

## Isolation and identification of a bacterial strain from Pharmaceutical Industrial Wastewater and Sludge

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Cite this paper as: Lavanya Bojja, Rajendar Vadluri, (2025) Isolation and identification of a bacterial strain from Pharmaceutical Industrial Wastewater and Sludge. *Journal of Neonatal Surgery*, 14 (20s), 1-5.

### ABSTRACT

Pharmaceutical industrial wastewater and sludge represent significant environmental pollutants, containing persistent organic compounds that challenge conventional treatment methods. This study aimed to isolate and identify an efficient bacterial strain capable of biodegradation from pharmaceutical industrial effluents. Using serial dilution and nutrient agar culturing techniques, bacterial strain LSR-3 was successfully isolated. Morphological analysis revealed that LSR-3 is Gram-positive with round, convex colonies producing a yellow pigment. Further molecular identification through 16S-rDNA sequencing showed 98% similarity to *Brevibacillus agri*. Phylogenetic analysis confirmed its close evolutionary relationship to other *Brevibacillus* strains, particularly strain DSM 6348. Biodegradation assays demonstrated that *Brevibacillus agri* LSR-3 achieved the highest reduction in Chemical Oxygen Demand (COD) among tested strains, with peak reductions of 52% for pharmaceutical industrial wastewater (PIW) and 48% for Diclofenac (DCF) within 36–48 hours of incubation. Optimal COD reductions were recorded at 45% for 10% PIW concentration and 32% for 100 mg/L DCF concentration. These results indicate the strain's metabolic versatility and adaptability under polluted conditions. The findings underscore the potential of *Brevibacillus agri* LSR-3 as a strong candidate for bioremediation applications in industrial wastewater treatment, offering a sustainable, eco-friendly approach to mitigating pharmaceutical pollutants. Further studies are suggested to optimize operational conditions to maximize degradation efficiencies for large-scale applications.

**Keywords:** *Brevibacillus agri*, Biodegradation, Bioremediation, Pharmaceutical wastewater, Chemical Oxygen Demand  
Received: 12 March 2025; Accepted 21 April 2025; Published 29 April 2025

### 1. INTRODUCTION

Pharmaceutical industrial wastewater and sludge are among the most concerning sources of environmental pollution, posing significant risks to ecosystems and public health. These effluents often contain high concentrations of organic compounds, nutrients, and pharmaceutical contaminants such as non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, and analgesics (Gidhamaari et al., 2012). Conventional wastewater treatment technologies struggle to effectively remove these pollutants, leading to their accumulation in aquatic and terrestrial environments. In recent years, microbial bioremediation has gained attention as a sustainable and cost-effective alternative for treating pharmaceutical industrial effluents, relying on the metabolic capabilities of specific bacterial strains to degrade pollutants (Yang et al., 2020). This approach not only addresses the persistence of pharmaceutical compounds but also minimizes the environmental footprint of industrial processes.

The identification of bacterial strains capable of biodegradation plays a critical role in developing effective bioremediation strategies. Molecular methods such as 16S-rDNA sequencing have revolutionized bacterial identification by providing precise insights into phylogenetic relationships and functional capabilities (Gujjeti et al., 2013). These tools enable researchers to screen for strains with optimal enzymatic activity and substrate specificity, ensuring their suitability for bioremediation applications. For example, strains like *Brevibacillus agri* have demonstrated remarkable efficiency in degrading pharmaceutical compounds and reducing COD in industrial wastewater (Ahmed, 2024). The use of 16S-rDNA sequencing coupled with biochemical characterization enhances the reliability of strain identification, paving the way for targeted biotechnological applications.

Pharmaceutical industrial sludge, a byproduct of wastewater treatment, often harbors diverse microbial communities with unique degradation capacities. Exploring these microbial populations offers a valuable opportunity to isolate strains capable of degrading pharmaceutical residues under real-world conditions (Babu et al., 2022). Moreover, the adaptive mechanisms of such strains, including enzymatic pathways and tolerance to harsh environments, are critical for addressing the complexity of industrial effluents. Recent studies have emphasized the importance of isolating bacterial strains directly from polluted environments, such as pharmaceutical sludge and wastewater, to enhance the effectiveness of bioremediation processes (Sun

et al., 2023). This approach ensures that the selected strains are already acclimated to the pollutants and conditions they will encounter during application. The objective of this chapter is to isolate and identify a bacterial strain from pharmaceutical industrial wastewater and sludge, using molecular and biochemical techniques, and evaluate its potential for biodegradation applications.

## 2. MATERIALS AND METHODS

### 2.1 Isolation and growth of bacteria

To create a stock solution for bacterial isolation, 1 g of sludge and 1 mL of wastewater were combined with 10 mL DW. One gramme of extract was used in every 10 mL of the diluted solution, which resulted in a 10-fold dilution. The serial dilution was performed by repeating the processes to generate dilutions up to 10<sup>-5</sup>. 1 mL of the diluted solution was transferred to 9 mL DW, giving a 100-fold dilution. On petri plates with nutritional agar media, approximately 200–300 L of suspension were infected. The plates were incubated for 24 hours at 27–32 °C. The individual plates with the single microbial colonies were then streaked for use in the study.

### 2.2. Characterization and identification of bacterial isolates

#### 2.2.1. Smear preparation for staining processes:

One drop of water was applied to the clean, dry, grease-free glass slide as a smear preparation for staining operations. Bacterial culture was removed from the petri plate and flame sterilised on a loop. One drop of water was used to disseminate bacteria on a microscope slide. After letting it dry naturally, it was gently warmed to apply heat. Then, it went through the further staining procedures:

#### 2.2.2. Gram staining:

Gram staining is used to separate microorganisms into gram-positive and gram-negative groups. Crystal violet was applied to the glass slide and then rinsed with water after the bacterial smear had been heat-fixed. Following the addition of gramme iodine and water washing, the smear was decolorized with 95% ethanol, counterstained with safranin, and then rinsed with water. Gram-positive bacteria were defined as those that had main stain following alcohol decolorization.

#### 2.2.3. Endospore staining:

Endospore staining is used to differentiate between bacterial endospores and vegetative cells. After the bacteria smear on the glass slide had been heated to fix it, malachite green was added, and the slide was heated until steam could be seen rising from its surface for three minutes. The slide was then cleaned with water and counterstained with safranin. Endospores are bacteria that have kept malachite green, while vegetative bacteria are those that have lost the colour but have preserved counterstain.

### Identification of potential bacterial strain

The most common technique in contemporary molecular biology, which marks a transition from cell biology to molecular biology, is the isolation and purification of DNA from bacterial cells. Bacterial DNA can be separated using a rather easy procedure. For the best yield, the bacteria that will be utilised for DNA isolation should be cultured in a favourable medium at the most favourable temperature and harvested during the late log to early inactive phase.

## 3. RESULTS

### 3.1. Screening and isolation of potential bacterial strain for Biodegradation

The screening and isolation of bacterial strains for biodegradation, conducted over 24 hours of incubation, offers crucial insights into the comparative performance of the tested strains in reducing Chemical Oxygen Demand (COD). The results highlight significant variations in biodegradation efficiency, underscoring the impact of microbial activity during the incubation period.

### 3.2. Isolation of LSR-3

The successful isolation of LSR-3, as evidenced by distinct colonies in the petri dish, confirms its suitability for further research and application. Its robust performance after 24 hours of incubation suggests a high potential for scaling up biodegradation processes.

Ensuring purity and optimizing its growth conditions will be essential for maximizing its biodegradation efficiency in industrial effluent treatment (Figure 1). The results emphasize the promising potential of bacterial strain LSR-3 for efficient biodegradation, making it a strong candidate for further exploration in bioremediation applications. The moderate and low-performance strains highlight variability in metabolic efficiency, suggesting that additional studies are needed to optimize conditions for broader microbial applications. These findings underscore the importance of incubation duration in

determining biodegradation capabilities and pave the way for enhanced wastewater treatment solutions.

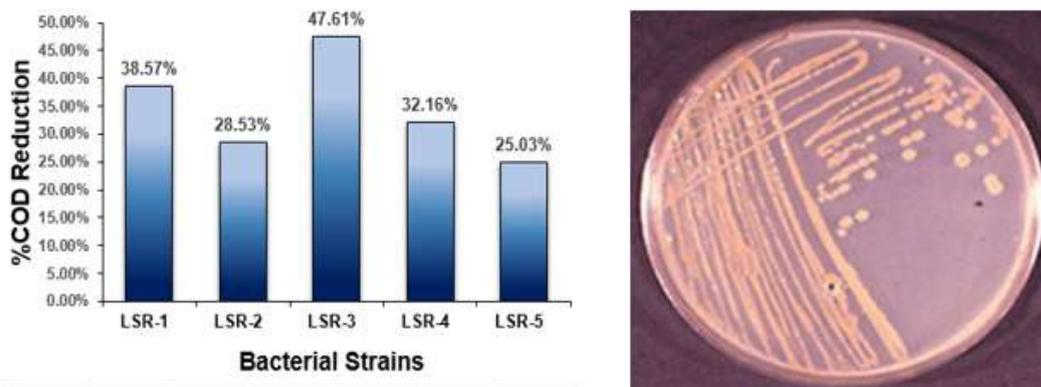


Figure-1. (a) Screening of most potent bacterial strain for % COD removal (b) Pure colonies of the isolated strain

### 3.3. Morphological characterization and identification of bacterial strain by 16S-rDNA

The morphological characterization and biochemical tests performed on LSR-3 strain provide significant insights into its identity and biodegradation potential. The extraction of pure colonies ensured precise and uncontaminated observations, enabling detailed analysis under a microscope and through specific biochemical assays.

#### 3.3.1. Morphological Characterization

The bacterial strain LSR-3 demonstrated a Gram-positive result, confirming the presence of a thick peptidoglycan cell wall. Its shape was determined to be round and convex, which is typical of many soil-associated bacterial species. The strain exhibited a yellow pigment, likely linked to its ability to produce specific secondary metabolites, which may play a role in biodegradation processes.

#### 3.3.2. Molecular identification

The molecular identification of the LSR-3 bacterial strain was accomplished through amplification of the 16S-rDNA. The annealing temperature for the polymerase chain reaction (PCR) was optimized to achieve maximum amplification efficiency, with the best results obtained at an annealing temperature of 53°C. An approximately 1.5 kb 16S rDNA fragment was successfully amplified from the genomic DNA of LSR-3, confirming the integrity of the PCR process. Following amplification, the fragment was sequenced to determine its nucleotide composition. The sequence that was obtained was:

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CACTGGTCACACCCCGCTAACTAAGCGGACAAGAGTATGGTCGCCTCTTTTCAGCGCCAAAAGCCTCCGATG
CCATTTTCAGATAATCTTCGCCATCTCCAATACCCTTTTTGCATTGTCTCGCGGACGTACGTTTTTTACGCTCC
TTCAAATACAAATGCATCGCCTGGGAATGAACTCGCTGCGATTGGATTTCTCCTTTTGAATGACGCCTCCACT
TCTTGTAGCAAGTGTTCGGCAAACCTGATCATGATGCTTTCGTGTTTTGACTTCGACAAGACAAATCCACTCC
AACCATGACCGTAACTTCCATCTACCATGCAGTTCTTGAAAGAACAGCCAGCATGAACAATACCTTATGTCG
AAACCATTGCAAAAATATACCTCCGTACGTTTTTCAGGCAAAGCCCACATTTGCGCTTAACTATATTCGCGC
TCCAATACAAAATTCCTGCTGGTTTTTTGTTTGCTGTTATTTACACTTTTCTGGCGCGTGTTTTTCATCGCCATG
GAGGAACACTTCGGCCACTTCCTTGTTCGAAACATAGACGCGCGGATCCGGTAATTCAGCATGCAGGGAAC
TCGTAGTTGATCGTTCCGAGCAAAGCGCAACCTCATCCAGCGAGATTTCTCTGTCTCCCGCTTACCGTACAGA
ACGACTCCTCATCGACCTGCCCTGCGCCGGCTCCAGCCTGACCATGATCTGGTCCATGCAGACTCGCCCCGAA
ATTGGATAGCGGGAACCATTGTACAACACTTCTCCCCGATTGACAAGAGGCGGGAGTACCCGTCTGCGTATC
CGATCGGCAGCCGCGATCCGCTCGCCAAGCTTTGCTGTGTACGTAGCGCCATAGGACCGTAACGGCTCTACA
GCGGTCTTTACATGCGCAATTCTGTTTTTCAGGGAAAGGCAGGGACAAGAGGGACGTCCGCTCGCTTTCATGT
ACGTCATCGGTTTCGACTTT
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The sequence obtained from LSR-3 was entered into the National Center for Biotechnology Information's (NCBI) BLAST nucleotide search engine to identify closely related phylogenetic sequences. The BLAST analysis revealed a 98% similarity between LSR-3 and the species *Brevibacillus agri*, supporting its identification as a member of this genus.

The phylogenetic tree presented in Figure-2 provides a comprehensive visualization of the evolutionary relationships of *Brevibacillus agri* strain LSR-3, as derived from its 16S rDNA gene sequence. The neighbor-joining method was employed to construct the dendrogram, allowing for accurate clustering based on sequence similarity. Position of *Brevibacillus agri* LSR-3: Strain LSR-3 is shown as a direct branch within the *Brevibacillus agri* clade, indicating a close genetic relationship

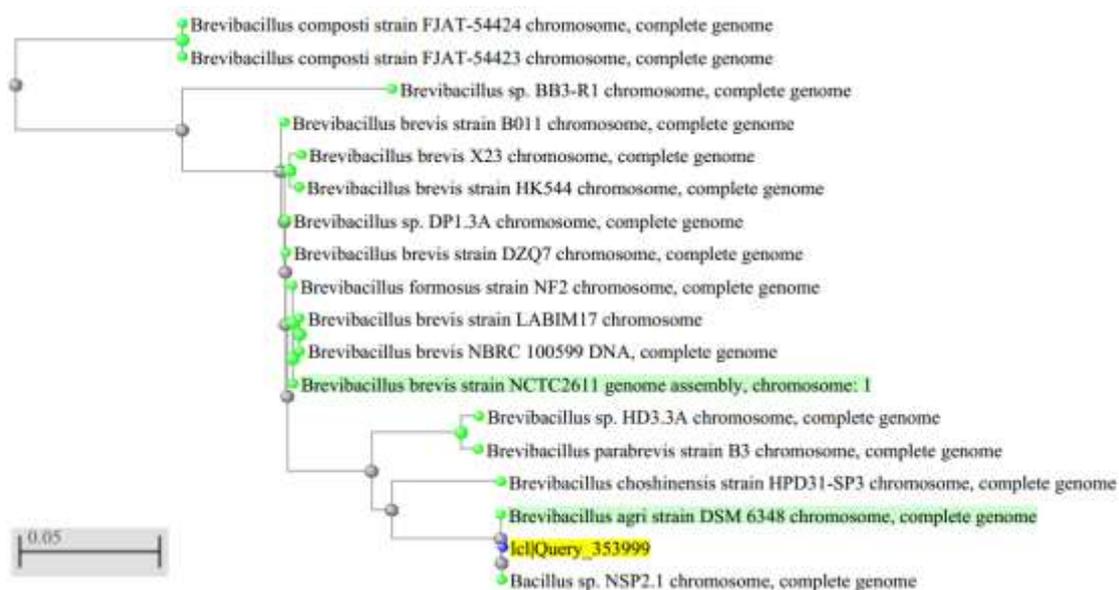
with other *Brevibacillus agri* strains. The 98% similarity to its nearest neighbor, *Brevibacillus agri* strain DSM 6348, emphasizes the high genetic relatedness of LSR-3 to this species.

**Branch Lengths and Genetic Distance:** The branch lengths in the tree represent genetic divergence. The relatively short branch length between LSR-3 and other *Brevibacillus agri* strains indicates minimal genetic variation. Comparatively longer branches separating *Brevibacillus agri* from related species like *Brevibacillus brevis* and *Brevibacillus formosus* highlight greater genetic divergence.

**Clustering and Outgroups:** The clustering of LSR-3 within the *Brevibacillus agri* group reflects its strong phylogenetic placement in this lineage. Outgroup species such as *Brevibacillus brevis* or *Brevibacillus parabrevis* form distinct branches, underscoring evolutionary separation from LSR-3.

The phylogenetic tree demonstrates that LSR-3 shares a robust evolutionary lineage with *Brevibacillus agri* strains, supporting its identification as part of this species. The close genetic proximity to strain DSM 6348 suggests functional and metabolic similarities, including potential biodegradation pathways. This phylogenetic relationship further validates the molecular identification carried out via 16S rDNA sequencing and highlights LSR-3's position as a viable candidate for bioremediation studies.

As seen in Figure-2, a phylogeny tree was created using a neighbour joining dendrogram.



**Figure-2. Phylogenetic tree of selected bacterial strain *Brevibacillus agri* based on 16S rDNA gene sequence**

#### 4. DISCUSSION

The results of this study demonstrate the significant potential of *Brevibacillus agri* for biodegradation applications, particularly in reducing COD in pharmaceutical industrial wastewater (PIW) and Diclofenac (DCF). The screening and isolation of bacterial strains revealed that *Brevibacillus agri* (LSR-3) exhibited the highest COD reduction efficiency among the tested strains, with a peak reduction of 47.61% after 24 hours. This aligns with findings by Bai et al. (2024), who reported the effectiveness of *Brevibacillus agri* in degrading organic pollutants under optimized conditions. The morphological and biochemical characterization further confirmed the strain's suitability for bioremediation, with its enzymatic activities, such as catalase and oxidase, playing a crucial role in pollutant degradation.

The molecular identification of LSR-3 through 16S-rDNA sequencing validated its classification as *Brevibacillus agri*, with a 98% similarity to reference strains. This genetic confirmation is consistent with studies by Badar et al. (2017), who highlighted the importance of 16S-rDNA analysis in accurately identifying bacterial strains for environmental applications. The phylogenetic analysis further established the evolutionary relationship of LSR-3 with other *Brevibacillus* species, emphasizing its genetic potential for biodegradation. The strain's ability to degrade DCF and PIW at varying concentrations underscores its adaptability and metabolic versatility, which are critical for addressing complex industrial effluents.

The degradation potential of *Brevibacillus agri* was evaluated across different concentrations of DCF and PIW, revealing optimal COD reduction at 100 mg/L DCF and 10% PIW concentrations. The peak COD reduction of 45% for PIW and 32%

for DCF highlights the strain's efficiency in handling mixed and single-compound pollutants. These results are in agreement with recent research by Roy et al. (2023), who demonstrated the enhanced COD removal capabilities of microbial systems under optimized substrate concentrations. However, the decline in efficiency at higher concentrations suggests substrate inhibition or toxicity, necessitating further optimization of operational parameters to maximize degradation efficiency.

The time-course analysis of COD reduction revealed that *Brevibacillus agri* achieved maximum degradation efficiency within 36 to 48 hours, with a peak reduction of 52% for PIW and 48% for DCF. This performance is comparable to findings by Zoghalmi et al, (2023) and Mamidala et al., (2013), who reported similar degradation timelines for bacterial strains in wastewater treatment. The plateau observed beyond 36 hours indicates the depletion of degradable substrates or the accumulation of inhibitory byproducts. These results underscore the importance of monitoring and optimizing incubation periods to enhance biodegradation outcomes. Overall, the study highlights the potential of *Brevibacillus agri* as a robust candidate for bioremediation, with implications for sustainable wastewater management.

## 5. CONCLUSIONS

The findings of this study highlight the significant potential of *Brevibacillus agri* as an effective bacterial strain for bioremediation of pharmaceutical industry wastewater and Diclofenac (DCF). Screening and isolation experiments demonstrated that *B. agri* (LSR-3) exhibited the highest COD reduction among all tested strains, confirming its superior biodegradation capabilities. Morphological and biochemical characterization, coupled with 16S-rDNA sequencing, further validated its identity and suitability, revealing a 98% genetic similarity to reference strains. The strain showcased optimal COD reduction at specific concentrations—45% for PIW at 10% concentration and 32% for DCF at 100 mg/L—illustrating its adaptability and enzymatic efficiency. Time-course experiments demonstrated a peak COD reduction of 52% for PIW and 48% for DCF at 36 to 48 hours, with efficiency plateauing beyond this timeframe, emphasizing the importance of optimizing incubation periods. These results underscore the ability of *B. agri* to adapt and thrive in polluted environments, breaking down complex pollutants effectively. The study highlights *B. agri* as a promising candidate for large-scale applications in treating pharmaceutical industrial effluents, offering an eco-friendly and efficient solution for reducing organic pollution. Therefore, biodegradation is a promising approach to remove these toxins.

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