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IN VITRO ANALYSIS OF ANTIBACTERIAL ACTIVITY OF VITAMIN C ALONE AND IN COMBINATION WITH ANTIBIOTICS ON GRAM POSITIVE ROD ISOLATED FROM SOIL OF A DUMPING SITE OF KOLKATA

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ABSTRACT

In today's fast moving world antibiotics resistance from soil borne infections isa major concern for us. So, medical practitioners have gained interest in treating infections with antioxidants containing dietary supplements, such as Vitamin C (Ascorbic acid) solely or in combination with antibiotics. In our investigation of soil samples containing various wastes showed the presence of Gram positive rods and 16s rDNA characterization confirmed the organism as Bacillus cereus strain KD125. In-vitro the antibiotic susceptibility test our isolate showed maximum sensitivity to Linezolid (LZ) and was found to be least sensitive or intermediate sensitive to Ampicillin (AMP). It is also found that Bacillus cereus strain KD125was sensitive to Vitamin C andin combination Vitamin C mostly decreased the antibacterial activity of Ampicillin (AMP), Linezolid (LZ), and Chloramphenicol (CHL) but had slightly increased the antibacterial activity of Azithromycin (AZT) and did not affect the antibacterial activity of LZ at higher concentration. This increase in antibacterial activity may be due to presence of flavonoids and phenolic present in Vitamin C. Moreover, it was also seen that observed LZ had maximum antibacterial activity in presence of Vitamin C. So, systematic use of Vitamin C alone or in combination can help to treat this food poisoning causing pathogen Bacillus cereus efficiently.

KEY WORDS

Antibiotics, rDNA, Vitamin C, Soil

INTRODUCTION

In this modern era antibiotics are the most efficient prescribed medication for any infections but unfortunately bacteria gained ability to destroy the effectiveness of these antibiotics [1] and efficiently survive within the human body. Therefore, antibiotic resistance is one of the major concerns of treatment in today's world. It has been seen that misuse[2] and overuse of antibiotics[3] have benefitted many bacteria by expressing their resistance genes[4] and lateral or horizontal gene transfer of these resistance genes[5,6] among the population of microbes and lead to development of multiple resistance[7] against each and every antibiotics introduced for their treatment. Moreover, antibiotic resistance evolves naturally because of the morphological structure of the bacteria [8] or may be due random mutation of the genome [9] or may be due to ability the microorganisms to gain a morphological structure during a stress that resists access of antibiotic or

drugs to destroy the microorganisms [10].Thus drugs are causing complication in treatment and increasing the cost of treatment [11]. So, to diminish the effect of antibiotic resistance researchers have introduced various semi synthetic antibiotics but microbes also gained antibiotic resistance against these semi-synthetic antibiotics along with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions[12]. So, this has forced the use of antioxidants or our dietary supplements to combat these side effects or enhance the antibacterial activity of the antibiotics [13]. In recent times, antioxidant, dietary supplements, such as Vitamin C (Ascorbic acid) is very effective against various bacterial infections solely or consumed with many antibiotics [14]. It is also found to have antibacterial and antiviral activity [15] and is found effective in treating infections that causes whooping cough, diphtheria, tetanus, polio or infections due to AIDS [16]. But, recently it is seen that consumption of Vitamin C or any other supplements with antibiotics sometimes decreases the antibacterial activity of many antibiotics [17]. So, this has prompted us to isolate a bacterium from soil contaminated from dumping ground and perform *in-vitro* antibacterial activity Vitamin C, commonly used antibiotics along with combination of Vitamin C with these antibiotics.

MATERIALS AND METHOD

(i) Sample Collection:

Soil sample was collected from dumping ground of South Kolkata (88.36^{0}E and 22.56^{0}N) in the month of January. The collection process was done aseptically from the surface layer (0-15cm). The temperature of the region during the time of collection was 15-16ºC and relative humidity was recorded as 42-48%. Other physical parameters like pH and Electrical Conductivity (EC) were also measured carefully [18].

(ii) Isolation Of Bacteria:

Soil sample was serially diluted using 10 fold dilution upto 10^{-6}[19] and this serially diluted soil sample was poured on a Mueller Hinton Agar (MHA, HiMedia, Mumbai) plate and after 24 hours of incubation at 37^{0}C, the colonies obtained were observed and an off-white irregular shaped colony was chosen for the isolation of pure colonies. The pure colonies were named as Sample 'S'.

(iii) Staining And Measurement:

Pure colonies of Sample S were taken for Gram Staining and the size of the bacterium was determined using stage and ocular micrometer [20].

(iv) Determination Of Susceptibility Of Sample S On Antibiotics And Vitamin C Alone And In Combination:

The antibiotic sensitivity test was carried by agar-diffusion method[21] by following the principle of Kirby Bauer 1959[22].The assay was carried out by spreading 100µl of 24 hours old culture of Sample S on MHA plates and then 30 µl and 50 µl of Vitamin C and commonly marketed antibiotics like Ampicillin (AMP), Linezolid (LZ), Chloramphenicol (CHL) and Azithromycin (AZT) were loaded in the well and there zone of inhibitions were calculated in millimeter (mm) and was compared with water as control (Cw). Similarly, AMP, LZ, CHL and AZT were combining with Vitamin C in equal volume and 30 µl and 50 µl were loaded in the well to observe the antagonism and synergism among these antibiotics.

(V) Identification of Sample S 16S rDNA:

Bacterial identification was carried out based on percent similarity of 16S rDNA using PCR technique, DNA sequencing and similarity analysis of rRNA genes. A direct comparison of 16S rDNA sequence is probably the most powerful tool for the identification of many bacteria [23]. So, DNA was isolated from pure culture of Sample S and its quality was evaluated on 1.2% Agarose Gel. Fragment of 16S rDNA gene was amplified by PCR from the isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel (Figure1). The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1297 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nr database of NCBI genbank database[24]. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment

software Clustal W and the phylogenetic tree was constructed using MEGA4[25].

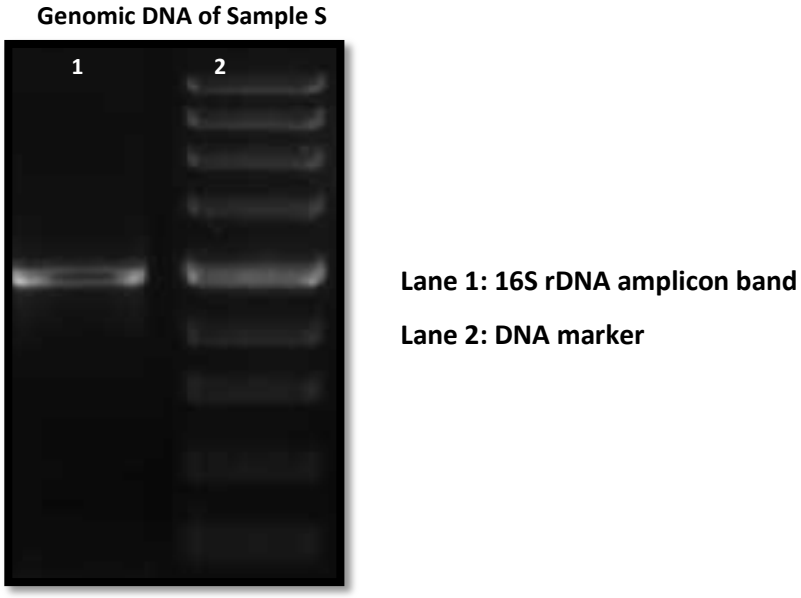


Figure 1: Gel Image Of 16S rDNA Amplicon of Sample S

RESULTS

1. Measurement of Physical Parameters of Soil Sample

The physical properties of the soil (Table1) are very much responsible for the kind of microorganism present. So the physical properties of the soil such as soil type, texture, pH and electrical conductivity was measured properly.

Table1: Physical parameters of Soil Sample

Soil Type	Texture	pH	Electrical Conductivity*(µs/cm)
Clay	Granular	6.07 ±0.3	280.3±0.05

***Results on the basis of three replicates/treatment**

2. Bacterial Characterizations

The Sample S were taken for colony characterization (Table 2), Gram staining and measurement of cell (Table2). The colony characterization showed it to be off white, irregular shaped colonies in MHA medium (Figure 2). The Gram Staining showed it to be Gram Positive short rods, measuring about 2.32±0.01µm in oil immersion (1000X) bright field microscope (Figure 3).

Table 2: Colony Characterization of Sample S

Colony shape	Colour	Gram Character and Morphology	Size (µm)*
Irregular	Off-white	Positive Short rods	2.2±0.4

***Results on the basis of three replicates/treatment**

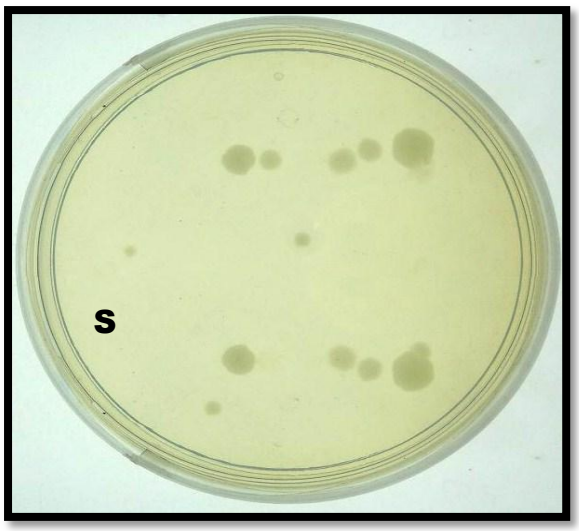


Figure 2: Pure Colonies of Sample S

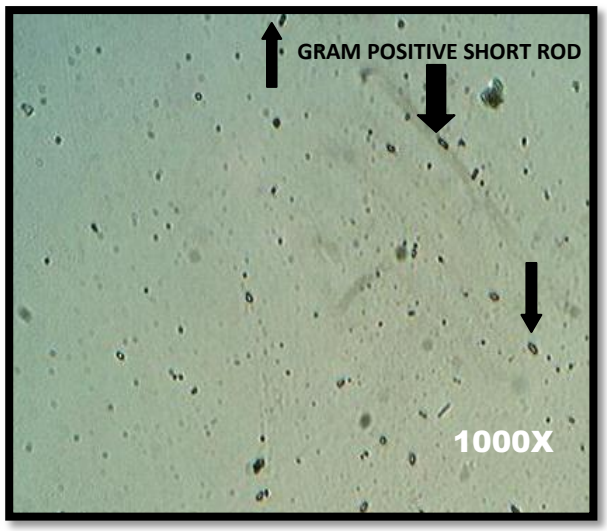


Figure 3: Gram Staining of Sample S

3. Determination of Susceptibility of Sample S on Antibiotics and Vitamin C Alone and In Combination

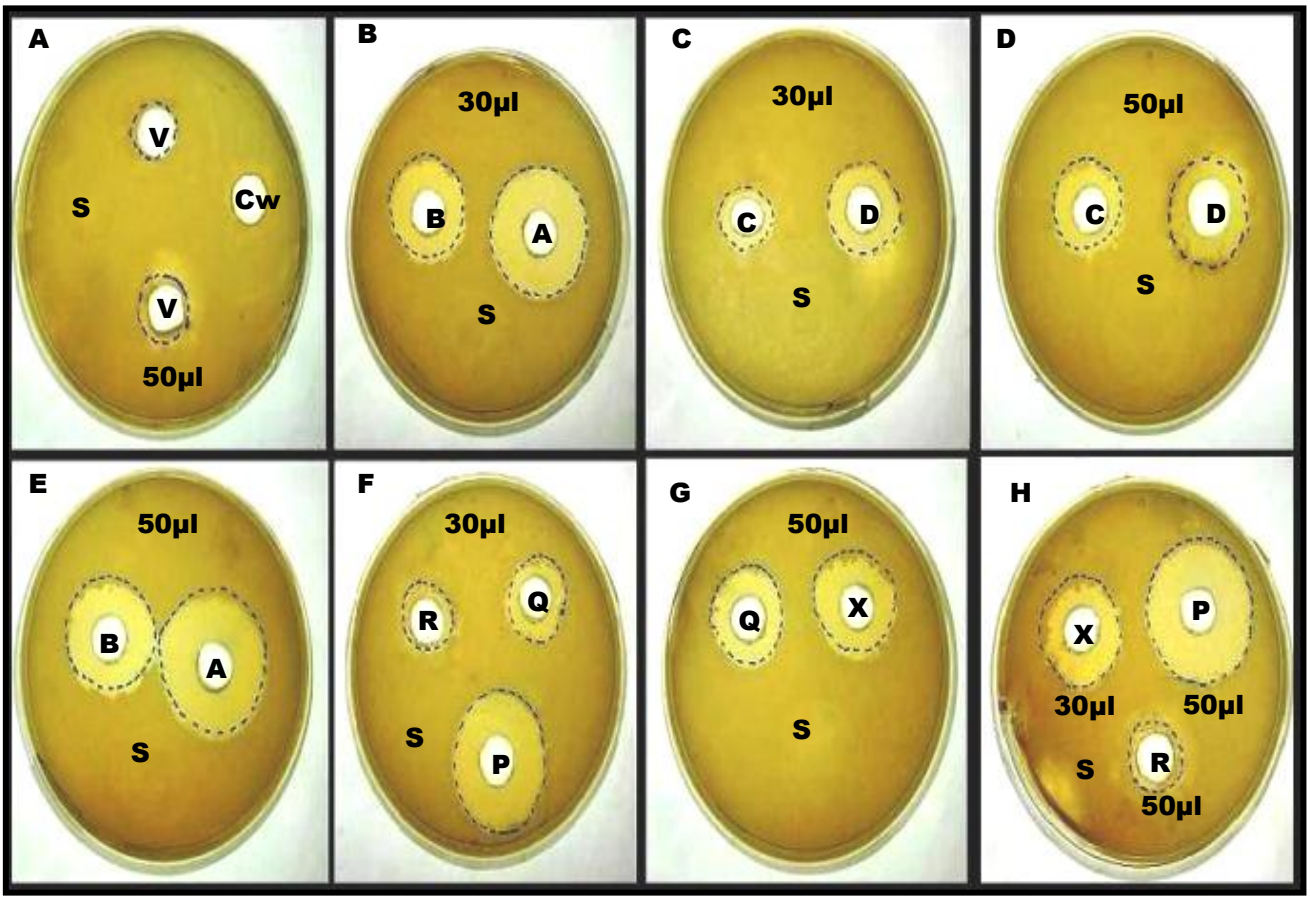
The susceptibility(Table3) of Sample S towards Antibiotics and Vitamin C alone and in Combination was determined by measuring the diameter of the zone of inhibitions obtained from Vitamin C, antibiotics and Vitamin C combined with antibiotics and the antibacterial activity was classified[26] into the following types:

>21 mm zone of inhibition is sensitive(S)

16-20 mm zone of inhibition is intermediate (I) and

<15 mm zone of inhibition is resistant(R)

Thus, from the classification it was seen that Sample S was maximum sensitive to LZ 50µl 31.6±0.4mm (Figure 4E) followed by LZ 30µl with zone of inhibitions 31.33±0.4mm (Figure4B) respectively. The Sample S had also shown sensitivity to 50 µl CHL and 50 µl AZT (Figure 4B & 4D) with zone of inhibitions respectively. It was seen that the isolate was also sensitive to 30 µl CHL(Figure 4B) and intermediate sensitive to 30 µl AZT, 30 µl and 50 µl of AMP(Figure 4D & 4C) with zone of inhibitions 24.0±0.5mm, 19±0.04mm, 16.3±0.4mm and 17.6±0.4mm respectively. This assay(Figure 4A) had also shown that 30 µl of Vitamin C did not produced any zone of inhibition and 50 µl of Vitamin C had produced zone of inhibition12.06±0.4mm but both of these concentrations were resistant to Sample S. Moreover, it was seen that 30 µl of Vitamin C was showing antagonism(Figure 4F,4H) against the antibacterial activity in combination with 30 µl of LZ,CHL,AMP and AZT and decreased the zone of inhibitions by 1.99 ± 0.4mm, 1.0 ± 0.5mm, 3.0 ± 0.4mm and 2.0 ± 0.4 mm compared to 30 µl of LZ, CHL, AMP and AZT (Graph1). Whereas, combination of 50 µl of Vitamin C with CHL and AMP (Figure 4G & 4H) also decreased the zone of inhibitions by 1.6±0.4mm and 3.00±0.4mm and did not produced indifference in zone of inhibition of 50 µl LZ and also showed synergism with 50 µl AZT (Figure 4G & 4H) and increased the zone of inhibition by 0.6±0.4mm (Graph1). But, fortunately, it was observed that all combinations of LZ and CHL with Vitamin C were sensitive on Sample S. It was also observed that combination of 30 µl of AZT with 50 µl of Vitamin C was found sensitive to Sample S but combination of 30 µl of Vitamin C was intermediately sensitive to our isolate and unfortunately all tested combinations of AMP and Vitamin C were resistant on Sample S.



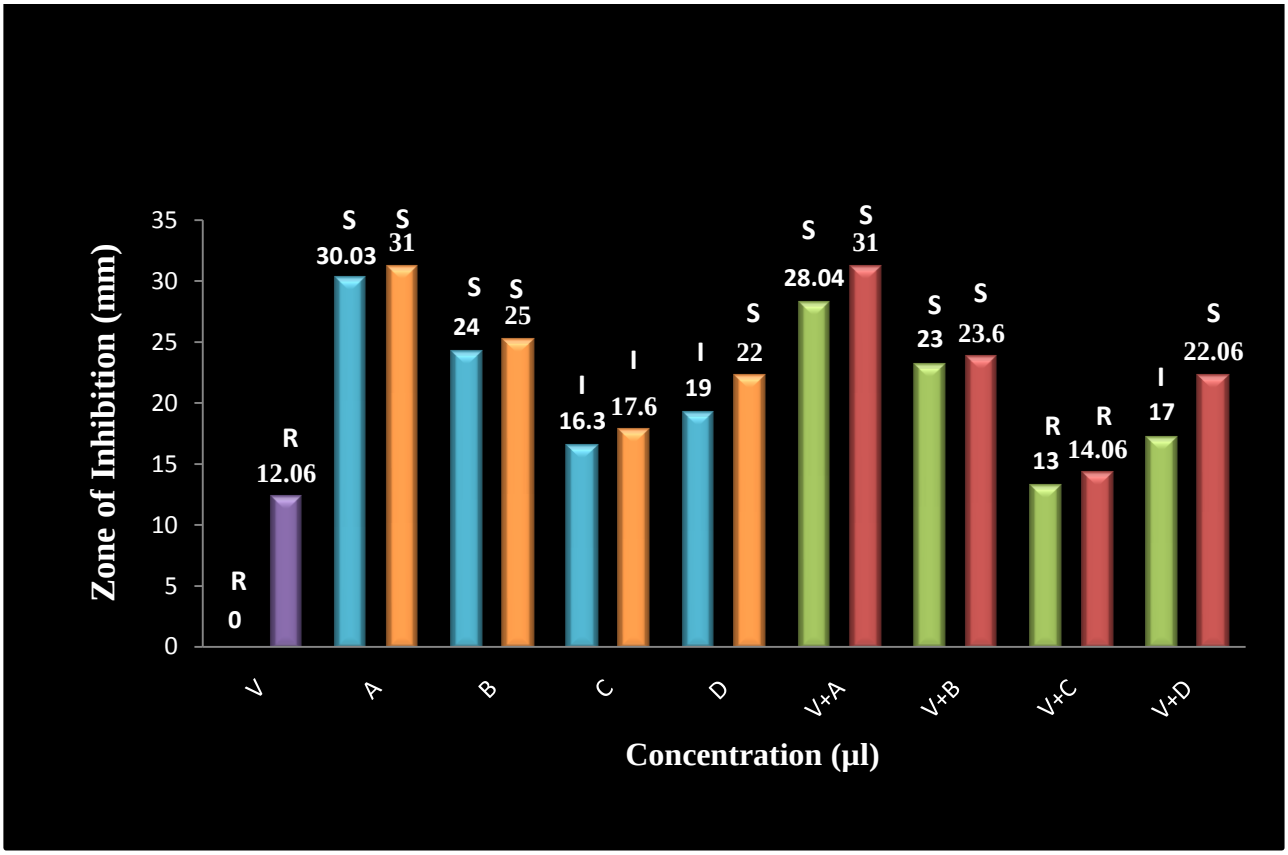
V- Vitamin C, A-LZ, B-CHL, C-AMP, D- AZT, P- Vitamin C+ LZ,Q- Vitamin C+ AZT, R- Vitamin C+ AMP, X- Vitamin C+ CHL and Cw- Control water.

Figure 4: Zone of Inhibitions of Commonly Used Antibiotics and Vitamin C Alone and In Combination on Sample S

Table 3: Determination of Susceptibility of Sample S on Antibiotics and Vitamin C Alone and In Combination

Sample	Zone of inhibition(mm)*		Inference	
	30µl	50 µl	30µl	50 µl
Vitamin C	0±0.0	12.06±0.4	R	R
LZ	30.03±0.4	31.0±0.5	S	S
CHL	24.0±0.5	25.0±0.5	S	S
AMP	16.3±0.4	17.6±0.4	I	I
AZT	19±0.04	22±0.4	I	S
Vitamin C +LZ	28.04±0.4	31.0±0.5	S	S
Vitamin C +CHL	23.0±0.4	23.6±0.4	S	S
Vitamin C + AMP	13±0.5	14.06±0.4	R	R
Vitamin C + AZT	17±0.5	22.06±0.4	I	S

*Results on the basis of three replicates/treatment



V- Vitamin C, A-LZ, B-CHL, C-AMP and D- AZT

[>21 mm- Sensitive(S), 16-20 mm-Intermediate (I) and <15 mm-Resistance (R)]

50µl VitaminC **30 µl Antibiotics** **50 µl Antibiotics**

30 µl Vitamin C + Antibiotics **50 µl Vitamin C + Antibiotics**

Graph 1: Comparative Analysis of Mode of Action of Commonly Used Antibiotics and Vitamin C Alone and In Combination on Sample S

4 Identification of Sample S by 16SrDNA

The identification of Sample S was carried out by BLAST (Figure 5) of consensus sequence 1297bp obtained from the alignment of the sequences of reverse and forward primers. The BLAST with the nr database of NCBI genbank database showed the distribution of 272 blast hits on the Consensus sequence(1297bp) matched the alignment scores ≥200 with100% similar to ***Bacillus cereus*** **strain KD 125**(GenBank Accession Number:**JQ580958.1**)and was also closely related to different strains of *Bacillus* species (Table 4). The E value (Expect value) of all the strains were found to be 0.0 which depicts that all the strains are homogenous to ***Bacillus cereus*** **strain KD 125** (GenBankAccession Number: **JQ580958.1**). The phylogenetic tree (Figure 6) constructed by Neighbor-Joining method [27] showed the SampleS was closely related to ***Bacillus anthracis*****strain APT25** (Gen Bank Accession Number: **KC519402.1**) and distantly related to ***Bacillus anthracis*** **strain APT24** (GenBank Accession Number: **KC519401.1**).

Description	Max score	Total score	Query coverage	E value	Max ident
Bacillus cereus strain KD125	2396	2396	100%	0.0	100%
Bacillus thuringiensis strain KUNi1	2353	2353	100%	0.0	99%
Bacillus cereus strain KD33	2340	2340	100%	0.0	99%
Bacillus sp. P014	2335	2335	100%	0.0	99%
Bacterium CulalnoE420	2335	2335	100%	0.0	99%
Bacterium CulalenE32	2335	2335	100%	0.0	99%
Bacterium CulvinoE21	2335	2335	100%	0.0	99%
Bacillus anthracis strain APT9	2335	2335	100%	0.0	99%
Bacillus anthracis strain APT25	2335	2335	100%	0.0	99%
Bacillus anthracis strain APT24	2335	2335	100%	0.0	99%

Table 4: Sequence Producing Significant Alignments

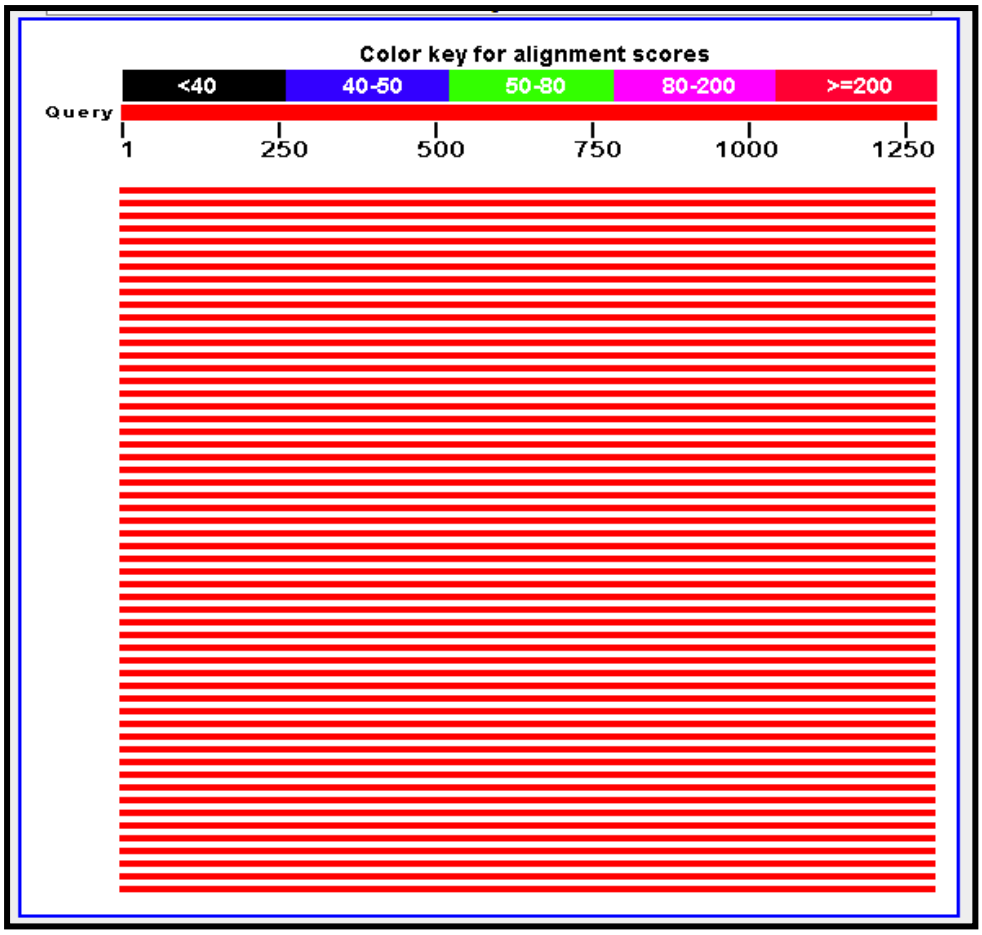


Figure 5: Distribution of 272 Blast Hits on the Query Sequence

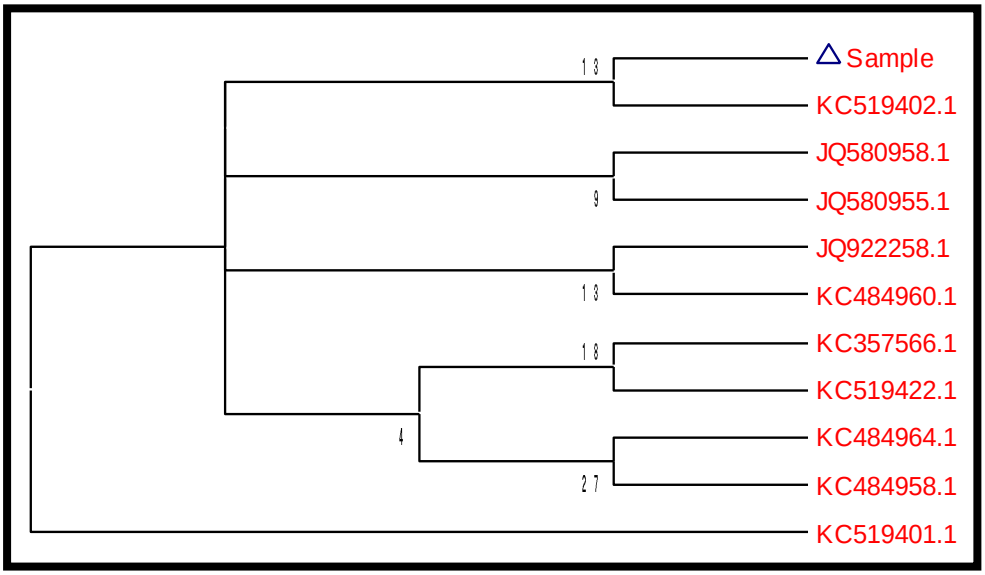


Figure 6: Evolutionary relationships of 11 taxa

DISCUSSION

In today's world treating a disease caused due to any infectious agent of soil, is a major problem, due to antibiotic resistance [28]. So, researchers have gained interest in treating these diseases using antioxidant compound, like Vitamin C alone and in combination with some antibiotics [29]. In our investigation of soil containing dumps also showed the presence of Gram Positive Rod and 16S rRNA characterization showed it to be 100% similar to *Bacillus cereus* strain KD125 (JQ580958.1). This may be due to fact that Gram positive Bacillus sp only survives in the pH(6-7) and our soil sample pH was around 6.7±0.2.This bacterium is more common in production of toxins in the rice product and in turn induces food poisoning in an individual[30].So, it is important to find possible treatment for this pathogen. In our investigation fortunately Sample S produced zone of inhibitions to all tested antibiotics with maximum sensitive to 50µl LZ least or intermediate sensitive to AMP 30µl.Sample S was also resistant to 50µl Vitamin C but the positive aspect is that it has produced a zone of inhibition and if we consume foods containing these natural compounds it may help resist many infections in human. This may be due to the fact that Vitamin C has antibiotic activity at much higher concentration. However, it is seen Vitamin C produced no effect on 50 µl Linezolid and increased the antibacterial activity of 50 µl AZT. This synersim in antibacterial activity may be due to presence of antioxidants, flavonoids and phenolics present in vitamin C (30µl) [31]. Unfortunately, it is also seen that Vitamin C has decreased the zone of inhibitions of of AZT(30 µl) , LZ(30 µl), AMP(30 µl and 50 µl) and CHL(30 µl and 50 µl) but the positive aspect of these combinations is that only AZT(30 µl) and AMP(30 µl and 50 µl) have shown intermediate sensitivity and resistant to our isolate and other antibiotics in combination of Vitamin C have showed sensitivity to Smple S. This may be due to th fact Vitamin C reduced the oxidative stress provided by the antibiotics and help to protect the pathogens. This may be the reason why antibiotics show decrease in the zone of inhibition on the Sample S when given in combination with Vitamin C. In such cases it is better to consume Vitamin C, 8 hours before or 4 hours after the antibiotic is taken and to prevent antibiotic resistance. So, our investigation has shown that although 50 µl AZT has slightly increased the zone of inhibition in combinatation with Vitamin C but it is seen that all tested concentration of LZ is the most effective antibiotic for treating this isolate alone and in combination with Vitamin C.

CONCLUSION

It is seen from this investigation has shown that *Bacillus cereus is highly sensitive to LZ and also* produced maximum antibiotic activity towards Sample S in presence of Vitamin C and hence can help in treating this disease causing pathogen. Lastly, further works are also needed to study the other stable antibiotics in presence of Vitamin C and increase the antibacterial activity of Vitamin C.

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