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ISOLATION, BIOCHEMICAL CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF ARSENIC RESISTANT BACTERIA ISOLATED FROM CONTAMINATED SITES OF UTTAR PRADESH, INDIA

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Abstract:

Heavy metals are responsible to create toxicity in environment and these causes serious challenge worldwide. Arsenic is a heavy metal that is non-essential but toxic to all living beings. Arsenic has always been under emerging issue due to its toxicity in living beings including human and animals. Arsenic (As) is a toxic pollutant released into the environment either by natural phenomena or anthropogenic activities. Arsenic is known as carcinogenic element that can harm not only human health but, plant and bacteria as well. Pesticides are the major source for accumulation of As in the agricultural soils. Currently available bioremediation techniques have major disadvantages such as secondary environmental pollution and are less cost effective. Therefore, the present study was aimed to isolate arsenic resistant bacteria from terrestrial environment Bahraich, Uttar Pradesh, India for their potential applications in bioremediation strategies. Total thirty eight isolated arsenic-resistant bacteria were isolated, in which three bacterial isolates BBAU/LP_3, BBAU/MMM_1, and BBAU/MMM_5 were taken for further studies due to their higher resistance ability to As and two isolates BBAU/LP_3 and BBAU/MMM_1 taken for molecular identification. The 16S rRNA gene sequence of the isolate BBAU/LP_3 belongs to the genera *Bacillus infantis* and BBAU/MMM_1 belongs to the genera *Bacillus litoralis*. The results revealed that our isolates BBAU/LP_3 and BBAU/MMM_1 encoded arsenite oxidizing gene and arsenate reducing gene respectively that will be useful for the development of efficient bioremediation strategies in the detoxification of arsenic from polluted environments.

Key Words: Arsenic, As-Resistance Bacteria, Biochemical, Molecular Identification Bacillus Infanti & Bacillus Litoralis

Introduction:

Heavy metals are responsible to create toxicity in environment and these causes serious challenge worldwide. Heavy metals are naturally occurring elements of the earth's crust that have high atomic weight as well as high density at least 5 times greater than that of water. They cannot be degraded or destroyed but can convert these metals from toxic to less toxic (Kabata-Pendias and Pendias, 1989). Arsenic is a naturally occurring and ubiquitous element with metalloid properties and highly mobilized element and mainly cycled by water in the environment. Arsenic is widely distributed in water, soil, rocks, sediments and metals ores in the form of oxyhydroxide or sulfide or compounds of various metals in the most part of world (Aronson, 1994). Arsenic naturally occurs in over 200 different mineral forms, of which approximately 60% are arsenates, 20% sulfides and sulfosalts and the remaining 20% includes arsenide's, arsenates, oxides, silicates and elemental arsenic (As). The soil arsenic level (13.12 mg/kg) crossed the global average (10.0 mg/kg), but within the maximum acceptable limit for agricultural soil (20.0 mg/kg) recommended by the European Union. The total arsenic concentration on food crops varied from 0.000 to 1.464 mg/kg of dry weight. The highest mean arsenic concentration was found in potato (0.456 mg/kg), followed by rice grain (0.429 mg/kg).The acceptable level of arsenic by WHO for maximum concentrations of arsenic in safe drinking water is 0.01 mg/L. The Bangladesh government's standard is a fivefold greater rate, with 0.05 mg/L being considered safe.

Arsenic have two forms inorganic and organic complexes in the environment, but the inorganic form of As is more toxic than organic. In inorganic form of As have two biological forms arsenate As(V) and arsenite As(III), which are inter convertible depending on the redox status of the environment (Asher and Reay, 1979). Arsenic is known to be a dangerous toxin that can lead to death when large amounts are ingested. Small amounts of arsenic exposure over long periods of time can also lead to numerous health problems, including abnormal heart beat, damage to blood vessels and a decrease of red and white blood cells, nausea and vomiting, and clearly visible irritations of the skin(Halim et al., 2009; Johnson et al., 2010). Arsenic is known as carcinogenic element that can harm not only human health but, plant and bacteria as well (Abernathy et al., 1999). Arsenic accumulates in the different tissues in different parts of the plant and adversely affects the growth and productivity of the plants (Zhao et al., 2010). The effect of arsenic on photosynthetic pigments, Chlorophyll-a and-b, growth behavior, and its accumulation in the tissues of different parts of onion plants (Allium cepa). Removal of arsenic from contaminated soil or water is important for providing safe drinking water. Remediation of arsenic from soil can be achieved mainly by chemical fixation, soil washing, electro-

remediation. Chemical fixation is viewed as one of the best remediation methods, as it suspends the mobilization of arsenic in soil. The development of Phytoremediation and bioremediation strategies for removal of heavy metals from contaminated soil is necessary. Phytoremediation is an inexpensive technology the use of plants for the removal of contaminants and metals from soil and water or to render then harmless. Bioremediation or conversion of inorganic arsenic to organic arsenic compounds with the application of microorganisms (molds, fungi and bacteria) is the simplest and most readily available remediation method. Extensive work on bioremediation has been done by many researchers. Electro-remediation is also a technique for removal of arsenic physically using direct current (DC) either by electro-migration, electro-osmosis, electrophoresis, landfill, thermal treatment and acid leaching are not suitable for practical applications, because of their high cost.

In the present study, As resistant bacteria were isolated from contaminated sites of paddy field from Bahraich (UP) and experiments were carried out to investigate a) the role of isolated resistant bacteria at different conc. of Sodium Arsenate b) show the morphology and biochemical parameters of isolates which was potential against Sodium Arsenate c) Show the molecular identification of potential isolate d) show the antibiotic sensitivity of potential isolates.

2. Method:

2.1 Sample Collection: Soil samples were collected from the different contaminated sites of paddy fields viz., Mari Mata Mandir, Govindpur, Rashulpur, Lalpur and Kochwa of Bahraich, Uttar Pradesh, India. The collected samples were filled into sterilize polythene bag, marked, kept in cold condition and carried to the departmental laboratory for further processing.

S.No	Sample Sites	Sample Code
1	Mari Mata Mandir, Bahraich	BBAU/MMM
2	Rashulpur, Bahraich	BBAU/RP
3	Lalpur, Bahraich	BBAU/LP
4	Govindpur, Bahraich	BBAU/GP
5	Kochwa, Bahraich	BBAU/KC

2.2 Isolation and Screening of as Resistance Bacteria: The soil sample (1g) was serially diluted with saline and plated on nutrient agar plate and minimal medium plate containing 1 mM of sodium arsenate (Na2HAsO4.7H2O). The plates were incubated at 37 °C for 72 hrs. After the growth was observed, the colonies were patched on fresh nutrient agar plate. A number of morphologically different colonies were randomly selected. Based on this preliminary screening, the colonies showing resistance to arsenate or arsenite were selected and used for further studies. The arsenic resistant bacteria was screened by MIC (Minimum inhibitory concentration) method (Calomiris et al. 1984) in medium containing sodium arsenate. A loopful of fresh culture (24 h) was streaked on nutrient agar plates supplement with different concentration of sodium arsenate (100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l, and 600mg/l). Then plates were incubated at 30^0C for 24-48 h, after incubation period, growth was observed for tolerance of arsenic resistant bacteria.

2.3 Biochemical Characterization of Bacteria: The bacterial isolates that could tolerate arsenate concentration were selected and identified by their morphological features and biochemical properties. The different biochemical characterization such as enzymatic activities (catalase, urease), methyl red test, Voges–Proskauer test and citrate utilization test were done for resistant isolated bacterial strain. Gram's stain test was performed as described by Vincent (1970), phosphate solubilization by Fiske and Subbarow, (1925) Indloe acetic acid by Bric et al, (1991), siderphore production by Schwyn and Neilands (1987) and other Biochemical characterization was determined by MR-VP test, Oxidase test, Starch hydrolysis, Gelatin hydrolysis, Triple sugar iron test, Mannitol salt agar, Dextrose, Sucrose, Maltose, Rhamnose, Arabinose and Sorbitol tests according to Bergey's Manual of Systematic Bacteriology, Claus and Berkeley (1986).

2.4 Determination of Antibiotic Resistance: Antibiotic sensitivity of the arsenic resistant isolates was determined by the disc diffusion method. Antibiotic-impregnated discs were placed on Nutrient Agar plates spread with bacterial culture and incubated at 37 °C for 24 hrs. Inhibition zone was noted after 24 hrs incubation, resistance was recorded as positive. The diameters of the inhibition zones around the discs were measured. The antibiotic concentrations of the disc used were Tetracycline (30 µg), Chloramphenicol (30 µg), Neomycin (30 µg), Penicillin (10 µg) and Streptomycin (10 µg).

2.5 16S rDNA Sequence Determination: Genomic DNA was extracted from BBAU/LP_3 and BBAU/MMM_1 as described by Sambrook et al. (2001). Bacterial 16S rRNA gene was amplified by PCR using the universal 16S rRNA primers, forward primer (5'-GGATTAGATACCCTGGTA-3') and reverse primer (5'- CCGTCAATTC MTTTRAGTTT-3'). PCR was carried out with 50 µL reaction containing 1X PCR buffer with 0.6 mM $MgCl_2$, 0.2 mM dNTP, *Taq* DNA polymerase 1U and 100 ng template DNA using a Gene Amp PCR system 2700 (Applied Biosystems) with the following cycling conditions, including initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 5 min. A negative control without the DNA template was used for amplification along with experiment. The PCR products were analyzed in 1.5% (w/v) agarose gel in 1X TAE

buffer, stained with ethidium bromide (0.5 mg/mL) and observed under ultraviolet light before being subjected to further analysis.

2.5.1 Nucleotide Sequencing: The purified products were sequenced by Aakaar Biotechnologies, Lucknow Pvt., Ltd, India. The BLASTn program (www.ncbi.nlm.nih.gov) was used for 16S rDNA based identification of the isolates and sequences submitted to GenBank. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M.1987). The optimal tree with the sum of branch length = 0.19467852 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein J.(1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K., Nei M., and Kumar S. 2004). And are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1375 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al. 2013)

3. Results:

3.1 Isolation and Screening of Arsenic Resistant Bacteria: Total 38 strains were isolated from these sampling sites which was resistant to different concentration of sodium arsenate. All these 38 isolated bacteria was grown on the different concentration of sodium arsenate about range from 100 to 600ppm but as the concentration of sodium arsenate was increase number of bacteria was decrease, that showed the reduction in minimum number of bacteria as conc. of sodium arsenate was increased. Out of 38 isolates, only two bacteria BBAU/LP_3 and BBAU/MMM_1 was observed at 600ppm and three bacteria BBAU/LP_3, BBAU/MMM_1, and BBAU/MMM_5 at 500 ppm while at 100, 200 and 300 ppm conc. generally grow so many number of bacteria (Table-1).

3.2 Biochemical Characterization of Bacteria: The preliminary identification of strains indicates that 12 isolates were Gram- negative rod shaped bacteria and 26 isolates were gram positive cocci. From these, three bacterial isolates (BBAU/LP_3, BBAU/MMM_1, and BBAU/MMM_5) were selected based on their higher arsenic resistance. The strains BBAU/LP_3 and BBAU/MMM_1 were found to be Gram-negative rode shape, non-motile and formed white colonies and pink colonies respectively while BBAU/MMM_1 was gram positive cocci, motile and formed yellowish colonies. In (Table-2) we shows the detailed analysis of morphological and biochemical characterization of three potential arsenic resistance strains BBAU/LP_3, BBAU/MMM_1, and BBAU/MMM_5.

3.3 Determination of Antibiotic Resistance: Antibiotic susceptibility test revealed that all three isolates BBAU/LP_3, BBAU/MMM_1, and BBAU/MMM_5 were sensitive to Streptomycin (10 µg), Neomycin (30 µg) and Chloramphenicol (30 µg) but resistant from Tetracycline (30 µg) and Penicillin (10 µg) (Table-3).

3.4 16S rDNA Sequences and Phylogenetic Analysis: The 16S rDNA sequences of BBAU/LP_3 and BBAU/MMM_1 isolates were subjected to nucleotide BLAST. BBAU/LP_3 showed 99% similarity to *Bacillus infantis* and BBAU/MMM_1 showed 98% homology to *Bacillus litoralis* (Figure 1 and 2). The 16S rDNA nucleotide sequence of BBAU/LP_3 and BBAU/MMM_1 were submitted in the NCBI.

4. Discussion:

The molecular approaches are frequently being used to investigate the specific microbial communities associated with metal-contaminated environments with increased sensitivity Miguez et al. (1997) PCR and gene probes are used to characterize the environment prevalence of microbial community associated with the polluted environment and development of genetic model system for efficient bioremediation strategies. The reduction and methylation rates of arsenic, necessary prerequisites to arsine production, vary greatly depending on soil properties, such as soil moisture and temperature, abundance of different species of arsenic and microbial populations in soil (Gao and Burau, 1997).

The isolation of arsenic resistant bacteria is the preliminary step for identification of potential candidates. In the present study, three arsenic resistant bacteria (MMM_1), (MMM_5) and (LP_3) were isolated from arsenic contaminated paddy soil. The soil profile analysis of samples (site MMM & LP) revealed relatively acidic pH and moderately low concentration of arsenic. The resistance limit to the highest concentration of sodium arsenate was evaluated based on the ability of MMM_1, MMM_5 and LP_3 cells to grow on sodium arsenate containing agar media. Isolates MMM_1, MMM_5 and LP_3 exhibited natural resistance up to 600 (ppm) for sodium arsenate in solid media. Notably MMM_1, MMM_5 and LP_3 strains showed the highest resistance to arsenic reported thus far. These bacterial isolates can be helpful to explore the diversity of arsenic resistance system genes in a variety of arsenic resistant bacterial groups. In our study soils, the microbial transformation of water soluble As(V). Under both aerobic and anaerobic laboratory conditions to As(III), MMAA and DMAA and to volatile TMA altogether was less than 0.5%, of which the production of TMA represented 0.02-0.3% . Friis et al. (1986) previously reported that reduction in growth is mainly because of the interaction between the cell surface and of metal cations along with phosphate, carboxyl, and hydroxyl and amino-groups. The 'life-time' of arsines in air is usually short since they are easily oxidized to MMAA, DMAA and TMAO and finally, to inorganic As(V), (Cullen and Reimer, 1989). In the present study, we have isolated and characterized three arsenic resistance bacterial isolates of MMM_1, MMM_5 and LP_3. The ability of the isolates MMM_1, MMM_5 and $PL_3$to oxidize the toxic As (III) to its less-toxic As **(**V) could be used for its potential application in

bioremediation processes, since the most suitable way to remove arsenic removal from environment is oxidizing arsenite into arsenate, which is less soluble and could be removed easily from the environment.

In previous studies have revealed that the application of bacteria resistant to arsenate (10.13 mM) in bioremediation processes, Takeuchi et al. (2007). Awais et al. (2011) have identified potential strains of *Klebsiella pneumoniae* (*K. pneumonia*) and *Klebsiella variicola* (*K. variicola*) with minimum inhibitory concentration of 26.6 and 24 mM against As (III). The MIC of arsenic in solid media was higher than those in liquid media due to the conditions of diffusion, complexation and availability of arsenic was different from those observed in solid media. To control risk management of arsenic contaminated soil and aquatic ecosystem is an important issue and a great challenge, its success is necessary to promote sustainable environmental health and also to minimize the adverse impact on humans and plants. These methods can reduce arsenic toxicity with help of arsenic resistant bacteria which is present in the fields and after the mass culture it is applied in the arsenic contaminated soil. There for it is imperative to search for the new bacterial strains, which are capable to tolerate high concentration of arsenic and can be used near future for the bioremediation of arsenic contaminated soil.

5. Conclusion:

The increase global population, industrialization and urbanization are the some major reasons to contaminate the environment. Arsenic contamination of soil and ground water is an alarming problem on a global scale. If these problems are not addressed, these could create disastrous effects on human and animal health as arsenic is carcinogenic in nature. In several parts of the world, biogeochemical processes have resulted in dissolution of naturally occurring as into soil. Many microorganisms have already evolved mechanisms to cope with this environmental challenge and these noble organisms could be exploited properly to remediate arsenic contaminated soil and water. The release of heavy metals from industries causing the serious health problem to human and other animals and also pollutes the environments due to their persistence and bio-accumulative nature. In this regards, bioremediation process provides and effective innovative measures for treatment of a wide variety of contaminants. Amongst the various known bioremediation process phytoremediation, rhizoremediation and bioremediation by microbes could be efficient methods to reduce the arsenic and lead contaminants of soil. Microbially mediated oxidation and reduction reactions may produce less mobile arsenic species and mixed solid phases capable of sorbing arsenic, thus enhancing the immobilization processes. The government should monitor industrial and agricultural activities leading to As pollution. The awareness of the population is deemed equally important in maintaining and choosing mitigation. However, even for well-aware population, the dilemma is often the ability to meet prohibitive costs versus the wish to improve their situation. Supervision departments should increase the frequency of sampling and analysis of the discharge from industrial plants. We sincerely hope that this paper will be of considerable interest to the readers. The paper reflects the latest state of the art on understanding of various interdisciplinary facets of the problem of arsenic in environmental realm, mechanisms of mobilization in groundwater, biogeochemical interactions, and the measure for remediation.

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7. Conflict of Interest:

The authors declare that there are no conflicts of interest associated with this work.

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Legends:

Table- 1: Presence of Bacteria in Sample at different concentration of Sodium Arsenate
Table -2: Morphological and biological characterization
Table -3: Antibiotic sensitivity test result

Figure Caption:

Figure 1: Electrophoretic analysis of amplified product and evolutionary relationships of taxa from isolates BBAU/LP_3 and BBAU/MMM_1.
Figure 2: Antibiotic sensitivity results of isolates from antibiotics Streptomycin (10 µg), Neomycin (30 µg), Chloramphenicol (30 µg), Tetracycline (30 µg) and Penicillin (10 µg). The sensitive antibiotic are Streptomycin (10 µg), Neomycin (30 µg).

Table 1: Presence of Bacteria in Sample at different concentration of Sodium Arsenate

Concentration of arsenate (mg/L)	Sample Code														
	BBAU/MMM				BBAU/LP			BBAU/RP			BBAU/GP			BBAU/KC	
	Site 1	Site 2	Site 3	Site 5	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2
100	+++	+ +	++	+++	+++	++	+++	+++	++	++	++	++	++	+++	++
200	+++	++	+	++	++	+	++	+	+	++	++	+	++	+	+
300	++	+	+	+	+	+	++	+	+	+	+	+	+	+	+
400	+	-	-	+	+	-	+	+	-	-	+	-	+	-	-
500	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-
600	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-

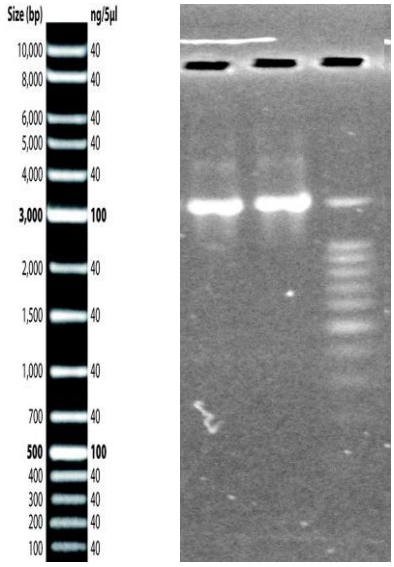
Table 2: Morphological and biological characterization

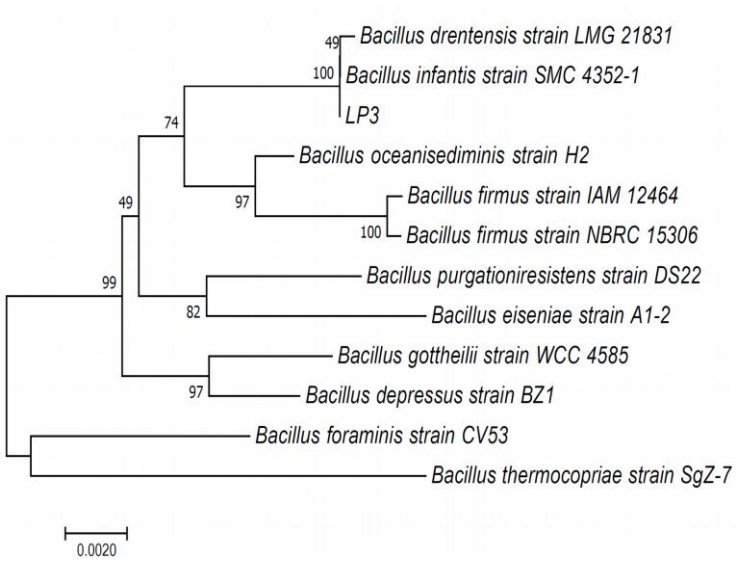
Biochemical Test's	Isolates		
	BBAU/ LP_3	BBAU/MMM_5	BBAU/MMM_1
Color	Whites	Yellowish	Pink
Gram stain	- ve	+ ve	- ve
Shape	Rod	Cocci	Rod

Motility	+	-	+
Methyl Red Test	+ weak	+	+ weak
Voges-Proskauer	+	-	+
Citrate utilization	+	-	-
Starch hydrolysis	-	-	-
Catalase test	+ weak	-	+ weak
Casein hydrolysis	-	-	-
Carbohydrate lactose	-	+	-
Sucrose	-	+	-
Mannitol	-	+	-
Urease	-	-	-
Indole acetic acid	-	+	-
Amylase	-	+	-
H_2S production	+	-	+

Table 3: Antibiotic Sensitivity Test Result

S.No	Antibiotic Used	Code disk Mcg	MMM_1	MMM_5	LP_3
1.	Streptomycin	(S10)	S	S	S
2.	Penicillin G	(P10)	R	R	R
3.	Neomycin	(NA30)	S	S	S
4.	Chloroamphenicol	(C30)	S	S	S
5.	Vancomycin	(VA 10)	R	R	R





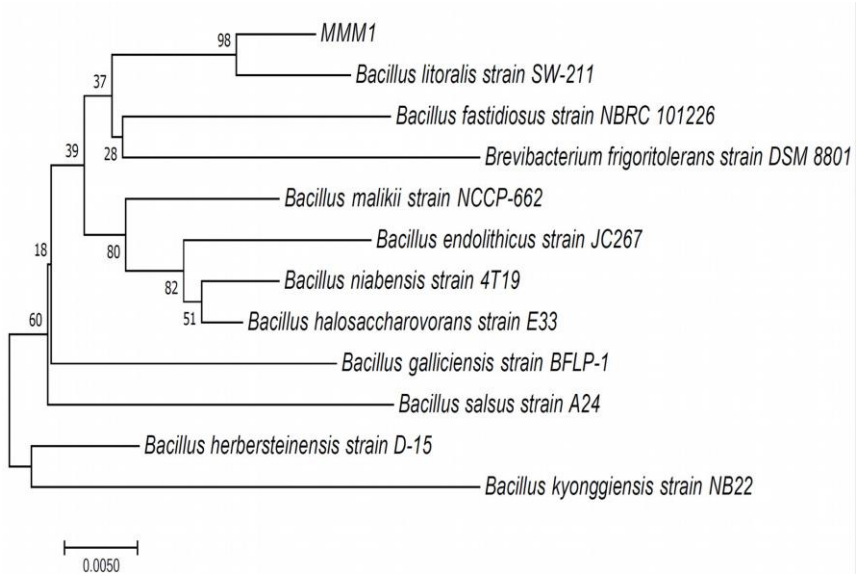


Figure 1: Electrophoretic analysis of amplified product and evolutionary relationships of taxa from isolates BBAU/LP_3 and BBAU/MMM_1.

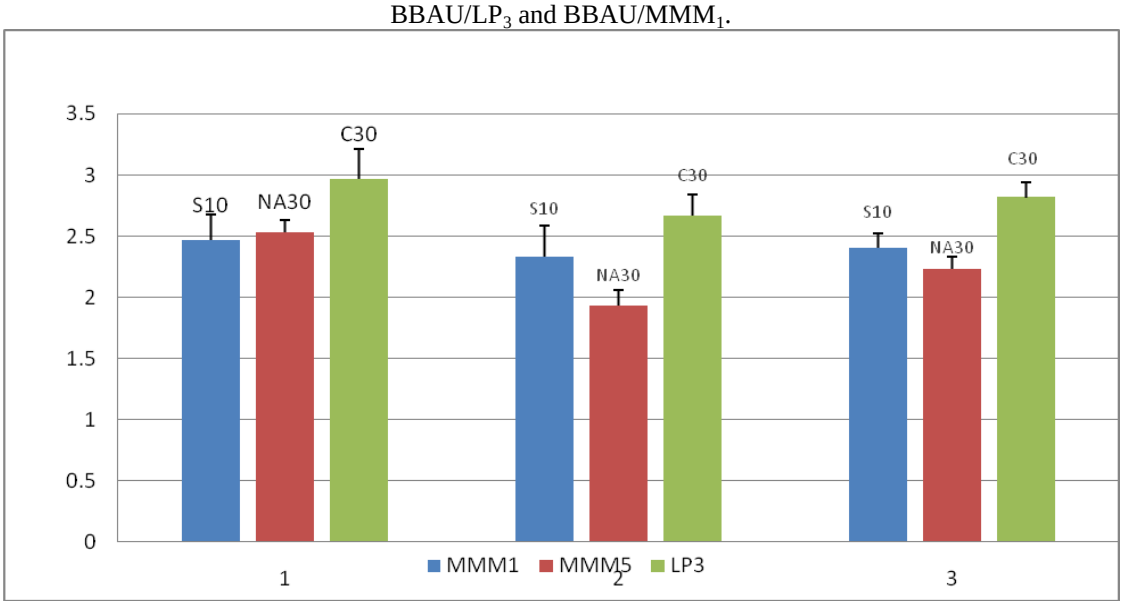


Figure 2: Antibiotic sensitivity results of isolates from antibiotics Streptomycin (10 µg), Neomycin (30 µg), Chloramphenicol (30 µg), Tetracycline (30 µg) and Penicillin (10 µg). The sensitive antibiotic are Streptomycin (10 µg), Neomycin (30 µg), and Chloramphenicol (30 µg).