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Table of Contents

Volume 3, Issue 2, March/April 2009.

Pages

19 - 30 Phylogenetic Analysis of the Potential Microorganism for Remediation of Heavy Metals from the Contaminated Environment
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Phylogenetic Analysis of the Potential Microorganism for Remediation of Heavy Metals from the Contaminated Environment

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ABSTRACT

The present research work has been carried out to study the waste disposal contaminated site for its physico chemical and microbial characterization and identification of potential microorganism capable of bioaccumulation and biodegradation of heavy metals. The ambient conditions present in the metal contaminated environment shows the values: pH(5.4),temperature(30℃), moisture(11.71%), nutrients; Nitrogen(0.2mg/l), phosphorus(22.65mg/l) and sulphur(559.3mg/l) respectively. The biological parameters studied indicate Dissolved oxygen (7.4mg/l), Biological oxygen demand (3.8 mg/l), Chemical oxygen demand (64.6 mg/l). The microbial consortium identified was found to survive and multiply in the present environmental conditions. Microbial consortium was sequenced and compared using Bioinformatics tools like BLAST, ClustalW and PHYLIP. In order to identify potential microorganism, microbial consortium was exposed to increasing concentrations of heavy metals viz 5mg/l, 25mg/l, 50mg/l, 100mg/l up to 800mg/l with special reference to Iron. At a concentration of 500mg/l, only one microorganism was found survived and multiplied. This shows that potential microorganism was only survived at higher concentration of iron. The 16SrRNA sequence and phylogenetic tree characterized the organism as Klebsiella pneumoniae, which was also confirmed by biochemical tests. The potential microorganism identified by BLAST technique can be used for remediation of the heavy metal from contaminated environment.

Keywords: bioremediation, bioaccumulation, heavy metals

1. INTRODUCTION

The small scale industries located at Mira Bhayander road, Mumbai, India carried out various operations which include grinding, mixing, plating, washing, milling, cutting, rubbing etc. The

waste generated contains heavy metals which are disposed off at the site near to industry. The waste characterized shows the presence of varying concentrations of physico-chemical parameters and survival of microbial consortium. The microbial consortium present in waste disposal metal contaminated site has been identified using the BLAST technique by sequencing and drawing phylogenetic tree. The microbial consortium present in such contaminated site will have the potential to remediate the contaminant by bioaccumulation, bioadsorption and biotransformation. The potential microorganism which can be versatile for remediation of the metals contaminant will have the high significance for the environmental clean up [11]. The scale up process technique has been used to expose the microorganism consortium at varying concentrations of metals viz 5mg/l, 25mg/l, 50mg/l, 100mg/l up to 800mg/l with special reference to Iron. At a concentration of 500mg/l only one type of colony was found survived, which was identified by sequencing and drawing phylogenetic tree. The 16SrRNA technique used for the identification of potential microorganism for the remediation of metals from the metal contaminated environment. The potential microorganism identified from the metal waste disposal contaminated site will be cultured for its beneficial use to remediate the toxic heavy metals. The bioinformatics tools with special reference to BLAST ,alignment of sequence using ClustalW and phylogenetic tree of potential microorganism has proved to identify the potential microorganism which could be useful for remediation of heavy metals for environmental clean up.

2. PRELIMINARIES

Material and methods:

The environmental contaminated sites located at Mira road, Bhayander have been selected for sampling the soil, sediments for their physico-chemical and microbial characterization. Samples were collected in sterilized sealed pack polythene bags. The physical and chemical parameters were analyzed as per described in standard methods for the examination of water and waste water, 17th edition, APHA [2].

Soil was air-dried, ground and passed through a 2mm pore size sieve and was stored in sealed containers at room temperature. Soil physico-chemical parameters organic carbon, Total Nitrogen, Sulfate, Phosphate including biological characterization such as Chemical oxygen demand, dissolved oxygen were analyzed [8].

Microbial analysis:

One gram of each sample was immediately used for microbial enumerations. The enumeration of bacteria and fungi was done according to a standard procedure [14]. Briefly, 1gm of soil was mixed with 10ml of sterile distilled water. An aliquot of 0.1 ml of dilutions for each soil samples was spread plated onto agar plates on to agar from the appropriate dilution tubes and incubated at room temperature [7]. The bacterial colonies were counted after every 24 hrs. Only the plates showing between 25 to 300 colonies were tallied, and the results were averaged for each soil samples. The fungal colonies were counted after 48-72 h. Samples were preserved at 4℃ for further microbial analysis [4]. Isolated colonies were further analyzed using specialized agar / 16S rRNA sequencing. The isolates were then identified based on the morphological, cultural and biochemical characteristics following Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The specialized agar (from Hi media) was used for identifying E. coli, Salmonella, Shigella, Vibriocholerae , Yeast and Mould, S. aureus, Clostridium, Pseudomonas , Streptococcus faecalis, Serratia [13]. Other microorganisms present were identified using 16SrRNA sequencing.

Metal analysis:

Each soil sample was digested was digested with 10ml of a mixture perchloric acid: nitric acid (HCLO4:HNO3-1:5 v/v) [15]. Acid digestion was carried out on a hot plate at 70-100C until yellow fumes of HNO3 and white fumes of HCLO4 were observed. The digestion process was continued until a clear solution remained after volatilization of acids, and was stopped when the residue in the flask was clear and white. The digested sample was dissolved in distilled water, filtered through Whatman no.1 filter paper to remove impurities and made up to the desired volume [1].

Isolation of potential microorganism:

Soil and sediments were serially diluted to 10,000 folds and plated on nutrient agar. 1ml. bacterial culture was inoculated in nutrient broth and further, in 250ml erlenmeyer flasks containing 100ml minimal media with a metal concentration of 5mg/l. Minimal media comprised of Na2HPO4, NH4Cl, glucose blended with 0.6ml. of trace elements solution. Trace elements solution contains CaCl2.2H2O, MgSO4, MnSO4.7H2O, and FeSO4.7H2O. Copper was added from 1000mg/l stock solution of CuSO4.5H2O. Flasks were kept in incubator shaker at37℃, 200rpm. pH was adjusted to 7.0 before inoculation. pH and optical density at 600nm were measured daily to analyze bacterial growth. Further, 1ml. bacterial culture was transferred to metal concentration of 25mg/l, and subsequently to 50mg/l, 100mg/l upto 800mg/l to get potential microorganism. 100µl of samples of each concentration were plated on minimal media containing metal solutions of respective concentration. Single colony was isolated at higher concentration and identified as potential microorganism for bioaccumulation iron.

Identification of isolated microorganism by 16SrRNA analysis:

16SrRNA analysis was done by using predetermined universal primers of 16SrRNA. DNA isolated from pure culture was used as template. PCR was performed with a 50µl reaction mixture containing primer 16S, DNA template buffer, MgCl2, dXTPs, Taq polymerase. PCR products were analyzed by electrophoresis in 1.8% agarose gel. PCR program was carried out in PTC-200 Peltier thermocycler which comprises of three steps; 1) Denaturation at 94ºC for 1minute; 2) Annealing at 55ºC for 1minute; 3) Extension at 72ºC for 1minute [3].

RNA sequences were compared with already submitted sequence in database using BLAST software. Further, most similar sequences were aligned by ClustalW and ClustalX software and phylogenetic tree was drawn using PHYLIP software to analyze evolutionary relationships among sequences of isolated microorganism and nearest neighbors [9].

Results and Discussion:

The metal comes in the natural environment from various sources like earth's crust, soil erosion, mining ,industrial discharge, urban runoff, sewage effluents, air pollution fallout ,pest or disease control agents applied to plants. The remediation of metal has become an increasing environmental problem and is/will continue to be a contaminated source unless the remediation techniques developed by identifying the versatile microorganism which has the capacity to bioaccumulated/ biotransformed or remediate the heavy metals. In view of these, the present research study has been carried out to characterize physico- chemical and microbial parameters of the metal disposal site. The physical parameters such as pH (5-6.2), temperature (28℃-30℃), moisture (2.14% - 17.3%) including the nutrients nitrogen (.004mg/l-0.44mg/l), phosphorus (19.8mg/l-26.5mg/l) and sulphur (59.5mg/l-1200mg/l) provide environmental conditions to microbial consortium for growing and multiplying. The chemical parameters studied include Dissolved oxygen (7.4mg/l), Biological oxygen demand (3.8 mg/l), Chemical oxygen demand (64.6 mg/l) (Table 1) also show that the potential microorganism can grow in the metal contaminated environment by bioaccumulation followed by remediation [12].

The identified microbial consortium was studied by sequencing and drawing their phylogenetic tree for evaluating evolutionary relationships with closest neighbors (Table 2). The 16SrRNA technique employed shows the presence of microbial consortium such as E. coli, Salmonella, Shigella, Vibriocholerae , Yeast and Mould, S. aureus, Clostridium, Pseudomonas , Streptococcus faecalis, Serratia. The microbial consortium characterized using biochemical tests. Further, the microbial consortium was exposed to the increasing concentrations of heavy metal such as 5mg/l, 25mg/l, 50mg/l, 100 mg/l upto 800mg/l using scale up process. pH and optical density at 600nm were measured daily to analyze bacterial growth (Table 6). At a higher concentration of Iron (500mg/l) only one type of colony was found remained present. This shows the survival of potential microorganism at a higher concentration of iron (Fe). This potential microorganism found surviving at a higher concentration was further analyzed by 16SrRNA technique and developed sequence and phylogenetic tree using BLAST technique (Figure 1 & 2). DNA sequences were compared with already submitted sequence in nucleotide databases

available at NCBI website using BLAST software [17]. Further, most similar sequences were aligned by ClustalW and ClustalX software and phylogenetic tree was drawn using PHYLIP software to analyze evolutionary relationships among sequences of isolated microorganism and nearest neighbors. Sequence of potential microorganism was closely matched with Klebsiella pneumoniae. The present research show that bioinformatics applied in bioremediation [9] has useful technique to identify microbial consortium followed by potential microorganism which has the capacity to bioaccumulate /biodegrade/biotransform heavy metals from the contaminated environment. The research study done by Chaudhari et al. 2009 also identified potential microorganism as pseudomonas pseudoalcaligenes for bioremediation of TBP from organic waste disposal contaminated area using bioinformatics tools.[5] The bioinformatics tools such as BLAST, ClustalW, PHYLIP etc. have been found useful for identification of potential microorganism for remediation of heavy metal with special reference to iron, which could be useful for remediation of other heavy metals too. The studies carried out by Sharma et al. 2000 confirmed that K. pneumoniae has potential to bioaccumulate cadmium at high concentrations [16]. The similar studies conducted by Chaudhari et al. 1998 also confirmed that K. pneumoniae has potential to remediate heavy metals from contaminated environment [6]. The BLAST technique using sequencing and phylogenetic tree of the microorganism would be useful technique to identify potential microorganism for its versatile characteristics to remediate the contaminant and clean up the environment.

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TABLE 1: Physical and chemical parameters

Sr. No.	Parameters	Sample				Avg.
		I	II	III	IV	
1.	pH	5.1	6.2	5	5.3	5.4
2.	Temperature	30	28	30	32	30
3.	Odor	Pungent	Odorless	Earthy	Pungent	Pungent
4.	Color	Black	Brownish	Black	Black	Black
5.	Bulk density	1.21	1.12	1.12	1.14	1.15
6.	Moisture content	2.14	1 7.3	12.12	15.25	11.71
7.	Alkalinity	172.6	240	250.6	138	200.3
8.	Dissolved oxygen	7.8	6.5	7.6	7.6	7.4
9.	Biological oxygen demand	4.7	2.5	2.5	5.5	3.8

10.	Chemical oxygen demand	54.6	52	129.3	22.6	64.6
11.	Phosphate	24.3	26.5	19.8	20.0	22.65
12.	Sulfate	374.5	59.5	1200	603	559.3
13.	Total organic carbon	.006	.007	.003	.012	.007
14.	Nitrogen	0.44	0.33	0.004	0.02	0.20
15.	Total organic matter	0.012	0.134	0.28	0.22	5.6

TABLE 2: Microbial Characteristics of contaminated soil sample

Sr. No.	Bacterial Identification Method	Description	16S rRNA Gene Sequence
1	16S rRNA	*Bacillus cereus* strain IMAUB1020	CGCGAAAGCGTGGGGAGAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTGAGTGCTAAGTG TTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAA GGCTGAAACTCAAAGGAATTGACGGGGGCCCCCAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGC GAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCAAATAGGGCTTCTCCTTCGGGAGCAGAGTGAC AGGTGGTGCTGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT GATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGG GATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGA
2	16S rRNA	*Cloacibacterium normanense* strain tu33	GTAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGCTAGATTCGGCTCAACCGTGGAGGGTCATTGGAA ACTGGGGTWWCTTGRGKGSAGAAGAGGAAAGTGGAATTCCRAGTGTAGCGGTGAAATGCGTAGATATT TGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCG CCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA CCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGGTGG TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAKCT TAGTTGCCAGCATTCAGTTGGGCACTCTAAGATGACTGYCGGTGACAAACCGGAGGAAGGTGGGGATGA CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA
3	16S rRNA	*Bacillus licheniformis* strain RH104	TAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAMYYMCMYMRGSKCAACCGGGGAGGGTCATTG GAAACTGGGGCTTGAGTGSAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGA GCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCG CCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA CCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTG CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTA GTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG
4	16S rRNA	*Citrobacter freundii*-BI359	GGGTCGCGGGCTAAAACATGCAAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGG ACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG CATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTA GCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAG GTGTTGTGGTTAATAACCACAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCT GTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTT

			GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA AGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACGGATTAAAACGCG GGGGGTCCACAAA
5	16S rRNA	*Buttiauxella izardii* , DSM 9397	GCTTGCGGGGTTCCTACATTGGAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCG GACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACC GCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATT AGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCAC ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA GCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAA GGCGTTAAGGTTAATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTC TGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTT GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGACCTGAGGAATACCGGTGGCGAA GCGGCCCCTGGACAAAGACTGACGCTCAGGTCGAAACCTGGGGAGCAAACAGGATAATACCCGGTAGCC CACCC
6	16S rRNA	*Klebsiella pneumoniae, MBRG 4.15.*	CACCGGCATCCTCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGG ACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG CATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTA GCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAG GCGTTAAGGTTAATAACCTTATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA GCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTG TCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGT AGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAG CGGCCCCCTGGACAAAGACTGACGCTCAGTGCGAAAGCGTGGGGAGCAAACAGATTAGTACCG

TABLE 3: Biochemical characteristics shown by Klebsiella pneumoniae

Sr. No	Characteristics	Result	Sr. No.	Characteristics	Result
1.	ONPG	–	15.	Esculin	–
2.	Lysine decarboxylase	+	16.	Arabinose	+
3.	Ornithine decarboxylase	–	17.	Xylose	+
4.	Urease	–	18.	Adonitol	_
5.	Deamination	–	19.	Rhamnose	+
6.	Nitrate reduction	+	20.	Cellobiose	+
7.	H2S production	–	21.	Melibiose	+
8.	Citrate utilization	+	22.	Saccharose	+
9.	Voges Proskauer's	–	23.	Raffinose	+
10.	Methyl Red	+	24.	Trehalose	+
11.	Indole	–	25.	Glucose	+
12.	Malonate	_	26.	Lactose	+
13.	Gram Characteristic	–	27.	Oxidase	+
14.	Motility	Motile	28.	Shape	Rods

TABLE 4: Comparative chart of potentiality of microorganisms from soil against different concentration of Fe

Sr. No	Concentration of Fe	Growth observed	Types of isolates found after spread plate	Colony identification using 16SrRNA gene sequence analysis
1)	10ppm	+++	Five colonies	*Bacillus cereus* strain IMAUB1020 *Cloacibacterium normanense* strain tu33 *Citrobacter freundii*- BI359 *Buttiauxella izardii* ,DSM 9397 *Klebsiella pneumonia, MBRG 4.15.*
2)	25ppm	+++	Three colonies	*Bacillus cereus* strain IMAUB1020 *Cloacibacterium normanense* strain tu33 *Klebsiella pneumonia, MBRG 4.15.*
3)	50ppm	++	Three colonies	*Bacillus cereus* strain IMAUB1020 *Cloacibacterium normanense* strain tu33 *Klebsiella pneumonia, MBRG 4.15.*
4)	100ppm	++	Two colonies	*Bacillus cereus* strain IMAUB1020 *Klebsiella pneumonia, MBRG 4.15.*
5)	200ppm	+	One colony	*Klebsiella pneumonia, MBRG 4.15.*
6)	500ppm	+	One colony	*Klebsiella pneumonia, MBRG 4.15*

FIGURE 1: 16SrDNA sequence of Klebsiella pneumoniae, MBRG 4.15.

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>BI361
CACCGGCATCCTCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGAC
GGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATA
ACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGT
AGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAGGTTA
ATAACCTTATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGA
AATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGCGGCCCCCTGGACAAAGAC
TGACGCTCAGTGCGAAAGCGTGGGGAGCAAACAGATTAGTACCG
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FIGURE 2: Phylogenetic Tree showing evolutionary relationships of Klebsiel*a pneumoniae*

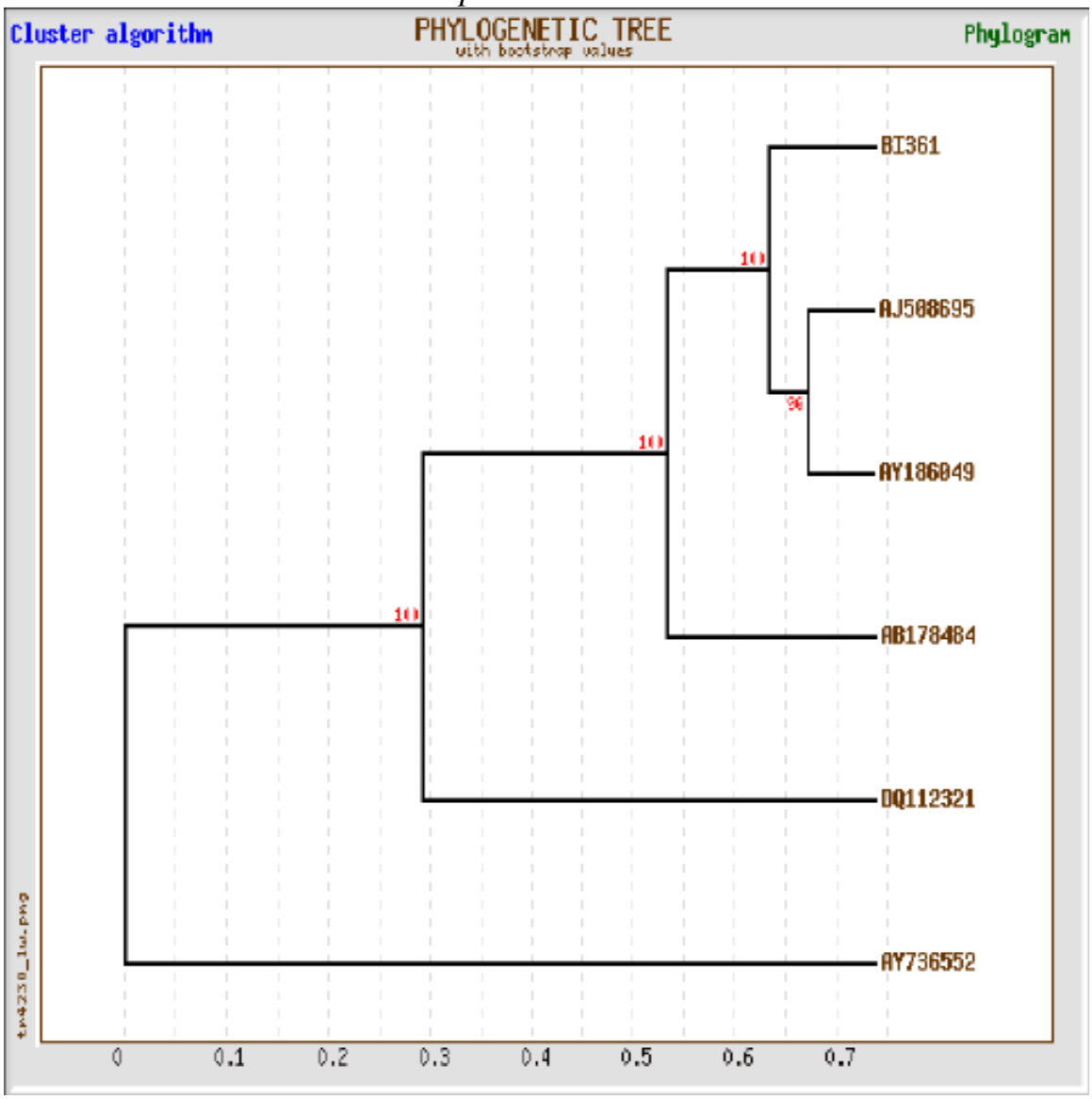


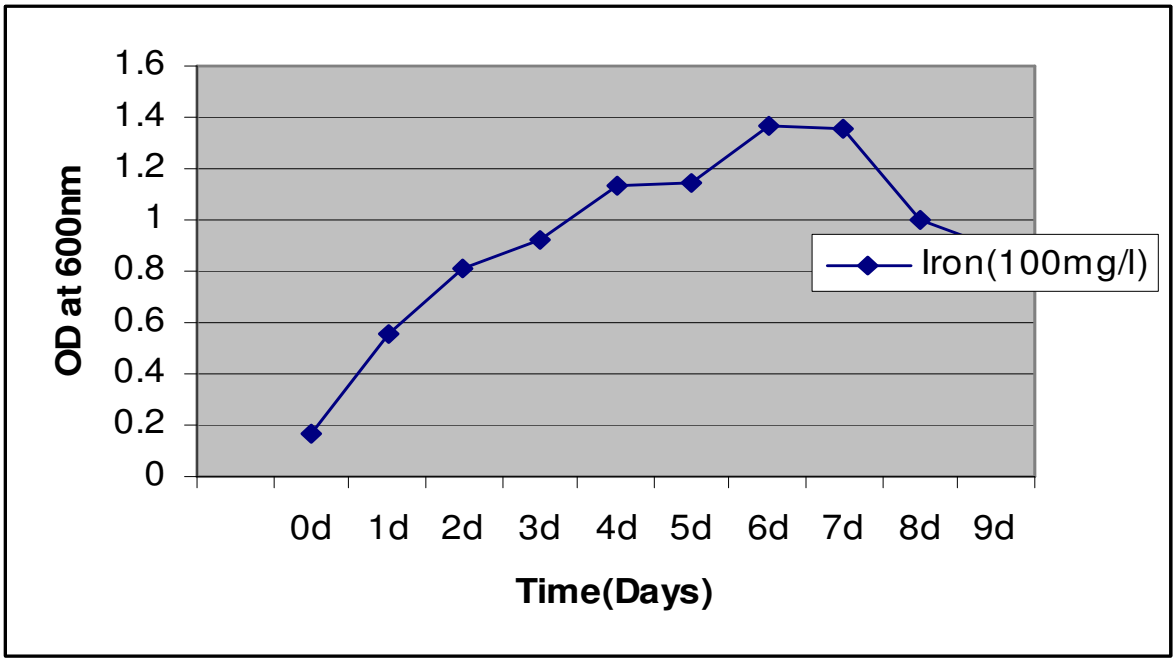
TABLE 5: Hit list and classification of Klebsiella pneumoniae showing evolutionary relationships

	Gene Bank Entry	Domain	Phylum	Class	Order	Family	Genus	Species
Sample BI 361	AJ508695	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	*pneumoniae*
	AY186049	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	*pneumoniae; m38.*
	AB178484	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	*pneumoniae*
	AY736552	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	*pneumoniae; OME1-3.*
	DQ112321	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	*granulomatis*

TABLE 6: pH observed during scale up process

Iron	**pH**
100mg/l	
0 day	7.0
1st day	6.4
2nd day	6.4
3rd day	6.2
4th day	5.1
5th day	4.9
6th day	4.7
7th day	4.7
8th day	4.6
9th day	4.9

FIGURE 3: Growth curve of Klebsiella pneumoniae



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