www.jmolecularsci.com

ISSN:1000-9035

# Biodegradation Potential of Brevibacillus agri for Diclofenac and Pharmaceutical Wastewater Under Optimized Conditions

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## Article Information

Received: 05-07-2025 Revised: 25-07-2025 Accepted: 18-08-2025 Published: 27-08-2025

### **Keywords**

Brevibacillus agri, Diclofenac, Biodegradation, COD, Pharmaceutical Wastewater, pH

### **ABSTRACT**

This study investigates the biodegradation potential of Brevibacillus agri for the removal of Diclofenac (DCF), a widely prevalent non-steroidal anti-inflammatory drug, and pharmaceutical industrial wastewater (PIW) under optimized conditions. The objective was to identify optimal growth parameters to enhance microbial degradation efficiency and improve wastewater treatment outcomes. Isolation and identification of the bacterial strain were performed using biochemical assays and 16S rDNA sequencing, confirming Brevibacillus agri as the effective degrader. The study systematically evaluated the influence of critical factors such as carbon and nitrogen source concentrations, pH, temperature, and inoculum size on the degradation process, with chemical oxygen demand (COD) reduction serving as the primary metric. Results demonstrated that optimal concentrations of 0.10-0.15% for carbon (dextrose) and nitrogen (peptone) sources significantly enhanced COD removal, achieving up to 71.8% efficiency within 6 hours. Neutral pH (7) and temperatures of 25°C and 35°C were identified as optimal environmental conditions facilitating maximum microbial activity and enzymatic function. Inoculum sizes of 2% and 4% further improved degradation rates. Physicochemical analyses of PIW before and after treatment revealed substantial reductions in pollutants, including over 80% decreases in sulfate, nitrate, total dissolved solids, and COD, confirming the effectiveness of the biodegradation process. The study concludes that Brevibacillus agri is a promising candidate for bioremediation of pharmaceutical contaminants, offering a sustainable and efficient approach for mitigating environmental risks posed by NSAIDs and complex pharmaceutical effluents. The findings underscore the importance of optimizing nutrient levels and environmental parameters to maximize microbial degradation and support large-scale application in wastewater treatment systems.

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### 1. INTRODUCTION:

The occurrence of emerging pollutants has become a global concern due to their adverse effects on non-target organisms in both aquatic and terrestrial ecosystems. Pharmaceuticals, particularly non-steroidal anti-inflammatory drugs (NSAIDs) such as Diclofenac (DCF), have been identified as significant contributors to environmental pollution. Their uncontrolled discharge into water bodies and persistence, even at trace levels, pose serious

ecological and health risks (Al-Odaini et al., 2020). These pollutants undergo partial transformation into metabolites upon release into the environment, but their hazardous nature often remains unchanged during treatment processes (Gunasekaran & Sathishkumar, 2024). By entering the food chain, these contaminants impact human health and disrupt ecosystems, necessitating urgent attention to their mitigation (Eapen et al., 2024).

NSAIDs, including acetaminophen, Diclofenac, and naproxen, are widely used over-the-counter medications. Diclofenac, in particular, has been linked to severe ecological consequences, such as the near-extinction of vultures in North India due to its veterinary use (Alvarenga et al., 2021). Persistent environmental contamination by DCF has been associated with reproductive and genetic alterations in mammals and other organisms (Khalidi-Idrissi et al., 2023). The unchecked infiltration of these pharmaceuticals into aquatic and soil environments poses a significant threat to biodiversity and ecosystem stability. Consequently, there is a growing body of research focused on understanding the sources, fate, and toxicological effects of these pollutants, as well as developing strategies for their effective removal (Ganiyu, 2019).

Experimental studies focusing on the optimization of microbial degradation processes involve a multifaceted approach that includes identifying suitable microbial strains, determining optimal growth conditions, and assessing the influence of various environmental factors on biodegradation rates. Factors such as temperature, pH, nutrient availability, and substrate concentration play pivotal roles in shaping microbial activity and degradation efficiency (Kumar et al., 2021). For instance, previous research has demonstrated that adjusting the pH and nutrient levels can significantly enhance the metabolic activity of specific bacterial strains, leading to improved degradation rates of pharmaceutical contaminants (Mishra et al., 2020). Additionally, the use of cosubstrates or bioaugmentation with effective microbial consortia can further optimize the biodegradation process, facilitating a more comprehensive treatment approach for complex wastewater matrices (Liu et al., 2022). This study aims to demonstrate the potential of Brevibacillus agri in degrading DCF and pharmaceutical industrial effluents under optimized conditions.

## 2. MATERIALS AND METHODS:

### 2.1 Chemicals:

Commercial-grade DCF tablets made by Abbott India Limited were acquired from a pharmacy in Hyderabad. The medication is sold under the name

Diclofenac-50. Chemicals utilised, including solvents, were of analytical quality. Chemicals of the analytical grade purchased from Sigma-Aldrich in the USA. Solvents of the HPLC grade were purchased from Merck in Germany. All additional chemicals for media and reagents were bought from Mumbai, India's Hi-Media Pvt. Ltd.

### 2.2. Isolation and Identification of bacteria:

Ten milliliters of DW were mixed with one gram of sludge and one milliliter of wastewater to make a stock solution for bacterial isolation. A 10-fold dilution was achieved by using one gramme of extract for every 10 millilitres of the diluted solution. The procedures were repeated to create dilutions up to 10-5 in order to carry out the serial dilution. A 100-fold dilution was achieved by transferring 1 mL of the diluted solution to 9 mL DW. 200–300 L of suspension were infected on petri plates containing nutritional agar media. The plates were incubated at 27–32°C for a whole day. For use in the investigation, the individual plates containing the single microbial colonies were subsequently streaked.

Bacterial isolates were characterized through staining and biochemical tests. For staining, heatfixed smears were prepared: Gram staining differentiated gram-positive (crystal retaining) and gram-negative (safranin-stained) bacteria, while endospore staining identified spores (malachite green-retaining) and vegetative cells (safranin-stained). Biochemical characterization assessed enzymatic activity using specific media. The oxidase test detected cytochrome oxidase (blue-purple color). Lactose fermentation was indicated by a phenol red broth shift to yellow. Gelatin hydrolysis was observed via liquefaction after incubation. SIM agar tested for indole (cherry red with Kovac's reagent) and H2S (black precipitate). Urease activity turned urea broth pink due to ammonia production. Catalase activity was confirmed by bubble formation upon H<sub>2</sub>O<sub>2</sub> exposure. Litmus milk reactions assessed lactose fermentation and casein digestion. These tests collectively identified bacterial metabolic traits, aiding genus-level classification before molecular analysis.

# 2.3. Effect of growth factors and process parameters for degradation of DCF by isolated strain:

The degradation efficiency of diclofenac (DCF) by the isolated bacterial strain was assessed by optimizing various growth factors and process parameters. The composition of the growth medium, particularly the concentration of selected carbon and nitrogen sources, significantly influenced microbial activity and COD removal

efficiency (Xiding et al., 2025; Hisam et al., 2024). Using minimal salt medium (MSM) supplemented with 0.1 g/L DCF, the effect of pH (6-9) was evaluated, with adjustments made using 0.01 M HCl or NaOH and incubation at 32°C and 120 rpm. The optimal concentrations of carbon and nitrogen were maintained throughout. Inoculum size was another critical factor, with volumes ranging from 1-4% tested under optimized nutrient and pH conditions. Temperature also played a key role, with experiments conducted at 20°C, 25°C, 30°C, and 35°C to determine the most favorable environment for bacterial growth and DCF degradation. The optimal conditions identified through these assessments were used in subsequent studies to maximize degradation efficiency and biomass yield.

# 2.4. Analysis of reduction in chemical oxygen demand (COD) in DCF containing media and PIW:

As an organic substance, diclofenac significantly raises the chemical oxygen demand (COD) unit of any solution to which it is added. COD is a reliable indicator for assessing how well effluent from the pharmaceutical industry is being bioremediated. The wastewater's organic and inorganic load is measured. PIW and medium containing DCF had their COD measured using the "open reflux method" (APHA Standard methods for the examination of water and wastewater). Before and after the degrading process, the part played by isolated bacteria in the percentage reduction of COD was investigated. To do this, the chosen bacterial population was injected into growth media containing the sample. At predetermined intervals (6, 12, 18, 24, 30, 36, and 48 hours), samples were taken and centrifuged for 20 minutes at 5000 rpm. A preliminary examination of biodegradation for the supernatant solution used a percentage COD decrease estimate. Inoculum-free control samples were employed.

# 2.5. Determination of physicochemical characteristics of pharmaceutical wastewater after degradation

Pharmaceutical wastewater was analysed for its physicochemical characteristics both before and after *B. agri's* biodegradation to determine its effectiveness. According to the Standard Methods for the Examination of Water and Wastewater handbook (APHA, 2011), the studied parameters included pH, colour, conductivity, TS, TDS, TSS, alkalinity, turbidity, hardness, COD, fluoride, chloride, nitrate, sodium, and other factors (See chapter 3 for complete details).

#### 3. RESULTS:

### 3.1. Identification of Bacterial strain:

Based on the biochemical tests and alignment with 16S-rDNA sequencing results, LSR-3 identified as Brevibacillus agri. This bacterial species is known for its role in biodegradation and organic matter decomposition. The combination of enzymatic activities, non-motility, and substrate utilization patterns positions Brevibacillus agri as a candidate for further studies bioremediation pharmaceutical of industry wastewater.

# 3.2. Effect of various growth factors and process parameters in COD removal:

Carbon and nitrogen concentrations play a pivotal role in enhancing bacterial metabolic activity and, consequently, the degradation of diclofenac (DCF). Experimental data (Figures 1 and 2) reveal that 0.15% concentrations of dextrose and peptone achieved maximum COD removal efficiencies of 68.3% and 71.8%, respectively, within just 6 hours. Bar graphs depicting different concentrations of carbon (0.05%, 0.10%, and 0.15%) show that higher concentrations significantly boost COD removal, particularly in the early stages. At 6 hours, 0.15% dextrose yielded around 65% COD removal, with 0.10% and 0.05% showing slightly lower efficiencies. However, by 12 hours, a slight decline was observed, potentially due to microbial saturation or byproduct accumulation, though 0.15% still led with 60% removal. Interestingly, at 24 hours, 0.10% dextrose recovered to 65%, matching 0.15%, suggesting improved microbial adaptation over time. Similarly, varying nitrogen (peptone) concentrations demonstrated that 0.15% showed superior performance at 6 hours (70%), but its efficiency declined slightly by 12 and 24 hours.

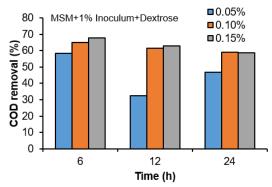


Figure-1. Effect of % concentration of carbon (dextrose) source on percent COD (Chemical oxygen demand) reduction of Diclofenac

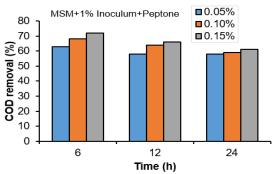


Figure-2. Effect of % concentration of nitrogen (peptone) source on percent COD (Chemical oxygen demand) reduction of Diclofenac

In contrast, 0.10% peptone showed consistent improvement, indicating that intermediate concentrations may better sustain microbial activity over longer durations. These findings highlight that while higher C and N levels initially enhance degradation, optimal concentrations like 0.10–0.15% are more effective for prolonged COD reduction in DCF-contaminated systems.

Overall, the results emphasize that while higher nitrogen concentrations (0.15%) enhance degradation in the short term, intermediate concentrations (0.10%) may provide more consistent performance across longer durations. Optimizing nitrogen source concentration is therefore essential to sustain microbial activity and maximize COD reduction efficiency in DCF degradation processes.

#### 3.3. Effect of Ph:

The bar graph in Figure-3 illustrates the effect of pH on COD (Chemical Oxygen Demand) removal during diclofenac degradation, highlighting pH as a critical factor influencing microbial efficiency. Tested across a range of pH values (6 to 9), the data show that COD removal was lowest at pH 6 (≈45%), indicating limited bacterial activity in acidic conditions, likely due to impaired enzymatic function. The highest COD removal, around 65%, was observed at pH 7, suggesting that neutral pH provides optimal conditions for microbial growth and enzymatic degradation. This peak performance confirms that neutral conditions best support the biological breakdown of diclofenac. At pH 8, COD removal declined slightly to approximately 60%, implying a modest reduction in enzymatic activity under mildly alkaline conditions. Further alkalinity at pH 9 led to a more noticeable drop in efficiency to about 55%, likely due to cellular stress or enzyme inhibition. These results demonstrate that extreme pH levels—either acidic or alkaline negatively impact microbial degradation potential. Overall, the analysis confirms that pH 7 is the most favorable for maximizing COD removal, highlighting the importance of maintaining neutral

pH in bioremediation processes to ensure optimal bacterial performance and effective pollutant breakdown.

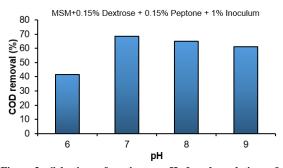


Figure-3. Selection of optimum pH for degradation of Diclofenac

### 3.4. Effect of Temperature:

Temperature plays a vital role in influencing microbial growth and enzymatic activity during biodegradation. In this study, the bacterial strain LSR-3 was tested for diclofenac (DCF) degradation at temperatures ranging from 20°C to 35°C under optimized conditions (MSM + 0.15% dextrose + 0.15% peptone, 2% inoculum, pH 7). As shown in Figure-12, COD removal was about 55% at 20°C, indicating moderate activity, possibly limited by slower enzyme kinetics. The efficiency peaked at 25°C with 70% COD removal, marking it as the optimal temperature due to favorable enzyme and microbial performance (Figure 4). A slight decrease to 60% was observed at 30°C, suggesting thermal stress may start affecting microbial stability. Interestingly, COD removal rose again to 70% at 35°C, indicating a second temperature optimum, likely due to strain-specific thermal tolerance. These results highlight 25°C and 35°C as ideal for effective DCF degradation and underscore the importance of temperature optimization bioremediation strategies.

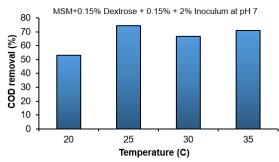


Figure-4. Selection of optimum temperature for degradation of Diclofenac

# 3.5. Analysis of physicochemical parameters before and after degradation:

The analysis of physicochemical parameters before and after degradation demonstrates the effectiveness of the biodegradation process in

enhancing water quality. A slight increase in pH from 7.1 to 7.5 (3.90%) suggests a stable, slightly alkaline environment post-treatment, favorable for microbial activity (Table 1). Major ionic pollutants such as sulfate and chloride were significantly reduced by 81.09% and 81.32%, respectively, indicating efficient removal of inorganic contaminants. Reductions in potassium (73.68%) and sodium (73.79%) further reflect the system's ability to lower salinity and associated toxicity. Physical parameters including turbidity (73.13%), total suspended solids (TSS; 81.30%), and total solids (TS; 81.83%) also showed marked improvement, enhancing water clarity. Nitrate levels decreased by 89.03%, indicating effective removal, critical nitrogen for preventing eutrophication. Electrical conductivity dropped by 91.20%, confirming substantial ion removal. Significant decreases in total hardness (81.24%), calcium hardness (81.70%), and magnesium hardness (81.45%) point to lower scaling potential. COD removal of 82.95% highlights strong degradation of organic matter. Additionally, total dissolved solids (TDS; 81.90%), salinity (82.01%), and alkalinity (83.05%) were significantly reduced. These results collectively affirm the biodegradation process's high efficiency in treating pharmaceutical wastewater and improving its suitability for safe discharge or reuse.

Table-1: Analysis of physicochemical parameters before and after degradation

after degradation			
Parameter	Before	After	%
	degradation	degradation	reduction
pН	7.1±0.81	7.5±0.54	3.90
Sulfate (mgL-1)	253±4.0	50.20±1.71	81.09
Chloride (mgL-	134±2.2	25.23±0.05	81.32
1)			
Potassium	42.8±0.64	10±0.02	73.68
(mgL-1)			
Sodium (mgL-	123±2.03	29±1.96	73.79
1)			
Turbidity	30.9±1.15	8.21±0.31	73.13
(NTU)			
Nitrate (mgL-1)	243.29±1.87	50.80±0.52	89.03
Conductivity	0.374±0.01	0.024±0.001	91.20
(S)			
TSS (mgL-1)	585±7.06	101.33±1.02	81.30
TS (mgL-1)	7726±10.20	1301±2.64	81.83
Ca hardness	192.6±0.67	31.9±0.11	81.70
(mgL-1)			
Hardness (mgL-	970±2.98	164.24±0.67	81.24
1)			
TDS (mgL-1)	7158±18.12	1217±5.16	81.90
Mg hardness	119.8±1.68	22.4±0.23	81.45
(mgL-1)			
COD (mgL-1)	1024±2.6	174.24±1.11	82.95
Alkalinity(mgL-	51±0.80	7.67±0.06	83.05
1)			
Salinity(mgL-1)	141±9.1	22.13±1.2	82.01
Alkalinity(mgL-	51±0.80	7.87±0.06	83.05
1)			

#### 4. DISCUSSION:

The results of this study demonstrate the significant influence of various growth factors and process parameters on COD removal efficiency during Diclofenac (DCF) and pharmaceutical industrial wastewater (PIW) degradation. The effect of carbon and nitrogen sources revealed that optimal concentrations (0.10% for carbon and 0.15% for nitrogen) enhanced microbial activity, achieving maximum COD reduction. This aligns with findings by Bai et al. (2023), who reported that balanced C/N ratios (6-7) support stable microbial networks and efficient pollutant degradation in constructed wetlands. Similarly, Sun et al., (2023) observed that increasing COD/ammonia ratios improved COD removal efficiency, emphasizing the importance of nutrient optimization in bioremediation processes. These results underscore the necessity of maintaining appropriate nutrient levels to maximize microbial degradation efficiency.

The analysis of inoculum size demonstrated that 2% and 4% inoculum concentrations achieved the highest COD removal, indicating the importance of microbial density in pollutant degradation. Zoghlami et al. (2023) similarly observed that higher inoculum sizes enhanced COD removal in slaughterhouse wastewater, although excessive microbial density could lead to substrate competition and reduced efficiency. These findings highlight the need to balance microbial load and substrate availability to optimize degradation processes. Hamdi et al., (2022) also reported that inoculum size significantly influenced COD reduction in electroplating effluent, further validating the results of this study.

### 5. CONCLUSIONS:

The bioremediation of NSAIDs, particularly Diclofenac and PIW, by Brevibacillus agri has been documented for the first time in the literature, making the current work unique in its kind. The findings of this study highlight the critical role of optimizing growth factors and process parameters for efficient degradation of Diclofenac (DCF) and pharmaceutical industrial wastewater (PIW). Carbon and nitrogen source optimization demonstrated that balanced nutrient levels (0.10% carbon and 0.15% nitrogen) significantly enhanced microbial activity, achieving maximum COD reduction. Environmental factors such as pH, inoculum size, and temperature were also shown to be key determinants of bioremediation efficiency, with neutral pH (7), inoculum sizes of 2% and 4%, and temperatures of 25°C and 35°C providing conditions optimal for degradation. before and after Physicochemical analysis degradation revealed substantial improvements,

including an 81–91% reduction in major pollutants such as sulfate, nitrate, total dissolved solids, and COD, underscoring the treatment's effectiveness. The study establishes *Brevibacillus agri* as a promising candidate for large-scale applications in the treatment of pharmaceutical contaminants and highlights the potential for sustainable and efficient wastewater management.

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