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Research Article

BIOLOGICAL DEGRADATION OF ANTIBIOTIC AZITHROMYCIN BY SELECTED BACTERIAL ISOLATES

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Abstract

Aim: The increasing presence of antibiotics in the environment has raised serious concerns about their impact on ecosystems and public health. Azithromycin, a widely used macrolide antibiotic, is frequently detected in soil and water, where it can contribute to antibiotic resistance. This study focuses on the biological degradation of Azithromycin by bacterial isolates obtained from soil samples collected in Gwalior, Madhya Pradesh, India.
Method: A total of sixteen soil samples were enriched, and bacterial strains capable of using Azithromycin as the sole carbon source were isolated and acclimatized. These isolates were tested for their growth at varying Azithromycin concentrations (50–500 ppm) and characterized based on morphological and biochemical properties. Further optimization of environmental conditions such as pH and temperature was conducted to enhance bacterial growth and degradation efficiency.
Results: Quantitative degradation studies showed significant reduction in Azithromycin concentration over a 72 Hrs period, confirmed by UV-Visible spectrophotometry. Efficient bacterial strains were further identified through 16S rRNA sequencing and phylogenetic analysis.
Conclusion: The findings of this study highlight the potential of indigenous bacterial strains for the bioremediation of Azithromycin-contaminated environments, offering an eco-friendly solution to reduce antibiotic pollution.
Keywords: Degradation, Azithromycin, Antibiotics, Biological, Bacterial.

1. Introduction

Antibiotics have revolutionized the treatment of infectious diseases, saving millions of lives globally. However, their extensive use and improper disposal have resulted in their accumulation in the environment, posing serious ecological and human health risks [1,2]. Among various antibiotic classes, macrolides like Azithromycin are increasingly found as persistent environmental contaminants due to their high usage rates and incomplete metabolism in humans and animals [3,4]. Azithromycin, a semi-synthetic macrolide derived from erythromycin, is characterized by a 15-membered lactone ring and exhibits broad-spectrum antimicrobial activity [5].

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Despite its clinical importance, Azithromycin has been consistently detected in surface water, soil, and even drinking water sources at concentrations ranging from ng/L to µg/L [6]. This occurrence is largely attributed to inefficient removal during conventional wastewater treatment processes and its environmental stability [7]. The accumulation of Azithromycin in the environment not only disturbs microbial communities but also promotes the emergence of antibiotic-resistant bacteria [8]. Therefore, developing effective and sustainable strategies for its removal is of critical importance to public and environmental health.

1.2 Environmental Impact of Azithromycin

Environmental contamination with Azithromycin presents multifaceted ecological threats. Even at low concentrations, Azithromycin can disrupt natural microbial ecosystems, inhibit algal photosynthesis, impair invertebrate development, and alter nutrient cycling [9]. Studies have demonstrated that sub-lethal concentrations of macrolides can modify bacterial diversity and increase the abundance of antibiotic resistance genes (ARGs) in aquatic systems [10].

Moreover, Azithromycin residues can bioaccumulate in aquatic organisms, leading to trophic transfer and potential biomagnification through food chains [11]. The World Health Organization (WHO) recognizes antibiotic resistance as one of the top global health threats, and environmental dissemination of antibiotics like Azithromycin significantly contributes to this crisis [12].

Recent studies indicate that environments contaminated with Azithromycin harbor resistant strains even in pristine ecosystems, demonstrating the compound's far-reaching ecological consequences [13]. Thus, the removal of Azithromycin from contaminated matrices is not merely desirable but an urgent necessity.

1.3 Biological Degradation as a Remediation Strategy

Traditional chemical and physical methods for antibiotic removal, such as advanced oxidation processes, ozonation, or adsorption techniques, although effective, are often cost-intensive and may generate harmful secondary pollutants [2, 14]. In contrast, biological degradation offers a promising eco-friendly and cost-effective alternative.

Microbial biodegradation utilizes the natural metabolic capabilities of bacteria, fungi, or algae to break down complex organic pollutants into simpler, non-toxic compounds [15]. Bacteria, in particular, possess enzymatic systems capable of cleaving the macrolide structure of Azithromycin through hydrolysis, oxidation, and demethylation reactions [16]. Several reports have identified native and acclimatized microbial communities capable of utilizing antibiotics as carbon or nitrogen sources, suggesting their potential for environmental bioremediation [17,18]. Given the complexity of antibiotic molecules, a consortium of microbial species is sometimes more effective than a single isolate, offering synergistic degradation pathways. Therefore, exploring indigenous soil bacteria capable of degrading Azithromycin can provide sustainable solutions for antibiotic remediation.

1.4 Soil as a Source of Azithromycin-Degrading Bacteria

Soil environments host the most diverse microbial communities on Earth, many of which have evolved mechanisms to degrade naturally occurring antibiotics produced by soil actinomycetes [19]. This evolutionary pressure has equipped soil bacteria with the enzymatic machinery to degrade not only natural antibiotics but also synthetic derivatives like Azithromycin.

Soil microbial communities exposed to anthropogenic pollution, such as agricultural runoffs and pharmaceutical waste, often develop specialized catabolic capabilities. Enrichment and acclimatization techniques can further enhance the degradation potential of these communities by selecting bacteria capable of surviving in environments with high antibiotic concentrations [20].

Previous studies have shown the successful isolation of antibiotic-degrading bacteria from contaminated soils, such as Bacillus, Pseudomonas, and Rhodococcus species, suggesting that soil is a rich reservoir for potential biodegraders [21]. Thus, isolating soil bacteria specifically adapted to Azithromycin presence is a strategic approach for developing effective bioremediation systems.

1.5 Importance of Molecular Characterization

Although morphological and biochemical characterization provides basic identification of bacterial isolates, molecular techniques, especially 16S rRNA gene sequencing, offer accurate and definitive bacterial classification [22]. The 16S rRNA gene is highly conserved among all prokaryotes but contains hypervariable regions that allow for differentiation at the genus and species levels.

Molecular identification not only confirms the taxonomic identity of bacterial isolates but also assists in understanding the phylogenetic relationships among different degraders. Furthermore, identification of genes related to degradation pathways offers insights into the metabolic mechanisms involved in antibiotic breakdown [23].

Phylogenetic analyses using bioinformatics tools like MEGA X software further strengthen the understanding of the evolutionary adaptations of bacteria toward antibiotic resistance and degradation [24].

Thus, combining classical microbiological techniques with advanced molecular methods ensures a comprehensive approach to selecting and utilizing potent Azithromycin-degrading bacteria.

3. Methodology

3.1 Collection of Soil Samples

A total of 16 soil samples were collected from various locations around Gwalior, Madhya Pradesh (26.22°N, 78.18°E). Samples were collected aseptically at a depth of 7 cm and stored at 4°C for further analysis. Soil pH and temperature were measured immediately at the collection site.

3.2 Enrichment and Acclimatization of Azithromycin-Degrading Bacteria

Enrichment was performed by suspending 1 g of soil sample in 9 mL of sterile distilled water, followed by serial dilution. The diluted sample was inoculated into Minimal Salt Medium (MSM) supplemented with 20 µg/mL Azithromycin as the sole carbon source. The cultures were incubated at 30°C, 150 rpm for 7 days.

Subsequently, bacterial strains were gradually acclimatized to increasing concentrations of Azithromycin (20–100 µg/mL) by sequentially transferring inoculum into MSM containing 40, 60, 80, and 100 µg/mL Azithromycin at 4-day intervals to obtain highly tolerant strains.

3.3 Isolation and Purification of Azithromycin-Degrading Isolates

After enrichment, serial dilutions of the final culture were plated on LB agar and incubated at 30°C for 48 hours. Morphologically distinct colonies were isolated

by repeated streaking and preserved at -20°C in 15% glycerol stocks.

3.4 Growth Studies of Isolates in Presence of Azithromycin

Growth studies were conducted in MSM supplemented with Azithromycin at varying concentrations (50–500 ppm) and incubated at 30±2°C, 120 rpm for 168 hours. Bacterial growth was monitored by measuring optical density (OD600 nm) at 24-hour intervals using a UV-Visible spectrophotometer.

3.5 Maximum Tolerance Level (MTL) Determination

Bacterial isolates were streaked on LB agar plates containing Azithromycin at concentrations of 50, 100, 200, 300, 400, and 500 ppm. Growth was visually categorized as no growth (-), mild growth (+), moderate growth (++), or maximum growth (+++) after 24–48 hours of incubation at 37°C.

3.6 Morphological and Biochemical Characterization

Morphological studies, including colony morphology and Gram staining, were performed. Biochemical tests included catalase, oxidase, indole production, methyl red, Voges-Proskauer, citrate utilization, carbohydrate fermentation, nitrate reduction, urease, and amylase activity to characterize the isolates.

3.7 Optimization of Environmental Factors

To optimize bacterial growth and Azithromycin degradation:

- pH Optimization: MSM with pH 3, 5, 7 (control), 9, and 11 was inoculated and incubated at 37°C. Growth was assessed by OD600 nm.
- Temperature Optimization: Cultures were incubated at 20°C, 30°C, 37°C (control), 40°C, and 50°C, and bacterial growth was monitored similarly.

3.8 Quantitative Degradation Studies

For quantitative analysis, bacterial inoculum (10^5 CFU/mL) was introduced into sterile nutrient broth supplemented with 50 mg/L Azithromycin. Cultures were incubated at 37°C, 150 rpm, for 7 days. At time intervals (0, 24 h, 48 h and 72 h), samples were withdrawn, centrifuged at 10,000 rpm for 5 minutes, and the antibiotic concentration in the supernatant was determined by UV-Visible spectrophotometry at 210 nm against a standard curve.

Percentage degradation was calculated using the formula:

$$\% \text{ Degradation} = [(C_0 - C_t) / C_0] \times 100$$

where C_0 = initial concentration; C_t = concentration at time t.

3.9 Molecular Identification

Efficient Azithromycin-degrading bacterial isolates were subjected to 16S rRNA gene sequencing. Genomic DNA was extracted using a spin column kit (HiMedia, India). PCR amplification was carried out using universal primers (F-8 and 1542R). Sequencing was performed by BioNGS Technologies Pvt. Ltd., Ghaziabad. Sequence similarity was analyzed by BLAST, and phylogenetic analysis was performed using MEGA7 software with Neighbor-Joining Method.

4. Data Analysis and Result

4.1 Sample collection

Sample collection was done from non-contaminated sites like normal dumping sites, suspected sites nearby region of pharmaceutical industries sites. Temperature of different samples varied in the range from 18°C to 43°C. The pH of the samples also varied from 6.75-8.73.

4.2 Enrichment and isolation of antibiotic degrading bacterial isolates

To isolate and select bacterial strains with potential for Azithromycin degradation, the enrichment culture technique was employed. Soil samples were inoculated into Minimal Salt Medium (MSM) containing Azithromycin as the sole carbon and energy source. These cultures were incubated at 37°C and 120 rpm and subjected to a series of sub-cultures at four-day intervals to promote the proliferation of Azithromycin-tolerant and degrading microbial populations.

Evidence of bacterial growth and antibiotic utilization was monitored by observing an increase in turbidity and a distinct color change in the medium, compared to an uninoculated control flask. This indicated the metabolic activity of enriched microbial communities utilizing Azithromycin.

Following successful enrichment, acclimatized cultures from the final flask were serially diluted (1:1000) using sterile distilled water and plated on MSM agar plates supplemented with Azithromycin. The plates were incubated at 37°C for 48 hours. Colonies displaying distinct morphological variations were selected, sub-cultured for purification, and maintained on MSM agar containing Azithromycin. These purified isolates were then labeled and stored at 4°C for further analysis.

As a result of this enrichment strategy, a total of 14 bacterial isolates capable of degrading Azithromycin were successfully obtained from soil samples. These isolates were later evaluated for their growth and degradation efficiency at varying concentrations of Azithromycin, as part of the antibiotic degradation assessment.

Table 1 : Sample code, pH and temperature of samples

S. No.	Samples code	pH	Temp.(°C)
1.	SS-1	7.69	30
2.	SS-2	8.73	18
3.	SS-3	7.20	19
4.	SS-4	7.60	19
5.	SS-5	7.20	18
6.	SS-6	7.00	19
7.	SS-7	7.30	19
8.	SS-8	7.46	35
9.	SS-9	6.75	40
10.	SS-10	7.75	42
11.	SS-11	8.60	43
12.	SS-12	7.78	39
13.	SS-13	7.10	41
14.	SS-14	7.20	40

4.3 Growth study of bacterial isolates at varying concentration of Azithromycin (AZI)

The growth pattern of azithromycin-degrading bacterial isolates was evaluated by measuring optical density at 600 nm. It was observed that at a concentration of 50 ppm, bacterial isolates such as AZI-4, AZI-6, AZI-7, AZI-9, AZI-10, and AZI-11 exhibited maximum growth, indicating their strong adaptability and potential for antibiotic degradation at lower concentrations. In contrast, isolates AZI-12 and AZI-13 demonstrated minimal growth under the same conditions. At a higher concentration of 500 ppm, only the AZI-7 isolate maintained notable growth, suggesting its enhanced tolerance to elevated antibiotic levels. Overall, most of the bacterial isolates showed an increasing trend in growth over time, with optimal growth typically occurring between 48 to 144 hours of incubation.

Table 2 : Sample collection sites and number of antibiotic degrading bacterial isolates obtained

S.No.	Sample code	Sample collection Area	Azithromycin degrading bacteria
1.	SS-1	Near Ranbaxy Laboratories, Malanpur	AZI-1
2.	SS-2	Ddumping site, DRDO, Gwalior	AZI-2
3.	SS-3	Dumping Site, Sharma Farm, Banmore	AZI-3
4.	SS-4	Dumping Site, Hazira, Gwalior	AZI-4
5.	SS-5	Hurawali Churaha, Sirol Road, Gwalior	AZI-5
6.	SS-6	Near Morar Anaj Mandi, Morar, Gwalior	AZI-6
7.	SS-7	Oppsit DD mall, Shinde ki Chhavni, Gwalior	AZI-7
8.	SS-8	Dumping site, Near Chhatri Mandi, Gwalior	AZI-8
9.	SS-9	ShreeRam Motor and repairs, Transport	AZI-9

S.No.	Sample code	Sample collection Area	Azithromycin degrading bacteria
		Nagar, Gwalior	
10.	SS-10	NavChetan Automobiles, Bahodapur, Gwalior	AZI-10
11.	SS-11	Shameem automobilesm and repair Shop, Thatipur	AZI-11
12.	SS-12	Dumping site, Near Sabji Mandi, Laxmi Ganj, Gwalior	AZI-12
13.	SS-13	BCG Petrochemical Plant, Banmore	AZI-13
14.	SS-14	Loco car wash, Hazeera, Gwalior	AZI-14
Total number of antibiotic degrading bacterial isolates			14

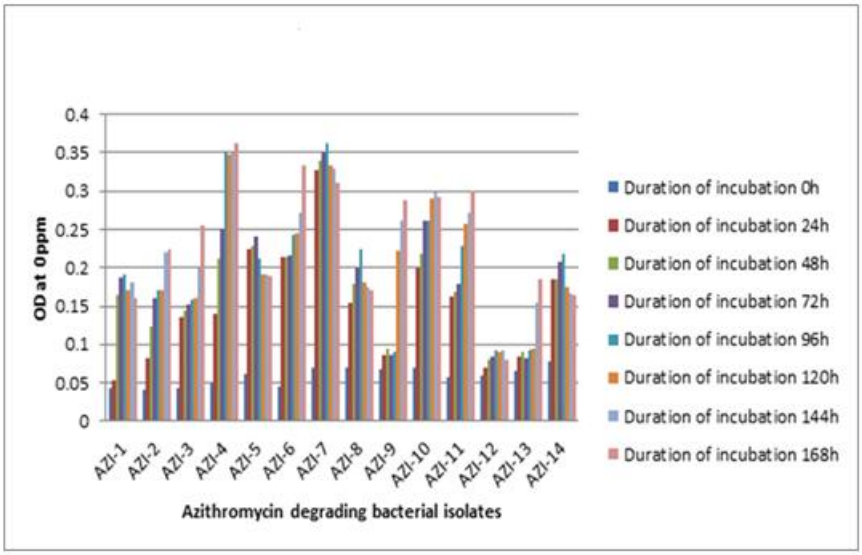

Figure 1: Relationship between growth and duration of incubation of Azithromycin degrading bacterial isolate at 50 ppm concentration of Azithromycin (OD at 600nm)

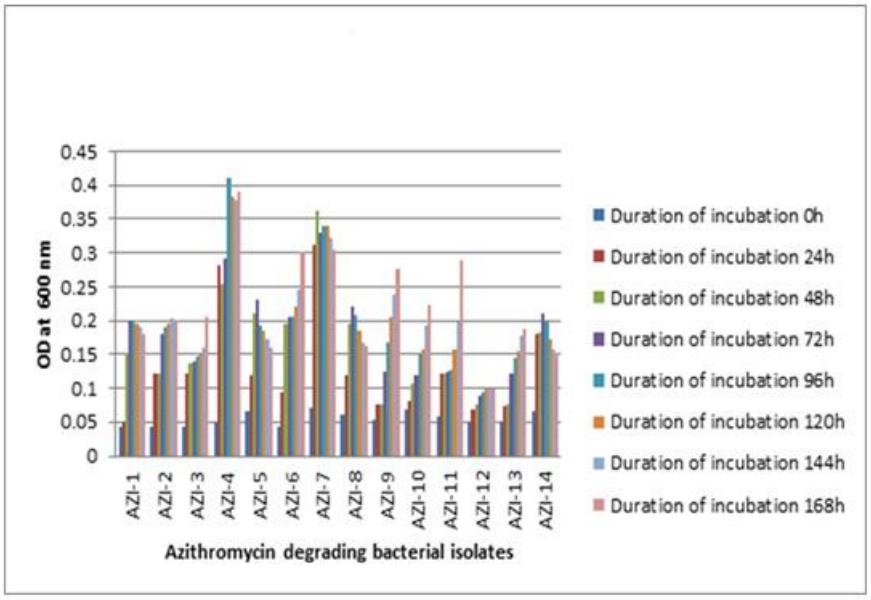

Figure 2: Relationship between growth and duration of incubation of Azithromycin degrading bacterial isolate at 100 ppm concentration of Azithromycin (OD at 600nm)

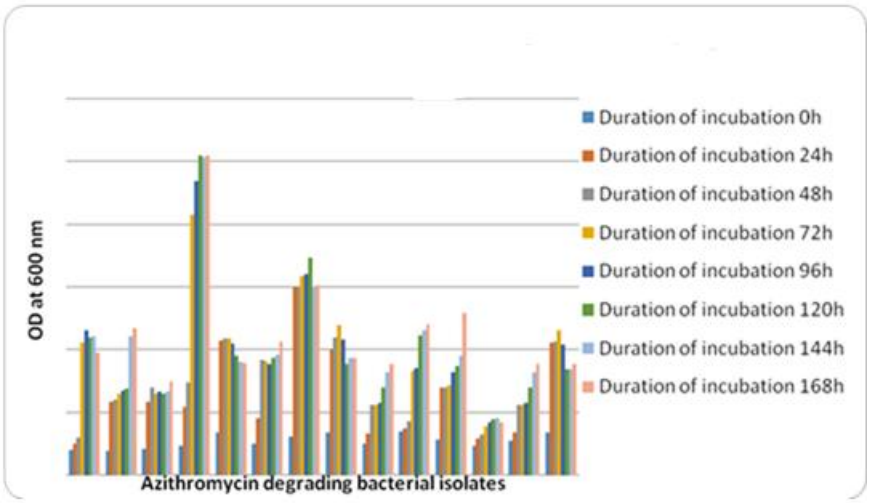

Figure 3: Relationship between growth and duration of incubation of Azithromycin degrading bacterial isolate at 200 ppm concentration of Azithromycin (OD at 600nm)

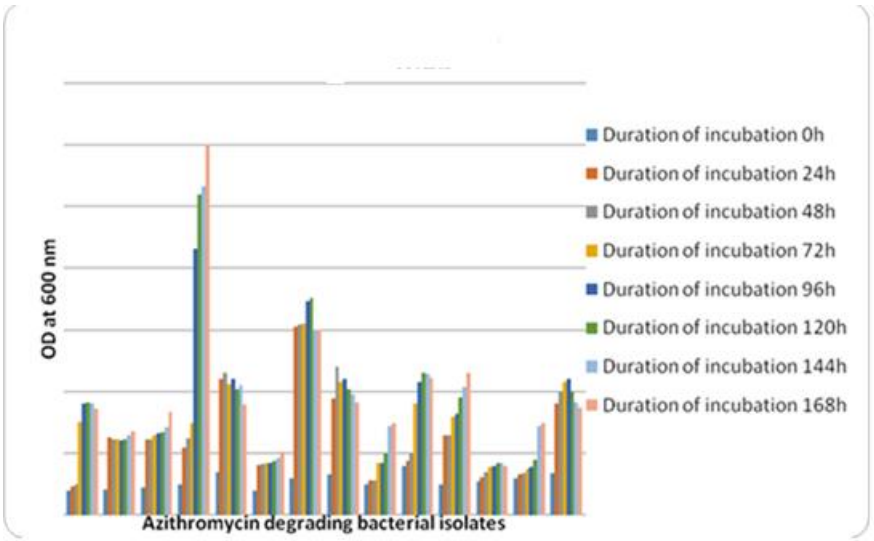

Figure 4: Relationship between growth and duration of incubation of Azithromycin degrading bacterial isolate at 300 ppm concentration of Azithromycin (OD at 600nm)

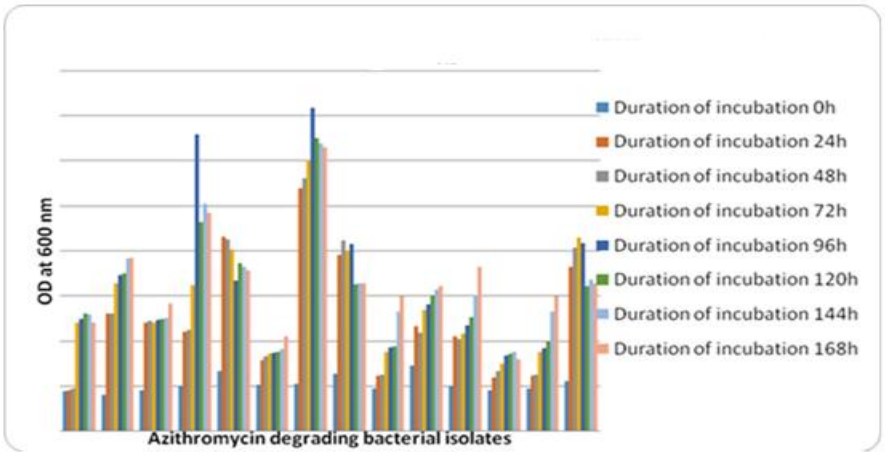

Figure 5: Relationship between growth and duration of incubation of Azithromycin degrading bacterial isolate at 400 ppm concentration of Azithromycin (OD at 600nm)

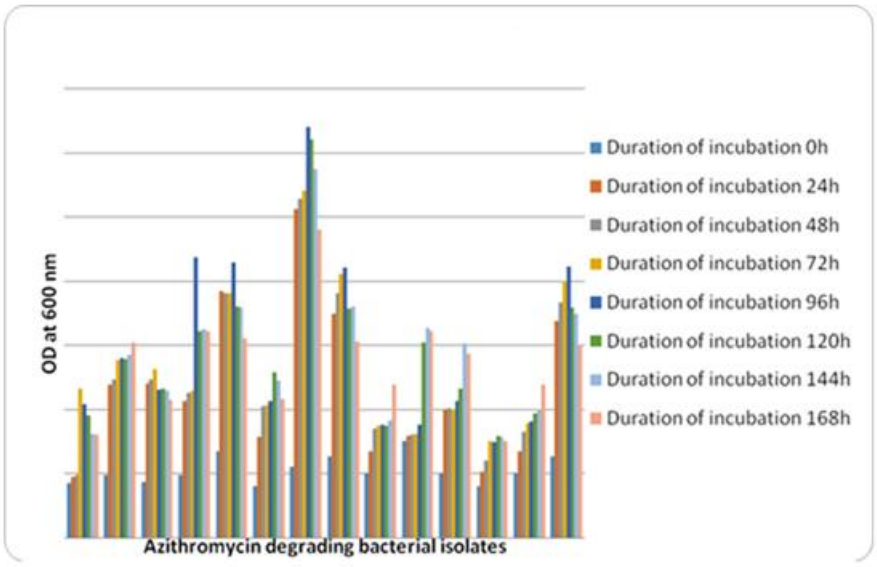

Figure 6: Relationship between growth and duration of incubation of Azithromycin degrading bacterial isolate at 500 ppm concentration of Azithromycin (OD at 600nm)

4.4 Screening of bacterial isolates for Maximum Tolerance Level (MTL) of Azithromycin (AZI)

Result concluded that all the 14 Azithromycin degrading bacterial isolates were able to tolerate upto 500ppm concentration of azithromycin. Bacterial isolates showed decrease ability to tolerate antibiotic as the concentration increased. Bacterial isolate AZI-7 showed maximum tolerance level upto to 400ppm concentration of azithromycin. Bacterial isolates AZI-2, AZI-5 and AZI-14 showed moderate growth upto 400ppm concentration. Two of the isolates named AZI-12 and AZI-13 showed no growth at 400ppm and 500ppm of concentration along with minimum growth at remaining concentration of antibiotics. Out of 14 isolates, 12 azithromycin degrading bacterial isolates showed tolerance upto 500ppm concentration of antibiotic.

Table 3: Maximum Tolerance Level (MTL) of Azithromycin degrading bacterial isolates at six successive concentrations of Azithromycin

Bacterial isolates	Bacterial growth at varying concentration of Azithromycin (AZI)					
	50 ppm	100 ppm	200ppm	300ppm	400ppm	500ppm
AZI-1	++	++	++	+	+	+
AZI-2	++	++	++	++	++	+
AZI-3	++	++	+	+	+	+
AZI-4	+++	+++	+++	+++	++	+
AZI-5	++	++	++	++	++	+
AZI-6	+++	+++	++	+	+	+
AZI-7	+++	+++	+++	+++	+++	++
AZI-8	++	++	++	++	++	+
AZI-9	+++	+++	++	++	++	+
AZI-10	+++	++	++	++	++	++
AZI-11	+++	+++	+++	++	++	+
AZI-12	+	+	+	+	-	-
AZI-13	++	++	+	+	-	-
AZI-14	++	++	++	++	++	+

(+)- Mild growth, (++)- Moderate growth, (+++)- Maximum growth and (-)- No growth

4.5 Heavy metal resistance of the Azithromycin degrading bacterial isolates

At 0.1% concentration of heavy metals maximum number of azithromycin degrading bacterial isolates showed resistance against copper (31%) followed by zinc (29%) and chromium (24%), while resistance against cadmium and mercury was found 8% only (Figure 5.13a). Isolates AZI-2 and AZI-8 showed resistance against all the five heavy metals, while isolates AZI-14 did not show resistance against any of the heavy metal.

At 0.5% concentration of heavy metals maximum number of azithromycin degrading bacterial isolates showed resistance against copper and zinc (38%) followed by chromium (17%), while resistance against cadmium and

mercury was found 4% and 3% respectively. Isolates AZI-8 showed resistance against all the five heavy metals, while isolates AZI-14 did not show resistance against any of the heavy metal.

At 1% concentration of heavy metals maximum number of azithromycin degrading bacterial isolates showed resistance against zinc (46%) followed by copper (42%), chromium (12%). On contrary none of the isolates showed resistance against cadmium and mercury on the 1% concentration of heavy metals. Isolates AZI-6, AZI-8 and AZI-11 showed resistance against maximum number of heavy metals (Zn, Cu and Cr), while isolates AZI-14 did not show resistance against any of the heavy metal.

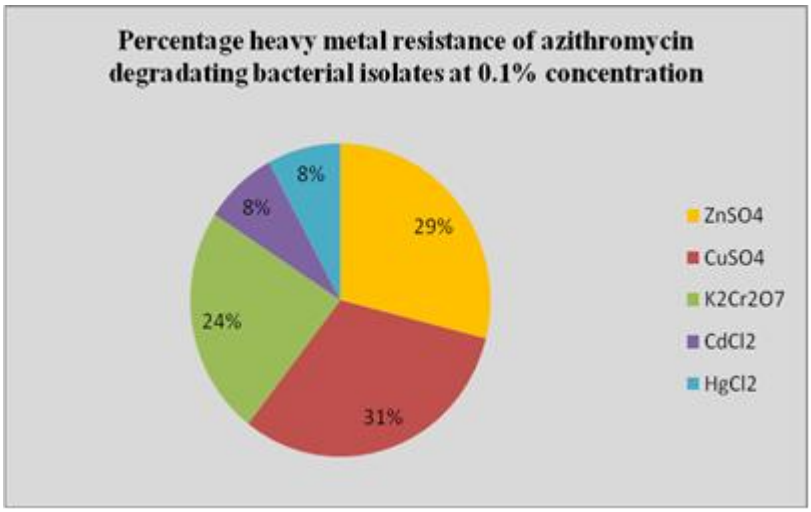

Figure 7: Percentage heavy metals resistance of the Azithromycin degrading bacterial isolates at 0.1 % metal concentration

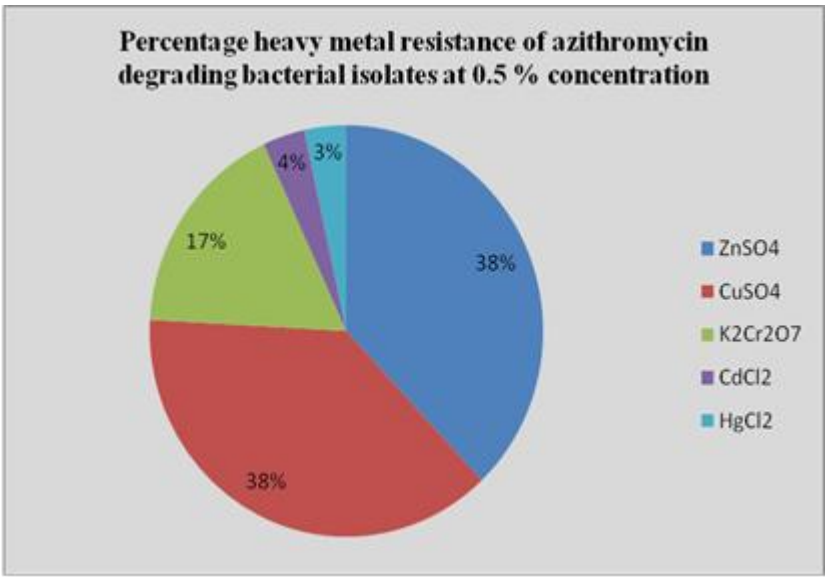

Figure 8: Percentage heavy metals resistance of the Azithromycin degrading bacterial isolates at 0.5 % metal concentration

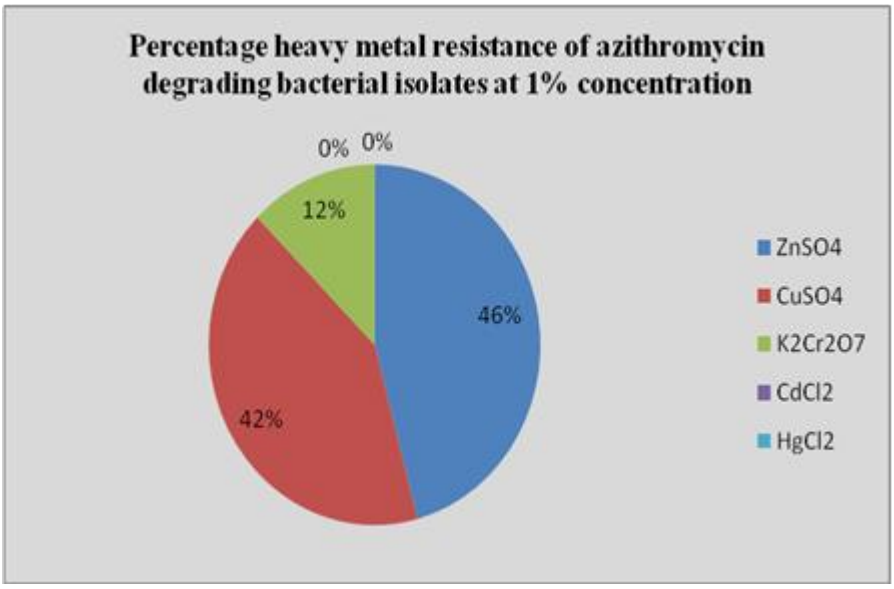

Figure 9: Percentage heavy metals resistance of the Azithromycin degrading bacterial isolates at 1 % metal concentration

4.6 Morphological and Biochemical Characterization of Azithromycin-Degrading Bacterial Isolates

A total of 14 Azithromycin-degrading bacterial isolates obtained through enrichment culture were subjected to morphological and biochemical characterization to assess their structural and physiological features.

4.6.1 Morphological Characterization of Azithromycin-Degrading Bacterial Isolates

Colony characteristics were observed on MSM agar plates, followed by standard staining techniques including Gram staining, endospore staining, capsule staining, and motility tests. Among the isolates, 12 were found to be Gram-positive and 2 were Gram-negative, with most displaying a rod-shaped morphology. Endospore staining revealed that only two isolates, AZI-4 and AZI-9, were capable of forming endospores, indicating their potential to survive in harsh conditions. Capsule staining showed no capsule formation in any of the Azithromycin isolates, while motility tests indicated that three isolates—AZI-1, AZI-7, and AZI-13—were motile in nature. These results suggest that the majority of Azithromycin-degrading bacteria are Gram-positive rods, with a few demonstrating endospore formation and motility, characteristics that may contribute to their environmental persistence and antibiotic degradation potential.

Table 4: Morphological characterization of Azithromycin (AZM) degrading bacterial isolates

S.No	Antibiotic degrading bacterial isolates	Colony characteristics	Gram's nature, shape and arrangement	Endospore staining	Capsule staining	Motility test
1.	AZI-1	Large, irregular, entire, rough, white	Gram positive, rod	-	-	Motile
2.	AZI-2	Pinpoint, circular, entire, smooth, cream	Gram positive coccobacilli	-	-	Non-Motile
1.	AZI-3	Large, irregular, undulate, glistening, cream	Gram positive, cocci	-	-	Non-motile
2.	AZI-4	Pinpoint, circular, undulate, smooth, white	Gram positive, Rod	+	-	Non-motile
3.	AZI-5	Large, circular, undulate, smooth,	Gram negative, Rod	-	-	Non-motile

S.No	Antibiotic degrading bacterial isolates	Colony characteristics	Gram's nature, shape and arrangement	Endospore staining	Capsule staining	Motility test
		white				
4.	AZI-6	Large, circular, undulate, rough, white	Gram positive, Rod	-	-	Non-motile
5.	AZI-7	Small, circular, entire, mucoid, cream	Gram positive, Rod	-	-	Motile
6.	AZI-8	Pinpoint, circular, entire, smooth, transparent	Gram positive, Long Rod	-	-	Non-motile
7.	AZI-9	Cream, large, entire, moist, opaque	Gram positive, Rod	+	-	Non-Motile
8.	AZI-10	Greenish, medium, wavy, moist	Gram positive, Bacilli	-	-	Non-motile
9.	AZI-11	White, small, entire, moist, opaque, raised	Gram negatve, Cocci	-	-	Non-Motile
10.	AZI-12	Yellow, punctiform, entire, moist, opaque, raised	Gram positive, Rod	-	-	Non-Motile
11.	AZI-13	White, small, entire, glossy, opaque, convex	Gram positive, Cocci	-	-	Motile
12.	AZI-14	Off white, medium fringes, glossy, mucoid,opaque,convx	Gram positive, Rod, chain form	-	-	Non-Motile

4.6.2 Biochemical characterization of Azithromycin degrading bacterial isolates

A total of 11 Azithromycin-degrading bacterial isolates were biochemically characterized to assess their metabolic traits and tentative classification. Among them, 4 isolates tolerated 6.5% NaCl, 9 were catalase-positive, and 4 showed oxidase activity. Esculin hydrolysis was positive in 6 isolates, amylase in 4, nitrate reduction in 5, and urease activity in 2. IMViC tests showed variable results: 2 isolates were indole-positive, 4 methyl red-positive, 7 Voges–Proskauer-positive, and 4 utilized citrate. Sugar fermentation tests revealed that all isolates fermented dextrose, most fermented maltose, while fewer fermented sucrose, lactose, fructose, mannose, inulin, inositol, raffinose, and rhamnose. Based on biochemical profiles, isolates were tentatively identified as belonging to genera including *Bacillus*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, and others.

Table 5: Biochemical Characterization of Azithromycin-Degrading Bacterial Isolates

Antibiotic degrading bacterial isolates	Growth in 6.5% NaCl	Catalase	Oxidase	Esculin hydrolysis	Indole	MR	VP	Citrate utilization	Nitrate reduction	Urease production	Amylase	Tentative Identification
AZI-2	+	+	+	+	-	-	-	-	+	-	-	*Micrococcus sp.*
AZI-3	+	-	-	+	-	-	+	-	-	-	-	*Enterococcus faecalis*
AZI-4	-	+	-	+	-	+	+	-	-	-	-	*Bacillus subtilis*
AZI-5	-	+	+	-	-	+	-	-	+	+	+	*Bacillus thuringiensis*
AZI-6	-	+	-	-	-	-	+	+	+	-	+	*Bacillus cereus*
AZI-7	-	+	-	+	-	+	+	-	-	-	-	*Bacillus subtilis*
AZI-8	+	+	+	-	-	-	+	+	-	+	+	*Bacillus subtilis*
AZI-9	-	+	-	-	-	-	+	+	+	-	+	*Bacillus cereus*
AZI-10	+	+	-	-	+	-	-	+	+	-	-	*Microbacterium*
AZI-11	-	-	+	+	+	-	-	-	-	-	-	*Ochrobactrum*

4.7 Characterization of environmental conditions for the optimal growth of Azithromycin-Degrading degrading bacteria

4.7.1 Effect of pH on the Growth of Selected Azithromycin-Degrading Bacterial Isolates

The growth behavior of nine selected Azithromycin-degrading bacterial isolates (AZI-2, AZI-4, AZI-5, AZI-7, AZI-8, AZI-9, AZI-10, AZI-11, and AZI-14) was assessed under varying pH conditions (3, 5, 7, 9, and 11) over a 72-hour incubation period at 50 ppm Azithromycin concentration. Growth was monitored by measuring optical density (OD) at 0, 24, 48, and 72 hours. Results indicated that pH had a significant impact on bacterial growth. Most isolates showed maximum growth at neutral pH (7), particularly AZI-2, AZI-5, AZI-7, AZI-8, and AZI-14. Meanwhile, isolates AZI-4, AZI-9, AZI-10, and AZI-11 demonstrated better growth under slightly alkaline conditions (pH 9). Growth was notably reduced at highly acidic (pH 3, pH 5) and strongly alkaline (pH 11) conditions, indicating that extreme pH levels adversely affected bacterial proliferation.

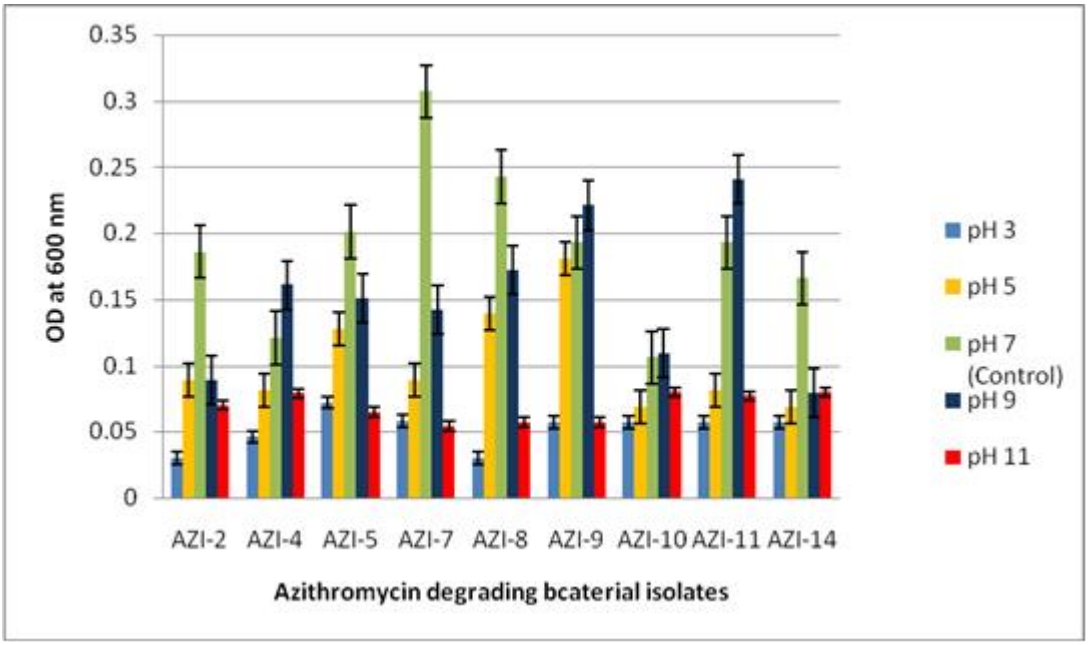

Figure 10: Growth of Azithromycin degrading bacterial isolates at different pH, at 50ppm Azithromycin concentration, after 72h of incubation period

4.7.2 Effect of Temperature on the Growth of Selected Azithromycin-Degrading Bacterial Isolates:

The effect of temperature on the growth of nine selected Azithromycin-degrading bacterial isolates (AZI-2, AZI-4, AZI-5, AZI-7, AZI-8, AZI-9, AZI-10, AZI-11, and AZI-14) was evaluated at five different temperatures—20°C, 30°C, 37°C (control), 40°C, and 50°C—over a 72-hour incubation period in the presence of 50 ppm Azithromycin. Optical density (OD) measurements were recorded at regular intervals (0h, 24h, 48h, and 72h) to monitor growth. The results indicated that 37°C supported the highest growth for isolates AZI-2, AZI-5, AZI-8, and AZI-11, while isolates AZI-4, AZI-7, AZI-9, and AZI-10 showed maximum growth at 30°C. Only one isolate, AZI-14, exhibited optimal growth at 40°C. No isolate showed peak growth at the extreme temperatures of 20°C or 50°C, suggesting that moderate temperature conditions favor Azithromycin-degrading bacterial proliferation.

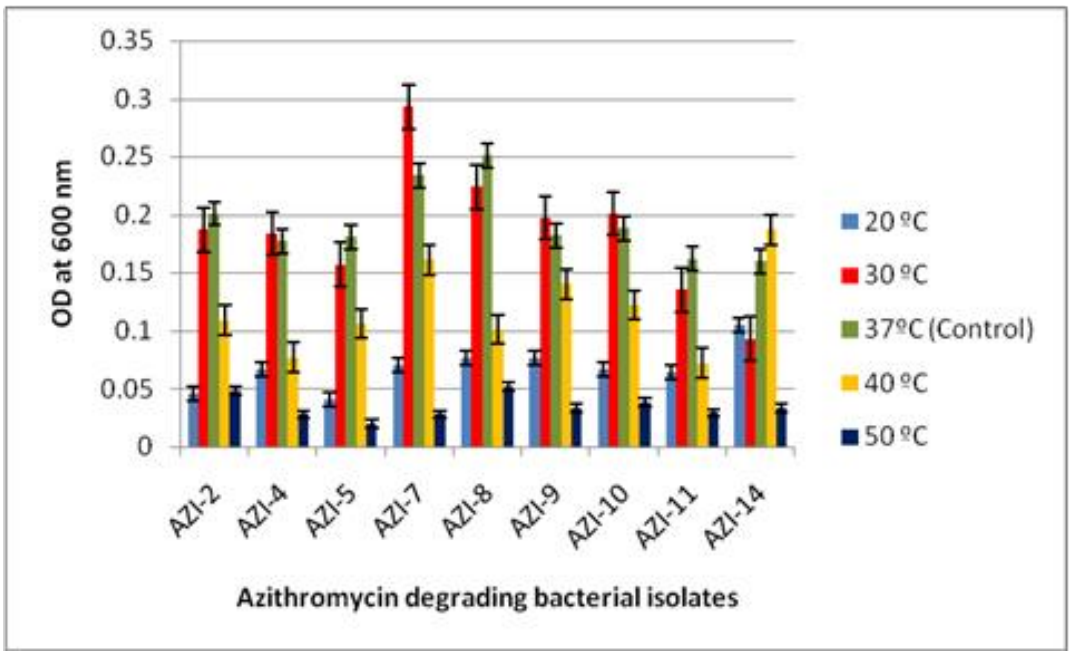

Figure 11: Growth of Azithromycin degrading bacterial isolates at different temperature, at 50ppm Azithromycin concentration, after 72h of incubation period

4.8 Quantitative Analysis of Azithromycin Degradation by Selected Bacterial Isolates:

Quantitative analysis of Azithromycin degradation was performed using two selected bacterial isolates, AZI-7 and AZI-8, under optimized growth conditions. A calibration curve was established using standard Azithromycin solutions, with the maximum absorbance (λmax) recorded at 208 nm. The regression equation derived from the curve was used to calculate the concentration of residual antibiotic in test samples collected at 0, 24, 48, and 72 hours. The percentage degradation was then computed accordingly. Among the two isolates, AZI-7 demonstrated the highest degradation efficiency, achieving 60.8% degradation after 72 hours of incubation, while AZI-8 showed 46.4% degradation in the same period. The results revealed time-dependent degradation patterns, with both isolates showing gradual reduction in Azithromycin concentration across the incubation intervals.

Table 6: Standard solution and absorbance for azithromycin at 208 nm

Concentration (µg/mL)	Absorbance at 208 nm
10	0.398
20	0.715
30	1.01
40	1.365
50	1.606

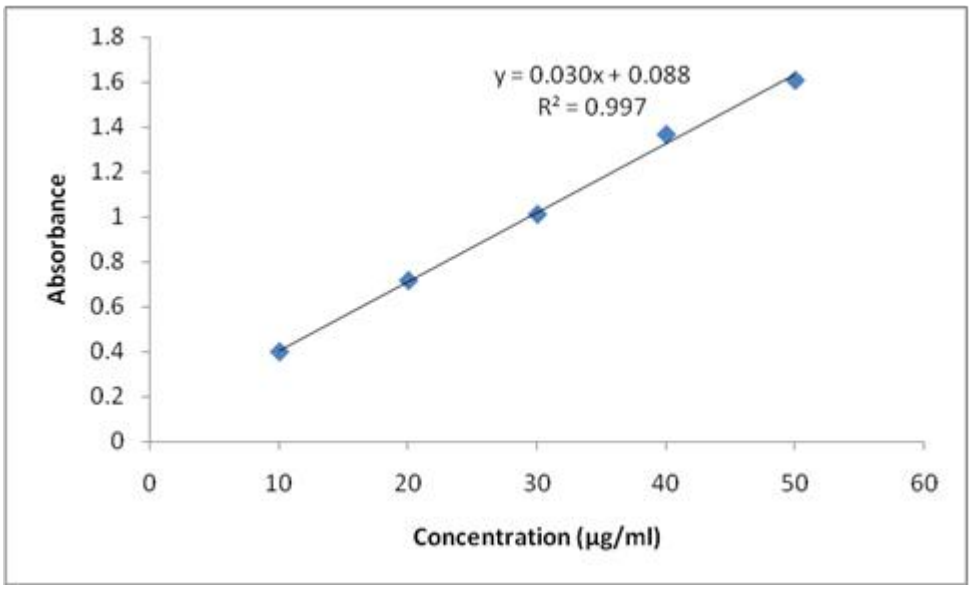

Figure 12: Calibration curve for Azithromycin

Table 7: Biodegradation of azithromycin by selected bacterial isolates

AZI-7				**AZI-8**			
Time (Hrs)	Absorbance at 208nm	Calculated concentration µg/mL	Percentage degradation	Time (Hrs)	Absorbance at 208nm	Calculated concentration µg/mL	Percentage degradation
0h	1.606	50.0	0%	0h	1.606	50.0	0%
24h	1.350	42.6	14.8%	24h	1.500	47.3	5.4%
48h	1.050	33.3	33.4%	48h	1.200	37.9	24.2%
72h	0.600	19.6	60.8%	72h	0.850	26.8	46.4%

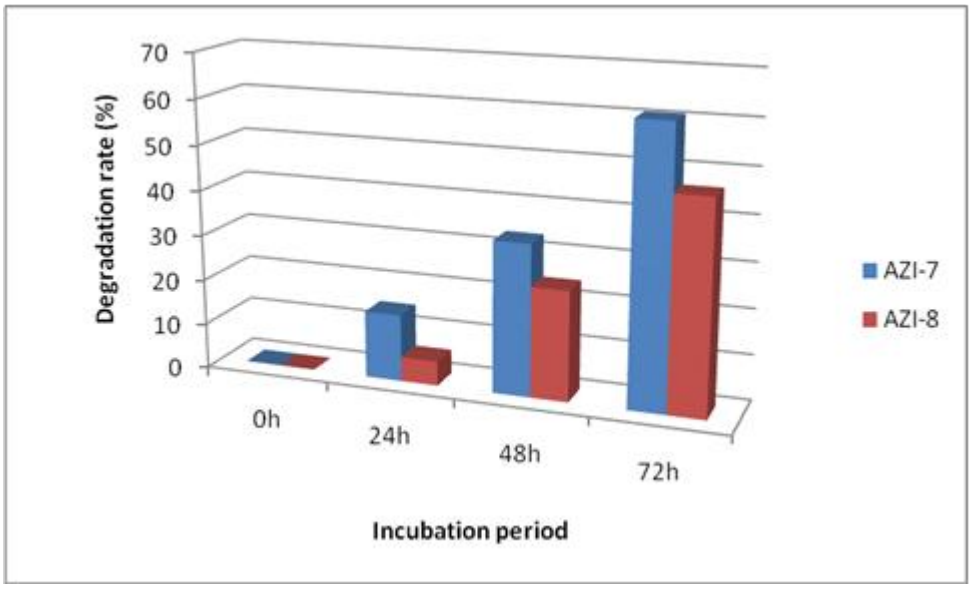

Figure 13: Biodegradation of Azithromycin by selected bacterial isolates at various incubation periods

4.9 Molecular Identification of Potential Azithromycin-Degrading Bacterial Isolate

Molecular identification of the most efficient Azithromycin-degrading bacterial isolate, AZI-7, was carried out using 16S rRNA gene sequencing. Genomic DNA was extracted, and the 16S rRNA gene was amplified using universal primers F8 (forward) and 1542R (reverse), resulting in an amplicon of approximately 1500 base pairs. The partial gene sequence of AZI-7 was analyzed through BLAST against the NCBI GenBank database to determine its closest taxonomic affiliation. The isolate AZI-7 showed 97.56% sequence similarity with *Bacillus subtilis* strain HAS31, suggesting its close phylogenetic relationship with this species. A phylogenetic tree was constructed using MEGA 11.0 software (Tamura et al., 2021) through the Neighbor-Joining method with 1000 bootstrap replicates to confirm the evolutionary placement of the isolate. This molecular identification validated the strain's classification and further supports its potential in Azithromycin biodegradation.

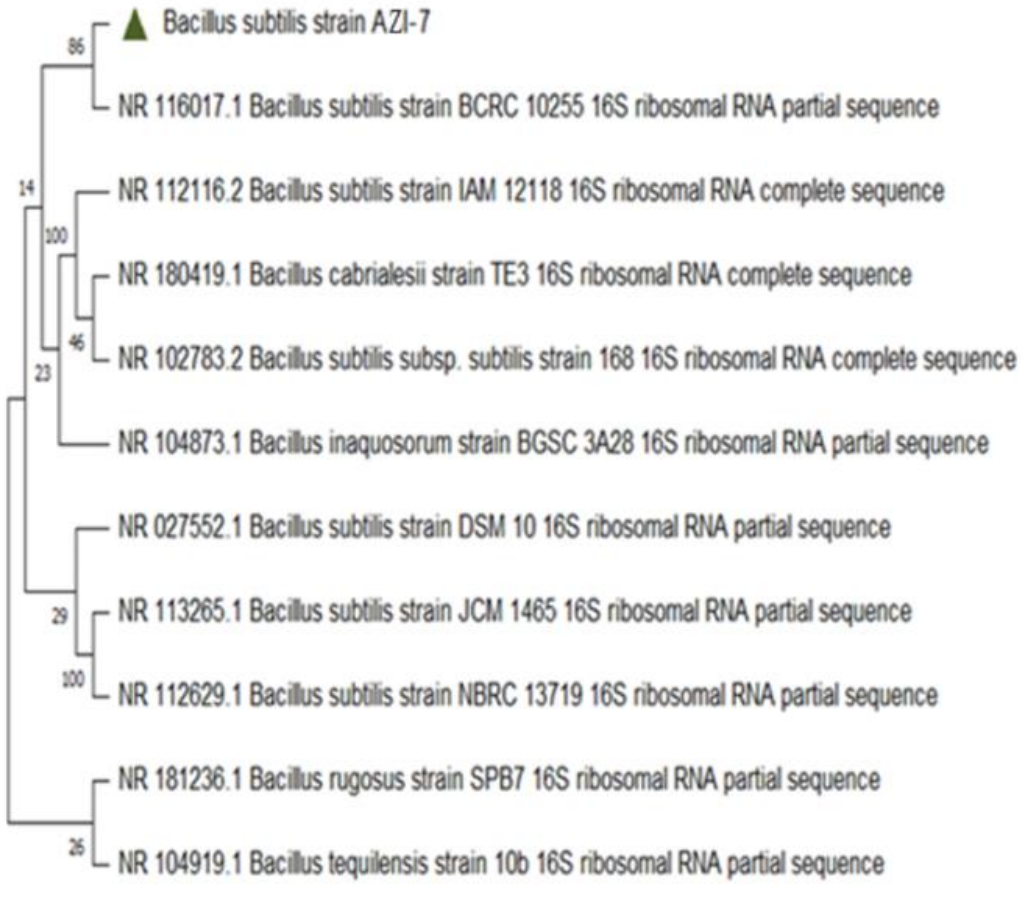

Figure 5.21: Phylogenetic tree of *Bacillussubtilis* strain AZI-7 constructed by neighbor joining method using MEGA

5. Conclusion

The increasing prevalence of antibiotics like Azithromycin in natural environments has become a pressing concern due to their persistence, ecotoxicological effects, and contribution to the spread of antibiotic resistance. Conventional treatment methods often fall short in effectively removing these pharmaceutical contaminants, necessitating the exploration of sustainable alternatives. In this context, biological degradation offers a promising, eco-friendly approach for mitigating antibiotic pollution.

The present study successfully isolated and characterized bacterial strains from soil capable of utilizing Azithromycin as a sole carbon and energy source. Through enrichment, acclimatization, and rigorous morphological, biochemical, and molecular identification techniques, efficient degraders were identified. Further, optimization of environmental factors such as pH and temperature enhanced bacterial growth and degradation efficiency, highlighting the adaptability of these isolates under variable conditions.

Quantitative analysis confirmed significant degradation of Azithromycin over a period of time, demonstrating the practical potential of selected bacterial strains in bioremediation applications. The use of indigenous soil bacteria for Azithromycin degradation not only represents a cost-effective remediation strategy but also aligns with the principles of environmental sustainability.

Overall, the findings of this study emphasize the importance of harnessing naturally occurring microbial communities for the bioremediation of antibiotic-contaminated environments. Future research should focus on scaling up the degradation process, elucidating the detailed metabolic pathways involved, and assessing the efficacy of bacterial consortia in real environmental settings to further enhance the biodegradation of persistent pharmaceutical pollutants.

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