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Research Article

Effect of Urea on Isolates of Winogradsky

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Abstract

The Winogradsky column, a classic experimental model, is widely used to study microbial ecology, metabolic diversity, and nutrient cycling in sedimentary environments. It provides a self-sustaining system where distinct microbial layers form based on oxygen, light, and nutrient gradients. Among the critical nutrients influencing microbial growth and composition, nitrogen—especially in the form of urea—plays a central role in microbial metabolism and ecosystem dynamics (Duppala & Kaladhar, 2019) [13]. Urea is a nitrogen-rich compound commonly found in agricultural runoff and wastewater, where it can alter microbial community structures and inhibit sensitive microbial populations.

This study investigates the effect of urea on microbial isolates obtained from different layers of a Winogradsky column, using the Minimum Inhibitory Concentration (MIC) method to determine tolerance thresholds. Isolates included cyanobacteria, green sulfur bacteria, and sulfate-reducing bacteria (SRB), each with distinct ecological functions. Serial dilutions of urea (0.1 to 5.0 mg/mL) were prepared, and growth inhibition was measured through optical density and colony count analyses following CLSI (2021) [8] microbroth dilution protocols.

The study underscores the importance of nitrogen management in aquatic and soil ecosystems, as excessive urea can shift microbial populations and affect nutrient cycling processes. Applying the MIC method in an ecological context bridges microbiological and environmental science, providing a quantifiable approach to assess chemical impacts on microbes. This research contributes to the understanding of nitrogen stress in microbial systems and its ecological implications.

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KEYWORDS: Winogradsky column, urea, Minimum Inhibitory Concentration (MIC), microbial ecology, cyanobacteria, sulfate-reducing bacteria (SRB), green sulfur bacteria, nitrogen metabolism, environmental microbiology, microbial isolation, nutrient cycling, Gram staining, microbial sensitivity, biogeochemical gradients, stratified microbial ecosystems.

1. INTRODUCTION

Microorganisms play an essential role in regulating nutrient cycling, ecosystem productivity, and the maintenance of environmental balance. Among various tools developed to study microbial ecology, the Winogradsky column remains one of the most effective and visually demonstrative systems. First introduced by Sergei Winogradsky in the late 19th century, this column creates a gradient-based microenvironment where diverse microbial communities stratify based on their metabolic capabilities and tolerance to oxygen and light (Duppala & Kaladhar, 2019) [13]. It serves as an in situ simulation of sedimentary environments, allowing researchers to observe microbial succession, interdependence, and response to changing chemical variables. Traditional remediation

techniques like soil excavation or chemical oxidation are costly, invasive, and often produce secondary pollution. This has driven the demand for sustainable, eco-friendly alternatives. However, natural attenuation is often too slow in highly contaminated or stressed environments. Hence, this study emphasizes bioaugmentation the introduction of indigenous new method degrading bacteria isolated from contaminated soil. Nitrogen, one of the most critical macronutrients in ecosystems, profoundly affects microbial growth and community composition. Urea, an organic nitrogen compound widely used in agriculture and wastewater treatment, is hydrolyzed by urease-producing bacteria into ammonia and carbon dioxide. This biochemical transformation can lead to elevated pH levels and ammonia toxicity, thereby influencing microbial survival, enzyme activity, and nutrient cycling pathways (Mobley & Hausinger, 1989). Despite its agricultural benefits, urea's ecological implications are a concern, especially in sensitive aquatic and soil ecosystems where microbial equilibrium is easily disrupted (Galloway *et al*., 2008) [19].

Understanding how different microbial isolates from the Winogradsky column respond to urea stress can reveal much about microbial resilience, metabolic flexibility, and the ecological roles of different functional groups. For instance, cyanobacteria, capable of photosynthesis and nitrogen fixation, may exhibit greater resistance to nitrogen influx, whereas anaerobic organisms like sulfate-reducing bacteria could be more susceptible due to their metabolic limitations and sensitivity to ammonia accumulation (Madigan *et al*., 2018).

To evaluate this, the Minimum Inhibitory Concentration (MIC) method—a standardized approach used in antimicrobial testing—can be employed. MIC testing helps determine the lowest concentration of urea that visibly inhibits microbial growth, offering a quantitative measure of microbial tolerance and susceptibility.

This study provides new insights into how nitrogen enrichment through urea affects environmentally significant microbes. The integration of classic ecological tools with microbiological techniques like MIC testing offers a comprehensive approach to studying the biochemical and ecological consequences of human-induced nitrogen perturbations.

2. LITERATURE REVIEW

A. National Status

D.S.V.G.K. Kaladhar and S. Duppala (2019) [13] emphasized the utility of the Winogradsky column in both education and microbial ecology research. T. Satyanarayana (2005–present) studied microbial enzymes, including urease, to understand nitrogen metabolism in extremophilic organisms. R.K. Jain (1990s) and M. Mahadevan (2008) investigated microbial biodegradation and nitrogen transformations in Indian soils, particularly the impact of synthetic fertilizers like urea**.** N. Raghuram (2007) highlighted the significance of nitrogen use efficiency and its influence on microbial health in agricultural ecosystems**.** A.K. Sarma (2010) and S. Ray (2013) explored microbial responses to nutrient enrichment in Indian freshwater and wetland environments, reinforcing the ecological relevance of studying MIC in stratified microbial communities. Collectively, these national contributions enhance the context for evaluating microbial responses to nitrogen stress in diverse Indian ecosystems**.**

B. International Status

Sergei Winogradsky (1890s), who introduced the Winogradsky column to study microbial stratification and nutrient cycling in sedimentary environments. Martinus Beijerinck (1901) contributed the concept of enrichment cultures, enabling the selective growth of microbial populations. Carl Woese (1977) revolutionized microbial classification using 16S rRNA sequencing, which remains central to identifying environmental isolates today. Selman Waksman (1940s) focused on soil microbiology and discovered antibiotic-producing actinomycetes, highlighting methods for microbial isolation. In more recent decades, David Stahl (1990s) and Donald E. Canfield (2010) have expanded the understanding of microbial roles in sulfur and nitrogen cycling, particularly in anoxic environments. Bruce Rittmann (2000s) contributed significantly to environmental biotechnology and microbial interactions in wastewater systems, while J. Tiedje (1980s–1990s) examined denitrification and microbial responses to nitrogen enrichment. Together, these international efforts provide foundational and modern frameworks for studying microbial responses to compounds such as urea using methods like MIC.

3. METHODOLOGY

This study was designed to evaluate the BTEX-degrading potential of indigenous bacteria isolated from petroleum-contaminated soil. All experimental procedures were conducted under aseptic and controlled laboratory conditions in a single primary phase comprising microbial isolation, screening, degradation testing, and biochemical characterization.

A. Sample Collection

Soil samples were collected from three ecologically diverse environments to ensure the representation of a wide range of microbial communities: riverbank sediment, garden soil, and petroleum-contaminated soil. The riverbank sediment was obtained from a shallow, organic-rich edge of a freshwater stream, known to support dense microbial diversity due to continuous exposure to fluctuating oxygen and moisture levels (Madigan *et al*., 2018). Garden soil, collected from the upper 10 cm layer of cultivated land, served as a representative of nutrient-rich terrestrial ecosystems with active nitrogen-cycling microbes (Prescott *et al*., 2005). Petroleum-contaminated soil was sourced from an industrial zone near a fuel storage facility to include microbes capable of hydrocarbon degradation and those adapted to chemically stressed environments (Timmis, 2002). All soil samples were collected using sterile spatulas and stored in sterile, labeled containers. The samples were then transported to the laboratory and kept at 4°C until they were processed for column preparation and microbial isolation.

Fig 1: Sample collection from river side.

B. Winogradsky Column Preparation

Fill transparent columns with sediment and water from the collection site. Amend with specific nutrients (e.g., cellulose, sulfate) to encourage microbial growth. Incubate under controlled light and temperature conditions for several weeks.

Fig 1: Observation after 1 week.

Fig 2: Observation After week 3

C. Sampling and Isolation of Microbial Isolates

Sampling from the Winogradsky columns was conducted weekly using sterile syringes or spatulas to collect biomass from specific layers and gram staining was performed weekly Top aerobic layer (green): Expected to contain cyanobacteria

and algae.Middle transition zone (purple, pink, or orange): Rich in phototrophic sulfur bacteria.

Bottom anaerobic zone (black): Likely containing sulfate-reducing bacteria (SRB) and fermenters. Samples were streaked on selective agar media to promote the growth of specific microbial groups: BG11 agar was used for cyanobacteria. Postgate's B medium supported SRB growth under anaerobic conditions. Chlorobium agar promoted the growth of green sulfur bacteria.

Plates were incubated at 30°C for 3–7 days. Anaerobic incubations were carried out in anaerobic jars using gas packs. Subculturing was done until pure isolates were obtained. Morphological features were documented through Gram staining and light microscopy.

D. Preparation of Urea Solutions

Stock urea solution (10 mg/mL) was prepared using analytical grade urea dissolved in sterile distilled water. Serial dilutions were made to generate concentrations ranging from 0.1 mg/mL to 5.0 mg/mL, which were then used for MIC assays. All dilutions were sterilized using 0.22 µm filters and stored at 4°C until use.

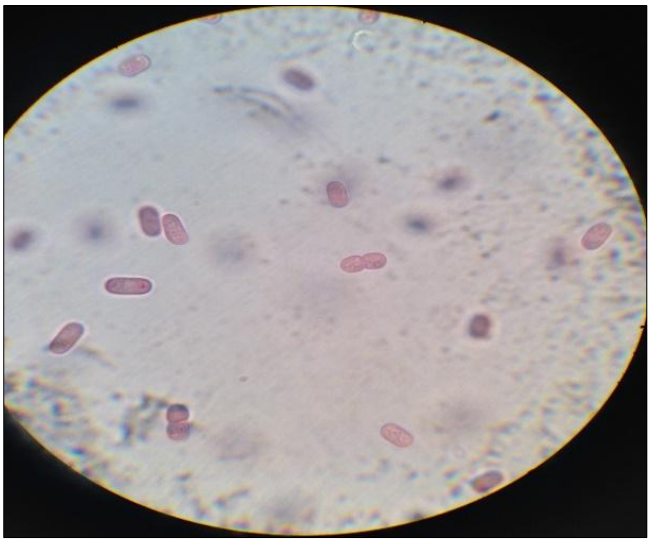

Fig 3: Microscopic view of Chromatium (purple- sulphur bacteria)

E. MIC Testing Procedure

The Minimum Inhibitory Concentration (MIC) of urea for each microbial isolate was determined using the microbroth dilution method, adhering to CLSI (Clinical and Laboratory Standards Institute) guidelines.

Step-by-step MIC procedure

Inoculum Preparation

Freshly grown isolates were suspended in sterile saline to achieve a turbidity equal to 0.5 McFarland standard (~1×10^8 CFU/mL). This suspension was diluted 1:100 in broth to reach a final concentration of ~1×10^6 CFU/mL.

Plate Setup

A sterile 96-well microtiter plate was used. Each well contained 100 µL of culture medium (e.g., nutrient broth or BG11 broth), 100 µL of urea solution at designated concentrations, and 10 µL of the microbial inoculum.

Controls: Positive control: Medium + inoculum (no urea) Negative control: Medium + urea (no inoculum) Sterility control: Medium only

Incubation

Plates were sealed and incubated at 30°C for 24–48 hours. Anaerobic isolates were incubated in anaerobic chambers to maintain appropriate environmental conditions.

Reading Results

After incubation, microbial growth was assessed visually (turbidity) and spectrophotometrically at 600 nm (OD600).

The MIC was recorded as the lowest concentration of urea where no visible growth or OD increase was observed compared to the control.

6. Data Recording and MIC Table Construction

MIC values for each isolate were recorded across multiple replicates for statistical consistency.

The isolates were grouped by type (e.g., cyanobacteria, green sulfur bacteria, SRB), and their average MIC values were tabulated.

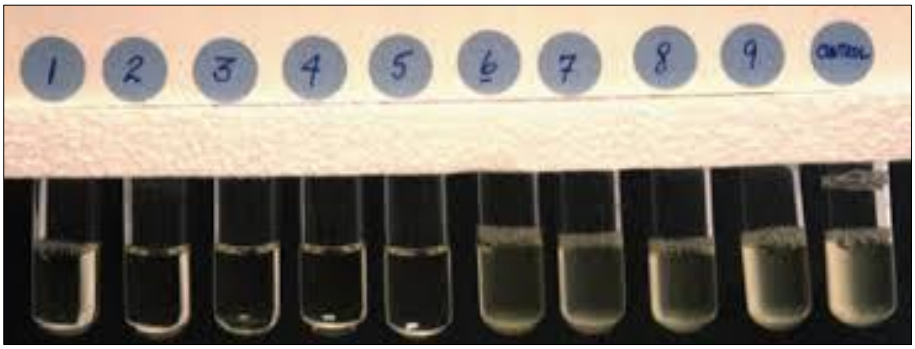

Fig 4: MIC Tubes

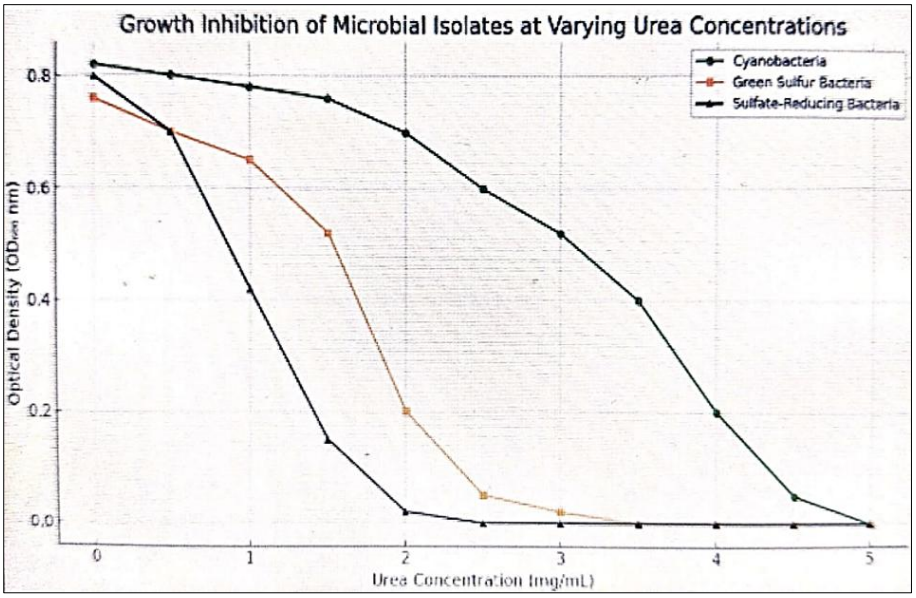

Fig 5: Graphical Representation of Urea Concentration Vs OD 600 nm

4. RESULTS

The Minimum Inhibitory Concentration (MIC) assays conducted on microbial isolates from the Winogradsky column demonstrated significant differences in the tolerance of various microorganisms to increasing concentrations of urea. The three major microbial groups analyzed—cyanobacteria, green sulfur bacteria, and sulfate-reducing bacteria (SRB)—responded differently to urea stress, indicating their varying metabolic capabilities and environmental sensitivities.

Cyanobacteria, isolated from the upper, oxygen-rich layer of the Winogradsky column, exhibited the highest resistance to urea, with an MIC value of 4.0 mg/mL. This finding suggests that cyanobacteria possess robust urease activity and can tolerate elevated levels of ammonia, the byproduct of urea hydrolysis. Their ability to carry out oxygenic photosynthesis and nitrogen fixation may also contribute to their resilience, enabling them to adapt to nutrient-rich environments without significant physiological disruption. The relatively high MIC for cyanobacteria indicates their potential dominance in nitrogen-enriched aquatic environments, where urea runoff is common.

Green sulfur bacteria, found in the mid-anoxic zone of the column, demonstrated moderate sensitivity to urea, with an MIC of 2.0 mg/mL. These organisms are obligate anaerobes and phototrophs that rely on reduced sulfur compounds for energy. Their sensitivity suggests that urea, or more specifically its hydrolysis product ammonia, interferes with their metabolic pathways or disrupts the delicate redox balance required for their survival. As a result, nitrogen enrichment could suppress their ecological role in sulfur cycling in natural sediment systems. The most urea-sensitive organisms in this study were the sulfate-reducing bacteria (SRB), with an MIC of just 1.5 mg/mL. Isolated from the anaerobic, deeper layers of the column, SRB rely on strict anoxic conditions and sulfate respiration. Ammonia accumulation resulting from urea breakdown may be toxic to these organisms, inhibiting essential enzymes or altering the pH of their microenvironment. Their high sensitivity indicates that excessive urea input into sedimentary or aquatic systems could severely hinder sulfate reduction and disrupt sulfur cycling processes.

The analysis of optical density (OD600) measurements and colony-forming units (CFU/mL) across varying concentrations further supports the MIC data. A clear decline in microbial growth was observed as urea concentration increased, particularly in SRB and green sulfur bacteria cultures. The growth curve plotted for each organism revealed a sigmoidal dose-response relationship, with a sharp decline in growth at concentrations near their respective MIC values.

In summary, the MIC results confirm that urea has a differential inhibitory effect on microbial communities, strongly dependent on their metabolic type and ecological niche. While some microbes like cyanobacteria can withstand high nitrogen levels, others, especially strict anaerobes, are vulnerable. These findings have broad ecological implications, especially concerning nitrogen pollution and its impact on microbial diversity, ecosystem functions like sulfur cycling, and the stability of natural microbial consortia

5. CONCLUSION

This study demonstrates that urea significantly affects the growth and metabolic activity of microbial isolates obtained from Winogradsky columns. The application of the Minimum Inhibitory Concentration (MIC) method provided a quantitative means to assess microbial sensitivity to varying concentrations of urea. Among the isolates tested, cyanobacteria displayed higher tolerance levels, whereas sulfate-reducing bacteria and green sulfur bacteria were more susceptible, with observable growth inhibition at lower concentrations. These findings suggest that excess nitrogen, particularly in the form of urea, can selectively suppress certain microbial groups while

allowing others to dominate, ultimately leading to ecological imbalance in natural environments.

The implications of this research are valuable for environmental microbiology, biogeochemistry, and agricultural management. It emphasizes the need for judicious use of nitrogen-based fertilizers to prevent unintended microbial shifts that may impact nutrient cycling and ecosystem health. Furthermore, this study reinforces the effectiveness of the Winogradsky column and MIC testing as tools for environmental impact assessment.

6. RECOMMENDATIONS

Based on the findings, future research should explore the genetic mechanisms underlying urea resistance in tolerant species, investigate long-term effects of urea exposure on microbial diversity, and assess the impact of combined nutrient exposures. It is also recommended to monitor field-scale microbial responses in agricultural and industrial settings where urea is commonly used. By integrating classical ecological models with modern microbial analysis, researchers can better predict and manage the microbial consequences of anthropogenic nutrient inputs.

ABBREVIATIONS

MIC- Minimum Inhibitory Concentration
SRB- Sulphate Reducing Bacteria
GSB – Green Sulphur Bacteria

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