industries, pigment manufacturing units, potteries, and battery manufacturing plants. Dumping of such wastes over a period of time has resulted in heavy contamination of this wetland **(Chatterjee *et al.,* 2006)**. Due to such anthropogenic activities, the heavy metal pollution has become a major concern today. The heavy metals are causing a major impact on soil and thus, leading to heavy metal toxicity in soil. Heavy metal toxicity hampers the ecological balance of nature resulting in risk to the survival of living beings. Heavy metal soil pollution has led to the accumulation of various heavy metals such as mercury, chromium, nickel, copper, aluminium and many more. The soil of East Kolkata wetlands contains various microorganisms such as *Bacillus sp,Aspergillus sp, Penicillium sp, Fusarium sp.* (Nath,& Kalam, 2014) *Arcella discoides* and *Diiflugia corona* **(Bindu *et al.,* 2013)**. The metals such as copper, nickel, chromium, cobalt, manganese are required but in trace amounts for microorganisms and therefore presence in higher concentrations leads to heavy metal toxicity and oxidative stress, which can cause damage of cellular DNA in microorganisms. To maintain a proper homeostatic balance the uptake and storage of various metals is quite important **(Chen et al., 2019)**. Biosensor thus, acts as a sensible element and helps the microorganisms to survive and and react to various metal concentrations **(Chiadò *et al.,* 2013)**. Siderophores play key roles of iron homeostasis in microorganisms, but there is increasing evidence implicating these chelators in the homeostasis of other metals **(Schalk *et al.,* 2011)**. Siderophores are produced by bacteria, fungi and plants to facilitate uptake in iron, they are low-molecular-weight chelation agents (approx. 200-2000Da) **(Singh et al., 2021; Chu *et al.,* 2010; Hider, & Kong, 2010)**. They are produced under iron-limiting conditions, but still they can potentially chelate other metals with lower affinity **(Braud *et al.,* 2010; Singh et al., 2021)**. The concentration of siderophore found in soil is wide and may range from tens of micromoles to a few mill moles per litre **(Hersman *et al.,* 1995)**. Iron is one of the most important metals required **(Saha et al., 2013)** for the growth and development of many living organisms. It plays a role as a catalyst in the fundamental enzymatic processes like, oxygen metabolism, DNA and RNA synthesis and electron transfer. Thus, to make iron available to microorganisms from minerals for extracellular solubilisation, siderophores play a very important role. Iron acts as an enzymatic cofactor, promotes the electron transfer or participate in oxygen metabolismbut its availability for microbial assimilation in the environment is extremely limiting, because it is mostly insoluble **(Singh et al., 2021; Sullivan, & Gara, 1992)**. During an infection the scenario seems to be often similar; bacteria produces siderophores to extract iron from organic substances and are considered to have virulence factors **(Lamont *et al.,* 2002)** and have affinity for iron with stability constants **(Albrecht-Gary *et al.,* 1994; Raymond *et al.,* 2003)**. Once the iron gets chelated, via energy-coupled transport involving TBDTs (TonB-Dependent Transporters) and the Ton complex, an inner membrane complex composed of TonB, ExbB and ExbD, the siderohpores deliver the iron to bacteria **(Schalk, 2008; Noinaj *et al.,* 2010)**. Through either enzymatic degradation or chemical modification of the siderophore and/or iron reduction iron gets released into the cytoplasm **(Hartman, & Braun 1980; Brickman, & McIntosh, 1992; Matzanke *et al.,* 2004)**. Screening with 16 different metals (Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Eu^{3+}, Ga^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Tb^{3+}, T^{l+} and Zn^{2+}) revealed that siderophores pyoverdine (PVD) is able to chelate all these metals **(Braud *et al.,* 2010)**. The siderophore produced here can be assumed as pyoverdine A which

codes for the enzyme L-ornithine N5-oxygenase and produces a green fluorescent colour. Various bioinformatics tools and softwares were used for the analysis of the pyoverdine. Tools such as BLAST (to find out the sequence similarity of the known protein of the species to the other species), Clustal Omega (to find out the phylogenetic relationship between the various species), ExPasy (to study the number of amino acids, molecular weight, isoelectric point, instability index, extinction coefficient, aliphatic index and grand average of hydropathicity), SOPMA (to study the secondary structure rates of the protein) **(Ishaku et al., 2019)**, SWISS MODEL (to study the sequence identity and GMQE) were used to study about the L-ornithine N5-oxygenase enzyme which codes for pyoverdine/siderophore A in *Bacillus cereus* including three-dimensional structure and Ramachandran plot analysis.

MATERIAL AND METHODS

Materials and Methods

Sample collection

Soil sample collection was done from different regions of East Kolkata Wetland area, Kolkata, India.

Soil sample preparation

The soil samples were collected from four different sites of East kolkata wetland in clean bags with the help a sterilized spatula and kept in the laboratory in room temperature for further use. East kolkata Wetland area generally have temperature ranging from 18°C-24°C, which is very much ideal for microbial growth. Using a mortar pestle the air dried samples were grinded. The sample was sieved properly using mesh sieve and stored for further processes.**(Firdous, & Chakraborty, 2018)**.

Isolation of bacteria

Each soil sample was diluted serially in normal saline water and inoculated in Luria-Bertani agar plate which contained about 500-2000 mg/l of potassium dichromate ($K_2Cr_2O_7$) and 200-800mg/l of nickel chloride ($NiCl_2$) concentration solutions by spread plating method. All prepared 48 plates were incubated at 37°C for 4 days. After four days of incubation single colonies were collected from Brahmi Root Soil and preserved in nutrient broth for further studies. Gram staining was performed to identify the morphology of the isolates **(Ilias *et al.,* 2011)**.

Biochemical screening and sequencing

The bacterial isolates were further tested for their biochemical characteristics and were identified using Bergey's manual of Determinative Bacteriology **(Ge *et al.,* 2013)**and further used for 16SrRNA sequencing **(Bru *et al.,* 2008)**.

Protein estimation

Protein estimation was done by using the Folin-Lowry method and then a bovine serum albumin (BSA) standard curve was made by calculating the protein concentration against optical density (OD) values at 660nm. Lowry method uses Folin–Ciocalteu as key reagent for the reaction as it involves reduction of the Folin–Ciocalteu reagent and oxidation of aromatic residues. The resultant molecule is blue in colour known as heteropolymolybdenum Blue. The concentration of the reduced Folin reagent (heteropolymolybdenum Blue) is measured by absorbance at 660 nm. As a result, the total concentration of protein in the sample can be measured. **(Everette et al., 2010).**

Isolation of siderophore producing bacteria

Qualitative assay

60.5 mg Chrome Azurol Sulphonate (CAS) was dissolved in 50 ml glass distilled water then it was mixed with 10 ml of iron (III) solution (1Mm $FeCl_3.6H_20$ in 10mM HCl). 40ml of distilled water to which 72.9 mg of hexadecyltrimethylammoniumbromide (HDTMA) and the solution made was added. The CAS reagent (dark blue colour) was then autoclaved for 15 minutes. This CAS reagent was added to Piperazine-N,N-bis (2-ethane sulfonic acid) (PIPES) agar medium (30.24 g ofPIPES buffer dissolved in 750ml of distilled water +15g of agar, whose pH was adjusted to 6.8 using 1.06g of NaOH pellets). The CAS agar plates which had solidified were punched with 2.5 to 5mm diameter holes by using a gel puncher. To each hole 25µl of the bacterial culture supernatant was filled and kept for incubation in dark at 28°C for 7days **(Jenifer *et al.,* 2015)**.

Quantitative assay (Cas-shuttle assay)

The quantitative estimation of siderophores, which are produced by the bacterial isolates taken from the sample can be done using CAS-shuttle assay **(Schwyn, & Neilands, 1987)**. The strain was grown in succinate medium and incubated for 24 to 30 hrs at 28ºC with constant shaking at 120 rpm on rotator shaking incubator. After incubation, the broth which was fermented was taken and centrifuged at 10,000 rpm in a cooling centrifuge at 4℃ for 10 minutes and cell-free supernatant was mixed with 0.5 ml CAS solution. The color obtained was determined using the Spectrophotometer at absorbance of 630 nm after 20 minutes of incubation with reference containing 0.5 ml CAS solution with 0.5ml uninoculated succinate medium. The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula

$[(Ar - As) / Ar] *100$

Where, Ar is the Absorbance of reference (CAS assay solution + uninoculated media)

and As is the Absorbance of the sample (CAS assay solution + cell-free supernatant) **(Jenifer *et al.,* 2015)**.

Determination of type of siderophore

Iron percholate assay

Iron percholate assay helps in detecting the presence of hydroxymate type strain. To a 0.5ml of culture supernatant, 2.5ml of Iron percholate solution (5mM $Fe(ClO_4)_3$ in 0.1M $HClO_4$) was added and allowed to incubate for 5min to develop the orange red colour solution and the absorbance was measured at 480nm. Desferal was used as standard **(Jenifer *et al.,* 2015)**.

Arnow's assay

For detection of catechols, 1ml of culture supernatant was mixed with 1ml of HCl, 1ml of nitrite molybdate (catechols produce yellow color),1ml of NaOH (color changes to red) followed by distilled water to make up the volume to 5ml. Absorbance was measured at 500 nm using 2,3 dihydroxybenzoic acid as the standard in UV-Visible spectrophotometer **(Jenifer *et al.,* 2015)**.

Statistical analysis

Various tools like NCBI, BLAST, CLUSTAL Omega, ExPasy, SOPMA, SWISS MODEL were used. The three-dimensional structural analysis and Ramachandran plot analysis were also done **(Ali, & Bala, 2016)**.

RESULTS AND DISCUSSION

Isolation of chromium and nickel resistant bacteria

The soil samples collected consists of a good amount of nutrients for plant nutrition **(Klimek, 2012)**, although presence of chromium and nickel increases the Electrical Conductivity in the soil **(Firdous, & Chakraborty, 2018)**. Thus, the microorganisms present in this soil have both metal resistant as well as metal reducing capacity. Microorganisms having metal resistance ability is helpful in many ways including waste water treatment by decomposition of organic matter **(Kavita, & Keharia, 2012)**. Primary study showed that all samples were having the capacity to grow by utilizing heavy metals present in their culture media **(Narasimhulu *et al.,* 2010; Nandi *et al.,* 2012; Nandi *et al.,* 2012; Nandi *et al.,* 2013)**. According to some reports ChrA and NerB genes are responsible for chromium and nickel resistance and the molecular weight varies from 43kDa to 66kDa. As per results the strains also showed bands between 48kDa to 65kDa which confirms the presence of the genes in bacterial sample **(Cervantes *et al.,* 1990)**. The soil sample was spread in 500mg/l to 2000mg/l concentrations for chromium and growth was observed in 1500mg/l concentration whereas no growth was observed in 2000mg/l concentration. The same sample was spread for nickel also using 200mg/l to 1000mg/l concentration and growth was observed till 800mg/l and no growth was observed in 1000mg/l. Thus, indicating that bacteria present in this sample can tolerate chromium and Nickel up to 1500mg/l and 800mg/l concentrations respectively.

Depending on the tests the colonies were identified to be gram positive and the 16S rRNA sequencing confirmed that the strain is *Bacillus cereus* (BF2). Using different concentrations of heavy metal of nickel and chromium, the bacterial growth was also observed and were studied **(Laila *et al.,* 2011; Garg *et al.,* 2012; Sevgi *et al.,* 2010)**. From the soil sample a total of four single colonies (Brahmi Root Soil-5,11,17 and 19) which were isolated after growing them under proper conditions in the laboratory were later checked under the microscope for morphological and biochemical characterization. The selected bacterial isolates found to be resistant to chromium and nickel were again subjected to identification using Bergey's manual of systemic bacteriology and found to have similar characteristics.

According to the result, bacterial isolate was gram positive, catalase positive, rod shaped, spore forming. In this study Genomic DNA was extracted from four stressed bacterial samples Brahmi Root Soil-5,11,17 and 19 including one unstressed bacteria *Bacillus cereus* (MTCC 430) and the samples were electrophoresed on 1% agarose gel against 1kb DNA ladder (Figure 1). This profiling is important to locate the gene and also to determine the genetic information. Protein profiling of the isolates was done to understand the molecular weight of the genetic material which also confirm the metal resistant genes **(Masood, & Malik, 2011)**. 16SrRNA sequencing further confirms the strain. **(Liu *et al.*, 2006)**.

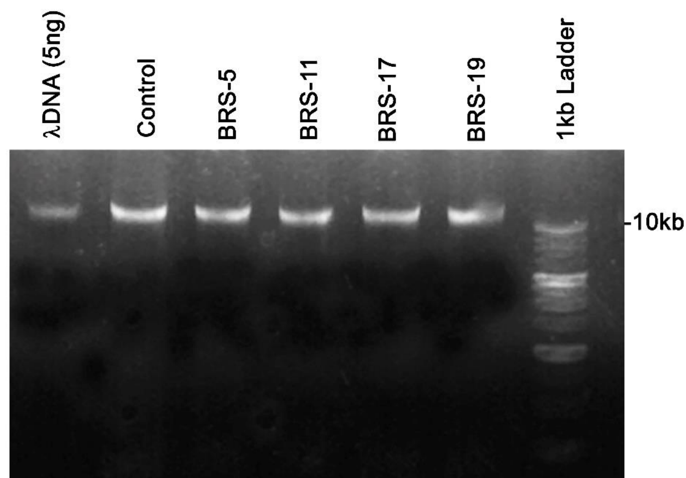

Figure 1 Genomic DNA of different bacterial isolates (Genomic DNA from each sample was electrophoresed on 1% agarose TAE gel and quantified against λ DNA 5ng and 1kb Ladder)

Protein Estimation

The protein analysis can be done using the BSA Standard Curve for various bacterial isolates **(Ernst, & Zor, 2010)**.The concentration of protein was determined against the OD values at 660 nm. The colour of the broth culture changed from yellow to green when the bacterial samples were exposed to stress conditions, showing some pigment releasing activity. Due to the colour change property the protein analysis was done using BSA standard curve of the different bacterial isolates (Brahmi Root Soil 5,11,17 and 19). Thus, the protein concentration of the isolates were measured, the colour change was also observed (from yellow to green). The Table 1 depicts the concentration of protein of the bacterial strain before and after the colour change took place.

Table 1 Protein concentration of bacterial isolates

Bacterial Isolates	Protein conc. before colour change of isolates (mg/l)	Protein conc. after colour change of isolates (mg/l)
BRS 5	0.81	0.84
BRS 11	0.83	0.89
BRS 17	0.79	0.86
BRS 19	0.87	0.91

The four stressed bacterial samples of overnight grown culture and one control unstressed sample *Bacillus cereus* (MTCC-430) were used for estimation of protein using SDS-PAGE using protein ladder (Figure 2).

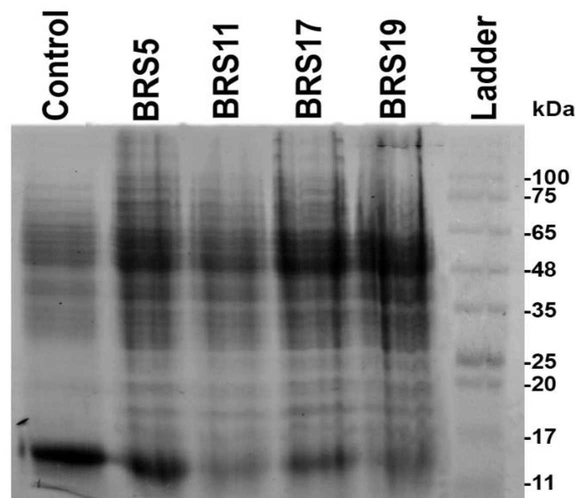

Figure 2 SDS PAGE analysis of different bacterial isolates

Morphology and biochemical identification and further 16srRNA sequencing confirms it as *Bacillus cereus* and the phylogenetic tree is given below (Figure 3). Since the bacterial strains showed the colour change from yellow to green fluorescent the siderophore production was confirmed. The bacterial strains were further studied using qualitative and quantitative assay, the siderophore were isolated from the bacterial strains and type of siderophore was also detected using iron-percholate assay and Arnow's assay.

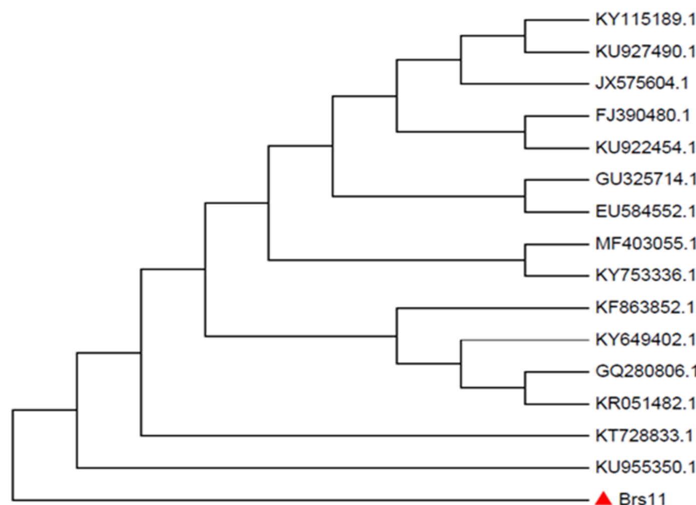

Figure 3 16SrRNA sequencing

Isolation of siderophore producing bacteria

Qualitative Assay and Quantitative Assay (CAS-Assay Shuttle)

On CAS agar plates, the four different bacterial isolates were screened for the production of siderophore. Isolates were siderophore positive with varying intensity of yellow zones. The percentage of siderophore produced varied from 30%, 36%, 36% and 37% for the bacterial colonies Brahmi Root Soil-5,11. 17 and 19 respectively (Figure 4). For the detection of siderophore, the CAS assay is the universal colorimetric method and is based on a siderophores high affinity for ferric iron. Chrome azurol Sulphonate (CAS) dye complexes with ferric iron and the CAS assay plates are blue in color. Iron bound to CAS is easily chelated by siderophores to produce a color change from blue to orange or yellow.

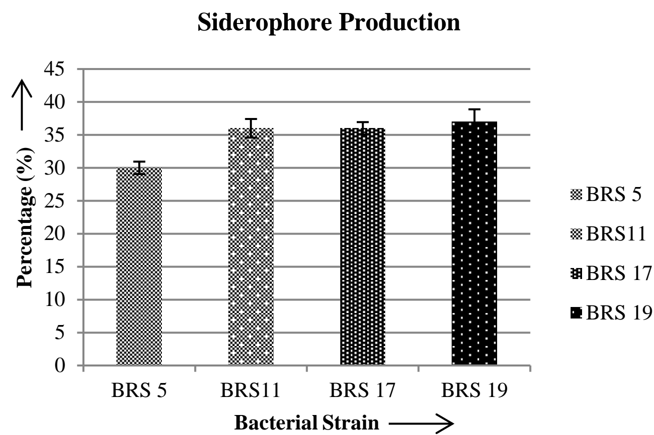

Figure 4 Quantitative assay for siderophore production

Determination of the type of siderophore

As earlier stated, for determination of the type of siderophore we use both iron percholate assay and Arnow's assay. Thus, after the assays were performed successfully the iron percholate assay was found to be negative while the Arnow's assay proved to be positive. Thus, the results of the positive (Arnow's) assay is given below:

Arnow's Assay

The type of siderophore produced by the four isolates were of Phenolate type (yellow colour) and the following table represents the presence of siderophore by the OD values. Using the above OD values, the bar graph was created of the different bacterial isolates BRS-5, BRS-11, BRS-17 and BRS-19 and then compared to the standard (Figure 5). All the assays were successfully performed and the bacteria *Bacillus cereus* strain (BF2) accession number KU955350.1 was found to produce siderophores. The molecular weight of the strain which we selected was quite similar to that of *Bacillus cereus* (Rock3-28). Thus, the statistical analysis of the siderophore the bacteria *Bacillus cereus* produced was studied by using various bioinformatics tools and softwares such as NCBI, BLAST, CLUSTAL Omega, ExPASy and SOPMA. The data of the bacteria *Bacillus cereus* (Rock3-28) which is stored in the database is compared with the strain which is derived from the soil sample because both of them have molecular weight within a range.

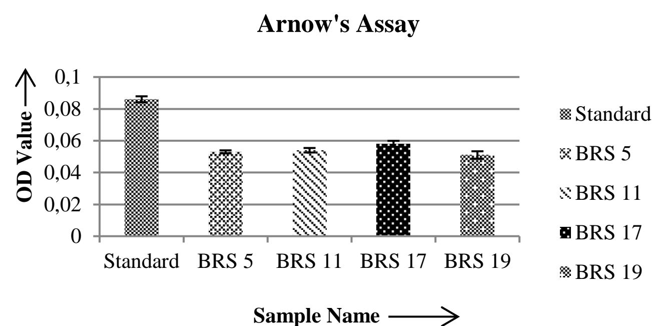

Figure 5 Arnow's assay of different bacterial strains

Statistical analysis

The statistical analysis were done using various bioinformatics softwares. The bacterial strain *Bacillus cereus* (Rock 3-28) was found to produce the siderophore which coded for the enzyme L-ornithine N5-oxygenase and was found to be similar to the strain *Bacillus cereus* (BF2) accession number KU955350.1.

Sequence Analysis of the bacterial strain *Bacillus cereus* (Rock 3-28)

The protein sequence of the enzyme L-ornithine N5-oxygenase **(Zwick *et al.*, 2012)**, present in the bacteria *Bacillus cereus* (Rock 3-28) which produced the siderophore A, was obtained from the database National Centre for Biotechnology Information (NCBI). A series of databases relevant to biotechnology and biomedicine which is an important resource for bioinformatics tools and services is housed by NCBI. The GenBank and PubMed (a bibliographic database) are the major databases for the getting information about the DNA sequences and biomedical literature. Using the online search engine, Entrez all these databases are available **(Pruitt *et al.*, 2009)**.

The search box was changed from all databases to protein sequence, to get the FASTA sequence (either nucleotide sequences or amino acid (protein) sequences represented in a text-based format) which the bacterial strain *Bacillus cereus* (Rock 3-28) contained. The sequence was thus retrieved using this software. The NCBI stores all the information regarding any gene, protein, and enzyme. Thus, the sequence was successfully retrieved and can consider the bacterial strain *Bacillus cereus* (Rock 3-28) has a similar sequence to that of *Bacillus cereus* (MTCC-430). The Figure 6 depicts the FASTA sequence of the enzyme L-ornithine N5-oxygenase which the bacteria produced, coding for the siderophore A. The accession number of the bacterial strain *Bacillus cereus* (Rock 3-28) is EEL31118.1.

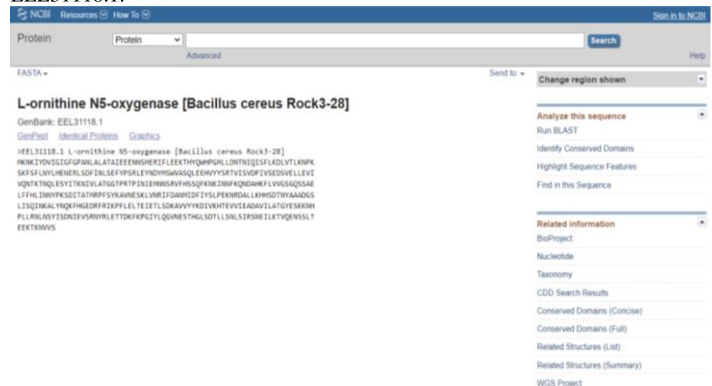

Figure 6 Sequence retrieval using NCBI of the enzyme (L-ornithine N5-oxygenase)

BLAST Analysis of *Bacillus cereus* (Rock 3-28) compared to other *Bacillus* strain

BLAST stands for Basic Local Alignment Search Tool. BLAST is used for aligning sequences **(Boratyn *et al.*, 2012)**. This is a bioinformatics software which is freely accessible to the users. The BLAST software helps in finding the sequence similarity between the sequence of interest and the other sequences of different or same species. The FASTA sequence of protein/ gene is first obtained from the NCBI database which stores all the information.

From the options given in the software, a type of BLAST needs to be selected according to the sequence we choose. The types of BLAST are: BLASTn (Nucleotide BLAST), BLASTp (Protein BLAST), BLASTx (translated nucleotide to protein) and tBLASTn (protein to translated nucleotide). The protein BLAST was selected, since the query sequence which was taken from the NCBI database is a protein sequence. The sequence was submitted and the results were displayed. This tool provided results from which the sequence identity was compared of that particular sequence, which was pasted in the query box from the species which was taken from others species. The software provided with a graphic summary of the sequence and there were alignment score given for each colour (Figure 7). The alignment scores for the colours are below 40 depicts black, 40-50 depicts blue, 50-80 depicts green, 80-200 depicts violet and 200 or more than that means red. The alignment score of this protein sequence taken from the bacteria *Bacillus cereus* (Rock 3-28) was equal to or more than 200 since, the lines were all red in colour **(McGinnis, & Madden, 2004)**.

Siderophore A

Figure 7 Graphic Summary of L-ornithine N5-oxygenase of *Bacillus Cereus* using BLAST software

The organism *Bacillus cereus*, accession number WP_000791092.1 was found to be 100% similar to that of *Bacillus cereus* (Rock 3-28) bearing the accession number EEL31118.1.

Phylogenetic Analysis of different strains of *Bacillus cereus* :

Multiple Sequence Alignment (MSA) **(Jurate *et al.,* 2013)** was carried out with the rhodopsin protein sequences from different mammals using a tool, Clustal Omega. Based on the differences of physical and genetic characteristics, a phylogenetic tree is constructed to show the evolutionary relationship among various biological species. Using a multiple sequence alignment software, the phylogenetic tree is constructed. The Clustal Omega software was used for phylogenetic tree since it is a multiple sequence alignment tool. The Clustal software helps to align sequences using a heuristic approach that progressively builds a multiple sequence alignment from a series of pairwise alignments. By analyzing the sequences as a whole, the method works, then utilizes the UPGMA (Unweighted Pair Group Method With Arithmetic Mean) /Neighbor-joining method (by generating a distance matrix). The scores of the sequences in the matrix was used to build a guide tree, then subsequently the multiple sequence alignment was built by progressively aligning the sequences in order of similarity.
The Clustal program accepts a wide range of input formats, including NBRF (National Biomedical Research Foundation)/PIR (Protein Information Resource), FASTA, EMBL (European Molecular Biology Laboratory) /Swiss-Prot, Clustal, GCC (Galaxy Community Conference) /MSF (Multiple Sequence Format Files), GCG9 RSF, and GDE. The output format can be one or many of the following: Clustal, NBRF/PIR, GCG/MSF, PHYLIP, GDE, or NEXUS. In the query box, set it as protein or gene according to the sequence to choose. The accession number of different strains of *Bacillus cereus* were taken from BLAST software and using the NCBI database the FASTA sequences were obtained. They were pasted in the query box of the software Clustal Omega and then multiple sequence alignment was executed. This software helps to create a phylogenetic relationship between different species (Figure 8) **(Sievers *et al.,* 2011)**. From Table 2, the accession number can be compared given in the table for understanding the phylogenetic tree.

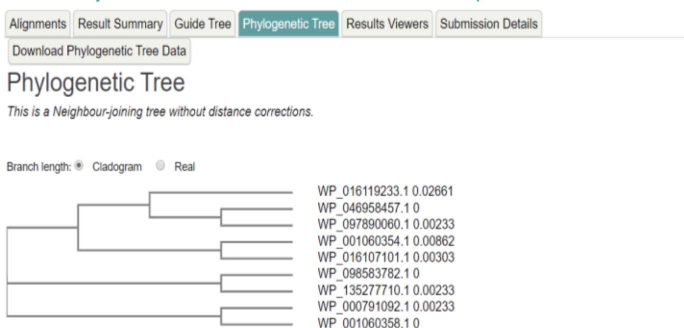

Figure 8 Phylogenetic tree using the software CLUSTAL Omega

Table 2 BLAST results showing percent identity between *Bacillus cereus* (Rock3-28) and other *Bacillus* strain **(Zwick *et al.,* 2012)**

Sr No.	Organism	Percent Identity	Accession No.
1.	*Bacillus cereus*	100%	WP_000791092.1
2.	*Bacillus cereus*	99.77%	WP_001060358.1
3.	*Bacillus cereus*	99.07%	WP_016107101.1
4.	*Bacillus cereus*	98.83%	WP_046958457.1
5.	*Bacillus cereus*	98.60%	WP_097890060.1
6.	*Bacillus cereus*	98.37%	WP_098583782.1
7.	*Bacillus cereus*	98.14%	WP_135277710.1
8.	*Bacillus cereus*	97.90%	WP_001060354.1
9.	*Bacillus cereus*	96.27%	WP_016119233.1

ExPASy Analysis

ExPASy is a bioinformatics tool for finding the physico-chemical properties of the protein, is operated by the Swiss Institute of Bioinformatics (SIB) and in particular the SIB Web Team. It is an extensible and integrative portal for accessing many scientific resources, databases and software tools in different areas of life sciences. The ExPASy software allows to find out the molecular weight, isoelectric point, extinction coefficient, aliphatic index, instability index and the grand average of hydropathicity of the protein. The UniProt KB software is used to find the accession number of the protein of the bacteria *Bacillus cereus* (Rock 3-28). The accession number is taken and pasted in the query box of the ExPasy software. The following results were obtained which are presented in Table 3. Physico-chemical properties like number of amino acids, molecular weight (M.WT) **(Tran et al., 2015)**, Isoelectric point (pI), Extinction coefficient (EC), Aliphatic index (AI), Grand Average Hydropathy (GRAVY) and Instability index (Ii) of the sequences were analysed.

Table 3 ExPasy results of the enzyme L-ornthine N5-oxygenase of *Bacillus cereus*

Organism	Accession No.	No. of Amino Acids	Mol.Wt	pI	EC	Al	Ii	GI
Bacillus cereus (Rock 3-28) **(Zwick*et al.,* 2012)**	EEL31118	429	49180.50	5.85	40800	91.10	39.02	-0.

The number of amino acids in the enzyme produced is 429. The molecular weight was found to be around 49180.50 Da (or 49.180 kDa). The isoelectric point was found to be around 5.85, the extinction coefficient was 40800, aliphatic index was 91.10, instability index was 39.02 and the grand average of hydropathicity was -0.423. Thus, these following results in Table 3 were obtained using the ExPASy software **(Gasteiger *et al.,* 2003)**.

SOPMA Results

SOPMA software helps in finding out the secondary structure rates of the protein that the species codes. This software helps in detecting the alpha helix, extended strand, beta turn and coil of the protein **(Bhattacharya et al., 2017)**. Secondary structure of proteins was predicted using SOPMA with the default parameters (window width: 17; similarity threshold: 8; number of states: 4) (Table 4).

Table 4 Secondary structure rates of the protein of the species using SOPMA software

Bacterial Species	Alpha Helix	Secondary Structure rate		Coil
		Extended Strand	Beta Turn	
Bacillus cereus (Rock 3-28) **(Zwick*et al.,*2012)**	161 is 37.53%	77 is 17.95%	21 is 4.90%	170 is 39.3%

The protein FASTA sequence is taken from the NCBI database and pasted in the query box. The sequence of protein from the bacterial strain *Bacillus cereus* (Rock 3-28) was taken and later the results were derived as given in the table below. The Table 4 detects the alpha helix, extended strand, beta turn and coil of the protein of *Bacillus cereus***(Geourjon, & Deleage, 1995)**.

SWISS MODEL of the protein coded by bacteria *Bacillus cereus* (Rock3-28)

Swiss Model is a homology-modelling server for protein structure which is fully automated, accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer). This tool makes protein modelling accessible to all life science researchers worldwide The software is accessed through the linkhttps://swissmodel.expasy.org/. Swiss model is a 3D structure prediction tool. It require a viewer such as DeepView - Swiss-PdbViewer, Rasmol, Cn3D v3.0 or WebMol Java PDB (Glaxo-Wellcome Experimental Research, Switzerland). Based on maximum similarity or identity with sequence the template selection is done **(Roy et al., 2017; Biasini *et al.,* 2014; Waterhouse *et al.,* 2018)**. After clicking on start modelling it takes to a page where the query protein sequence is pasted and then click on build model. The following information given in Table 5 was provided by the software along with the ligands, three-dimensional structure of the protein and the Ramachandran plot results **(Schwede*et al.,* 2003)**. Figure 9 shows the structure of the protein. The oligo-state of the protein is a homo-tetramer.

Table 5 Results of the protein of *Bacillus cereus* (Rock 3-28)

Species	Template	Seq. Identity	GMQE	QMean
Bacillus cereus (Rock 3-28) **(Zwick*et al.,*2012)**	5cku.1.A	34.94%	0.70	-1.59

GMQE- Stands for Global model quality estimate

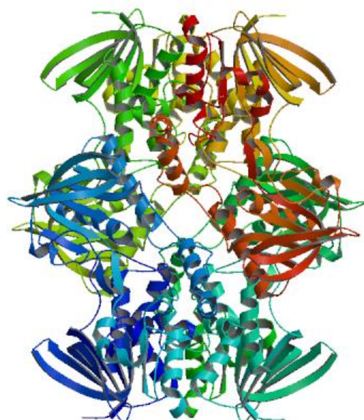

Figure 9 Three dimensonal structure of L-ornithine N5-oxygenase of *Bacillus cereus* (Rock 3-28)

Ramachandran Plot Analysis using the SWISS-MODEL software

Protein conformation was studied properly by Ramachandran plot analysis. Torsional angles (ϕ, ψ) of the Ramachandran plot describe the polypeptide as well as protein conformation **(Hollingsworth *et al.,* 2010)**. Ramachandran plot analysis was done with the help of SWISS MODEL software. The results of the species *Bacillus cereus (*Rock 3-28) which were derived from the SOPMA software are given in Table 6.

Table 6 Showing the results of the protein of *Bacillus cereus* (Rock 3-28)

Species	MolProbity Score	Ramachandran Favoured	Ramachandran Outliers	Rotamer Outliers
Bacillus cereus (Rock 3-28) **(Zwick*et al.,* 2012)**	1.84	93.24%	1.33%	0.79%

The Ramachandran plot is a plot of the torsional angles - phi (φ) and psi (ψ) - of the residues (amino acids) contained in a peptide. The protein structural scientists make a Ramachandran plot to determine the torsional angles which are permitted and can obtain insight into the structure of peptides. Figure 10 shows the Ramachandran plot of the enzyme L-ornithine N5-oxygenase present in the bacteria *Bacillus cereus* (Rock 3-28).

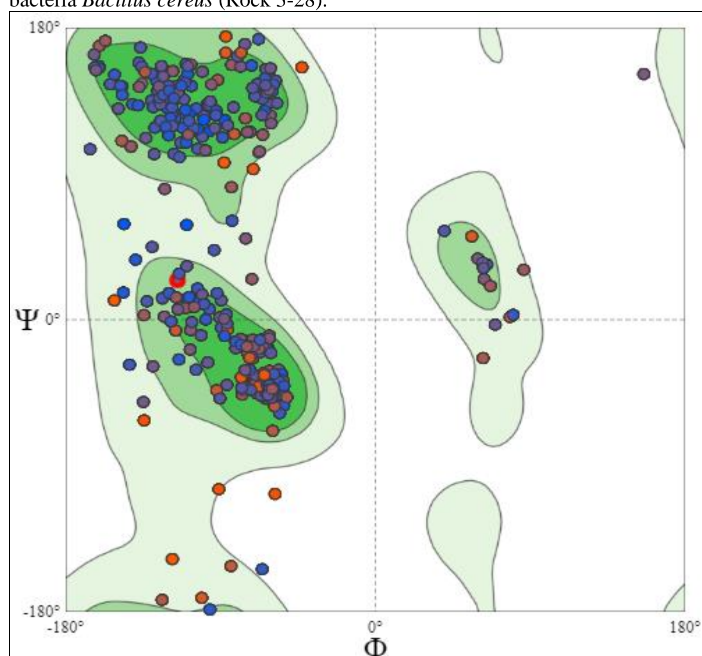

Figure 10 Ramachandran plot of L-ornithine N5-oxygenase of *Bacillus cereus* (Rock 3-28)

CONCLUSION

The soil sample was found to have the presence of the bacteria *Bacillus cereus* which was Chromium (Vi) and Nickel (II) resistant and produces siderophores. Siderophores facilitate the iron uptake content in bacteria under iron-limiting conditions. The CAS blue agar test when performed on the bacterial strain produced a fluorescent yellow colour which later changed to green producing a pigment containing siderophores. The various assays like iron-percholate assay and Arnow's assay were used to find out the type of siderophore the bacteria produced. The siderophore produced was found to be pyoverdine, coding for the gene pvd A and the enzyme L-ornithine N5-oxygenase. It was a study of transcriptional profiling of copper stressed bacteria. The experiment was done under metals nickel and chromium with a different genera group. Since, the bacteria isolated from the soil sample *Bacillus cereus* (BF2) accession number KU955350.1 was found to be similar to that of *Bacillus cereus* (Rock 3-28) bearing the accession number EEL31118.1 because of the molecular weight being within the range of 48 kDa -65 kDa, various statistical analysis were done using the bioinformatics tools. The protein sequence was isolated, sequenced with confirming it with various assays and later doing a brief computational approach study. The use of various bioinformatics tools and softwares helped in the study of the protein sequence in detail followed by homology search, physico-chemical properties, three dimensional study and phylogenetic analysis. The bacterial strain isolated from the sample was found to be similar to that of the bacterial strain *Bacillus cereus* (Rock 3-28) that was already stored in the national database of NCBI. The protein sequence was later compared to the other bacterial strains using the BLAST software which provided the graphic summary of the sequence and the sequence identity percentage. Then, using the Clustal Omega software the phylogenetic tree was constructed with nine sequences of the bacteria *Bacillus cereus* each having different accession number. The ExPASy software provided the information about the siderophore pyoverdine of the bacteria *Bacillus cereus* (Rock 3-28), accession number EEL31118.1, which detected the physico-chemical properties. SOPMA, another bioinformatics software detected the secondary structure rates of the protein such as alpha helix, beta turn, extended strand and coil. SWISS-MODEL software modelled the protein structure providing with three-dimensional structure along with sequence identity, QMean, GMQE and the template of the enzyme/ siderophore produced by the bacteria. This software also helped in the Ramachandran plot analysis. The sequence retrieved of the protein from *Bacillus cereus* (Rock 3-28) also helped predicting the isolated strain Bacillus cereus (BF2).

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REFERENCES

Aksu, Z. (2002). Equilibrium modelling of individual and simultaneous biosorption of chromium (VI) and nickel (II) onto dried activated sludge. Water Research, 36, 3063-3073. https://doi.org/10.1016/s0043-1354(01)00530-9.

Albrecht-Gary, A. M., Blanc, S., Rochel, N., Ocacktan, A. Z., Abdallah, M. A. (1994) Bacterial iron transport: coordination properties of pyoverdinPaA, a peptidic siderophore of *Pseudomonas aeruginosa*. Inorganic Chemistry, 33, 6391–6402.https://doi.org/10.1021/ic00104a059.

Ali, Z., Bala, S. B. (2016). Basic statistical tools in research and data analysis. Indian Journal of Anaesthesia, 60, 662-669.https://doi.org/10.4103/0019-5049.190623

Bhattacharya, M., Hota, A., Kar, A., Sankar Chini, D., Chandra Malick, R., Chandra Patra, B., Kumar Das, B. (2018). In silico structiral and functional modelling of Antifreeze protein (AFP) sequences of Ocean pout (Zoarces americanus, Bloch & Schneider 1801). Journal of Genetic Engineering and Biotechnology, 16, 721-730. https://doi.org/10.1016/j.jgeb.2018.08.004.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Gallo, T., Cassarino, Bertoni, M., Bordoli, L., Schwede, T. (2014). SWISS-MODEL: modellingprotein tertiary and quaternary structure using evolutionary information. Nucleic Acids Research, 42, W252-W258.https://doi.org/10.1093/nar/gku340.

Bindu, L., Chitra, J., Sanyal, A.K. (2013). Notes on some protozoa and zooplankton of sewage systems in Kolkata. Marine Biology Regional Centre. Zoological Survey of India, 113, 141-143.

Boratyn, G.M., Schäffer, A.A., Agarwala, R., Altschul, S.F., Lipman, D.J., Madden, T.L. (2012). Domain enhanced lookup time accelerated BLAST. Biology Direct, 7, 12.https://doi.org/10.1186/1745-6150-7-12.

Borkar, S. (Ed.). (2015). Bioprospects of Coastal Eubacteria. https://doi.org/10.1007/978-3-319-12910-5.

Braud, A., Geoffroy, V., Hoegy, F., Mislin, G.L.A., Schalk, I.J. (2010). Presence of the siderophores pyoverdine and pyochelin in the extracellular medium reduces

toxic metal accumulation in *Pseudomonas aeruginosa* and increases bacterial metal tolerance. Environmental Microbiology Reports, 2, 419–425.https://doi.org/10.1111/j.1758-2229.2009.00126.x.

Brickman, T.J., McIntosh, M.A. (1992). Overexpression and purification of ferric enterobactin esterase from *Escherichia coli*. Demonstration of enzymatic hydrolysis of enterobactin and its iron complex. Journal of Biological Chemistry, 267, 12350–12355.

Bru, D., Martin-Laurent, F., Philippot, L. (2008). Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S r RNA gene as an example. Applied and Environmental Microbiology, 74, 1660–1663.https://doi.org/10.1128/aem.02403-07.

Cervantes, C.M., Hadjeb, N., Newman, L., Price, C.A. (1990). ChrA is a carotenoid-binding protein in chromoplasts of *Capsicum annuum*. Plant Physiology, 92, 1241-1243.https://doi.org/10.1104/pp.92.4.1241.

Chatterjee, S., Chattopadhyay, B., Mukhopadhyay, S.K. (2006). Trace metal distribution in tissue if cichlids (Oreochromisniloticus and O. mossambicus) collected from wastewater-fed fishponds in East Kolkata Wetland a ramsar site. ActaIchthyologica etPiscatoria, 36, 119-125.https://doi.org/10.3750/aip2006.36.2.05.

Chen, A., Hao, X., Xiao, Y., Zou, K., Liu, H., Liu, X., ... Liang, Y. (2019). Responses of *Acidithiobacillus thiooxidans* A01 to Individual and Joint (Ni2+) and Ferric (Fe3+). Minerals, 9, 82. https://doi.org/10.3390/min9020082.

Chiadò, A., Varani, L., Bosco, F., Marmo, L. (2013). Opening Study on the Development of a New Biosensor for Metal Toxicity Based on *Pseudomonas fluorescens* Pyoverdine. Biosensors,3, 385-399.https://doi.org/10.3390/bios3040385.

Chu, B.C., Garcia-Herrero, A., Johanson, T.H., Krewulak, K.D., Lau, C.K., Peacock, R.S. (2010). Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. BioMetals, 23, 601–611.https://doi.org/10.1007/s10534-010-9361-x.

Dutta, S., Firdous, K., Chakraborty, S. (2021). Screening of synergistic and antimicrobial effect of Cr (VI) and Ni (II) tolerant bacteria *Bacillus cereus*. Journal of Applied Biology and Biotechnology, 9, 69-77. https://doi.org/10.7324/jabb.2021.9409.

Ernst, O., Zor, T. (2010). Linearization of the Bradford Protein Assay. Journal of Visualized Experiments, 38, 1918.https://doi.org/10.3791/1918.

EveretteJaceD,BryantQuintonM,GreenAshlee M. ,Abbey Yvonne A. , WangilaGrant W. , and Walker Richard B (2010). A thorough study of reactivity of various compound classes towards the Folin-Ciocalteu reagent. J Agric Food Chem., 58(14): 8139–8144.https://doi.org/10.1021/jf1005935.

Firdous, K., Chakraborty, S. (2018). Correlation of different soil parameters and evaluation of its effect in a ramsar site East Kolkata Wetland. International Journal of Scientific Research in Science and Technology, 4, 966-976.

Gail, T.M., Geddie, A., De Long, S.K., Kirisits, M.J., Whiteley, M., Parsek, M.R. (2006). Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. Journal of Bacteriology, 188, 7242-7256.https://doi.org/10.1128/jb.00837-06.

Garg, S.K., Tripathi, M., Srinath, T. (2012). Strategies for chromium bioremediation of tannery effluent. ReviewsofEnvironmental Contamination and Toxicology, 75-140.https://doi.org/10.1007/978-1-4614-2329-4_2.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., Bairoch, A. (2003). ExPASy: the proteomics server for in-depth protein knowledge eand analysis. Nucleic Acids Research,31, 3784–3788.https://doi.org/10.1093/nar/gkg563.

Geourjon, C., Deleage, G. (1995). SOPMA: significant improvements in proteinsecondary structure prediction by consensus prediction from multiple alignments. Bioinformatics, 11, 681-684.https://doi.org/10.1093/bioinformatics/11.6.681.

Ge, S., Zhou, M., Dong, Z., Lu, Y., Ge, S. (2013). Distinct and effective biotransformation of hexavalent chromium by a novel isolate under aerobic growth followed by facultative anaerobic incubation. Applied Microbiology and Biotechnology, 97, 2131–2137.https://doi.org/10.1007/s00253-012-4361-0.

Hartman, A., Braun, V. (1980). Iron transport in *Escherichia coli*: uptake and modification of ferrichrome. Journal of Bacteriology, 143, 246–255.https://doi.org/10.1128/jb.143.1.246-255.1980.

Hersman, L., Lloyd, T., Sposito, G. (1995). Siderophore promoted dissolution of hematite. Geochimica et CosmochimicaActa, 59, 3327–3330.https://doi.org/10.1016/0016-7037(95)00221-k.

Hider, R.C., Kong, X. (2010). Chemistry and biology of siderophores. Natural Product Reports, 27, 637–657.https://doi.org/10.1039/b906679a.

Hollingsworth, S.A., Karplus, P.A. (2010). A fresh look at the Ramachandran plot and the occurrence of standard structures in proteins.BioMolecular Concepts, 1, 271–283.https://doi.org/10.1515/bmc.2010.022.

Ilias, M., Rafiqullah, I.M., Debnath, B.C., Mannan, K.S., MozammelHoq, M. (2011). Isolation and characterization of chromium (VI) reducing bacteria from tannery effluents. Indian Journal of Microbiology, 51, 76–81.https://doi.org/10.1007/s12088-011-0095-4.

Ishaku, G.A. (2019). In-silico structural annotation of methylthioadenosine nucleosidase protein Zm00014a_031618 in maize (Zea mays L.). Journal of Applied Life Sciences International, 1-8. https://doi.org/10.9734/jalsi/2019/v21i330108.

Jenifer, C.A., Sharmili, A.S., Anbumalarmathi, J., Umamaheswari, K., Shyamala, K. (2015). Studies on siderophore production by microbial isolates obtained from aquatic environment. European Journal of Experimental Biology, 5, 41-45.

Jurate, D., Aisling, O.D., Roy, D.S. (2013). An Overview of Multiple Sequence Alignments and Cloud Computing in Bioinformatics. ISRN Biomathematics.1-14.https://doi.org/10.1155/2013/615630.

Kavita, B., Keharia, H. (2012). Reduction of hexavalent chromium by *Ochrobactrum intermedium* BCR400 isolated from a chromium contaminated soil. 3 Biotech, 2, 79–87.https://doi.org/10.1007/s13205-011-0038-0.

Klimek, B. (2012). Effect of long-term zinc pollution on soil microbial community resistance to repeated contamination. Bulletin of Environmental Contamination Toxicology, 88, 617–622.https://doi.org/10.1007/s00128-012-0523-0.

Laila, M.A., Wagdy, K.B., Thanaa, H.A. (2011). Heavy metal resistance and gene expression analysis of metal resistance gene in gram- positive and gram- negative bacteria present in Egyptian soils. Journal of Applied Sciences in Environmental Sanitation, 6, 201–211.

Lamont, I.L., Beare, P.A., Ochsner, U., Vasil, A.I., Vasil, M.L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences, 99, 7072–7077.https://doi.org/10.1073/pnas.092016999.

Liu, Y.G., Xu, W.H., Zeng, G.M., Li, X., Gao, H. (2006). Cr(VI) reduction by *Bacillus* sp. isolated from chromium landfill. Process Biochemistry, 41, 1981–1986.https://doi.org/10.1016/j.procbio.2006.04.020.

Masood, F., Malik, A. (2011). Hexavalent chromium reduction by *Bacillus* sp. strain FM1 isolated from heavy-metal contaminated soil. Bulletin of Environmental Contamination and Toxicology, 86, 114–119.https://doi.org/10.1007/s00128-010-0181-z.

Matzanke, B.F., Anemuller, S., Schunemann, V., Trautwein, A.X., Hantke, K. (2004). FhuF, part of a siderophore reductase system. Biochemistry, 43, 1386–1392.https://doi.org/10.1021/bi0357661.

McGinnis, S., Madden, T. L. (2004). BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Research, 32, 20–25. https://doi.org/10.1093/nar/gkh435.

Momand, J., McCurdy, A. (2017). Concepts of Bioinformatics and Genomics. Oxford University Press.

MukhopadhyaySubhra Kumar, Chattopadhyay Buddhadeb, GoswamiAbhishek Roy and ChatterjeeAsitava (2007). Spatial variations in zooplankton diversity in waters contaminated with composite effluents. J. Limnol., 66(2): 97-106. https;//doi.org/10.1.1.816.7788.

Nandi, S., Srivastava, R.C., Agarwal, K.M. (2012). Bioaccumulation of heavy metal in *Brassica oleracea* effects on human health. International Journal of Medical Sciences, 5, 21-23.

Nandi, S., Srivastava, R.C., Agarwal, K.M. (2013). Heavy metal concentration in surface water of East Kolkata Wetland. Indian Journal of Public Health Research & Development, 4, 227. https://doi.org/10.5958/j.0976-5506.4.4.179.

Nandi, S., Srivastava, R.C., Agarwal, K.M. (2012). Lead and Cadmium accumulation in fresh water fishes *Labeo rohita* and *Catla catla*. Journal of Environment Research and Development, 6, 748-753.

Narasimhulu, K., Sreenivasa, P., Vinod, A. (2010). Isolation and identification of bacterial strains and study of their resistance to heavy metals and antibiotics. Journal of Microbial & Biochemical Technology,02, 074–076.https://doi.org/10.4172/1948-5948.1000027.

Nath, S., Kalam, A. (2014). Study of fungal diversity of some selected natural spot of east Kolkata wetland. Indian Journal of Microbiological Research, 1, 60-71.

Noinaj, N., Guillier, M., Barnard, T.J., Buchanan, S.K. (2010). TonB-dependent transporters: regulation, structure, and function. Annual Review of Microbiology, 64, 43–60.https://doi.org/10.1146/annurev.micro.112408.134247.

Pruitt, K., Tatusova, T., Klimke, W., Maglott, D. (2009). NCBI Reference Sequences: current status, policy and new initiatives. Nucleic Acids Research, 37, D32-D36.https://doi.org/10.1093/nar/gkn721.

Raymond, K.N., Dertz, E.A., Kim, S.S. (2003). Enterobactin: an archetype for microbial iron transport. Proceedings of the National Academy of Sciences, 100, 3584–3588.https://doi.org/10.1073/pnas.0630018100.

Roy, A. (2017). In silico analysis and characterization of fresh water fish ATPases and homology modeling. Annals of Proteomics and Bioinformatics, 1, 018-024. https://doi.org/10.29328/journal.hpbr.1001003.

Saha, R., Saha, N., Donofrio, R.S., Bestervelt, L.L. (2012). Microbial siderophores: a mini review. Journal of Basic Microbiology, 53, 303-317. https://doi.org/10.1002/jobm.201100552.

Schalk, I.J., Mélissa, H., Armelle, B. (2011). New roles for bacterial siderophores in metal transport and tolerance. Environmental Microbiology, 13, 2844–2854. https://doi.org/10.1111/j.1462-2920.2011.02556.x.

Schalk, I.J. (2008). Metal trafficking via siderophores in Gram negative bacteria: specificities and characteristics of the pyoverdine pathway. Journal of Inorganic Biochemistry,102, 1159–1169.https://doi.org/10.1016/j.jinorgbio.2007.11.017.

Sevgi, E., Coral, G., Gizir, A.M. (2010). Investigation of heavy metal resistance in some bacterial strains isolated from industrial soils. Turkish Journal of Biology, 34, 423–431.

Schwede, T., Kopp, J., Guex, N., Peitsch, M.C. (2003). SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Research, 31, 3381–3385.https://doi.org/10.1093/nar/gkg520.

Schwyn, B., Neilands, J.B. (1987). Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry, 160, 47-56.https://doi.org/10.1016/0003-2697(87)90612-9.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., Higgins, D.G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular System Biology, 7, 539.https://doi.org/10.1038/msb.2011.75.

Singh, S.K., Singh, P.P., Gupta, A., Singh, A.K., Keshri, J. (2019). Tolerance of heavy metal toxicity using PGPR strains of *Pseudomonas* species. PGPR Amelioration in Sustainable Agriculture, 239-252. https://doi.org/10.1016/b978-0-12-815879-1.00012-4.

Singh, S.K., Verma, H., Singh, V.K., Ghosh, S., Giri, D.D., Mishra, M., Kumar, A. (2021). Sustainable agricultural practices using microbial strains for crop production. Food Security and Plant Disease Management, 357-370. https://doi.org/10.1016/b978-0-12-821843-3.00002-7.

Sullivan, D.J.O., Gara, F.O. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiological Reviews, 56, 662–676.https://doi.org/10.1128/mmbr.56.4.662-676.1992.

Tran, N., Liu, H., Jakovlić, I., Wang, W.-M. (2015). Blunt snout bream (*Megalobrama amblycephala*) MyD88 and TRAF6: Characterization, comparative homology modelling and expression. International Journal of Molecular Sciences, 16, 7077-7097. https://doi.org/10.3390/ijms16047077.

Ünsal, A., Elif, K., Sevilay, T., Zümriye, A. (2004). Individual and simultaneous biosorption for Chromium (VI) and Nickel (II) onto dried activated sludge. Chemical Engineering Communications, 191, 1589-1605. https://doi.org/10.1080/00986440490472616.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., Beer T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Research, 46, W296-W303.https://doi.org/10.1093/nar/gky427.

Zwick, M.E., Joseph, S.J., Didelot, X., Chen, P.E., Bishop-Lilly, K.A., Stewart, A.C., Willner, K., Nolan, N., Lentz, S., Thomason, M.K., Sozhamannan, S., Mateczun, A. J., Du, L., Read, T.D.(2012). Genomic characterization of the *Bacillus cereus*sensulato species: backdrop to the evolution of *Bacillus anthracis*. Genome Research, 22, 1512-1524. https://doi.org/10.1101/gr.134437.111.