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Bioprospecting Of Soil Microflora For Urease Activity And Their Role In Urea Detoxification From Wastewater

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ABSTRACT

Urea-rich wastewater from agricultural and industrial sources poses a significant threat to environmental sustainability due to nitrogen accumulation and eutrophication. In this study, soil samples were collected from agricultural farmland and petroleum-contaminated soil to explore and harness native urease-producing microflora for bioremediation applications. Bacterial colonies were isolated and screened for urease activity using nutrient agar plates. Among the isolates, a potent urease-producing strain, designated WUA_1, identified as Pseudomonas panipatensis and subjected to further characterization through gram staining, 16S rRNA sequencing confirmed the identity of the isolate. Both qualitative and quantitative assays, including Nessler's reagent method, were employed to evaluate urease activity. Growth optimization studies were conducted under varying parameters such as temperature, pH, salinity, and incubation periods to determine conditions that maximize urease production. For enhanced practical application, cells were immobilized in alginate beads, and immobilization efficiency was assessed. Application studies demonstrated that the immobilized microbial beads effectively hydrolyzed urea in industrial effluent, confirming their potential for sustainable and eco-friendly urea detoxification. The results underscore the importance of bioprospecting soil microflora as a viable approach for wastewater treatment through enzymatic degradation of urea.

Keywords: *Urease-producing bacteria, bioremediation, wastewater treatment, urea degradation, immobilized cell reactor*

INTRODUCTION

The global reliance on urea as a nitrogen fertilizer has resulted in a significant influx of urea into terrestrial and aquatic environments, primarily through agricultural runoff and industrial waste. Once in water bodies, urea undergoes enzymatic hydrolysis, releasing ammonia, which can raise pH and lead to severe ecological imbalances such as algal blooms and hypoxia (Glibert et al., 2016; Zhang et al., 2020). Addressing this environmental challenge requires sustainable, biologically driven solutions.

Urease (EC 3.5.1.5), the enzyme responsible for urea hydrolysis, is produced by a range of organisms including plants, fungi, and bacteria. Among these, bacteria are particularly significant due to their

rapid growth, adaptability, and widespread presence in soil ecosystems. Urease catalyzes the conversion of urea into ammonia and carbon dioxide, a key process in the nitrogen cycle that also contributes to nutrient bioavailability for plants (Mobley & Hausinger, 1989). Bacterial genera such as Sporosarcina, Bacillus, Proteus, Klebsiella, and Pseudomonas have been widely recognized for their high urease activity (Krajewska, 2018; Kumar et al., 2021).

The presence and activity of urease-producing bacteria are strongly influenced by environmental parameters including soil pH, temperature, moisture content, and the availability of organic matter. Optimal urease activity typically occurs between pH 6.5 and 8.0 and under mesophilic temperatures. Organic amendments such as compost and biochar have been found to enhance microbial urease activity by improving nutrient availability and microbial biomass (Wang & Xiong, 2022).

In response to environmental challenges posed by urea pollution, research has increasingly focused on the potential application of urease-positive microorganisms in wastewater treatment systems. These microbes can effectively hydrolyze urea in contaminated effluents, including those from fertilizer industries, livestock farms, and municipal wastewater systems. Their metabolic capacity not only reduces urea concentration but also transforms it into forms usable by plants or other microorganisms, thereby contributing to nutrient recycling (Srinivasan et al., 2015; Bhavsar et al., 2020).

To improve the operational stability of these bacteria in treatment systems, strategies such as immobilization in biocompatible matrices (e.g., alginate beads) have been employed. Immobilized bacterial systems demonstrate enhanced enzyme activity, increased resilience to environmental stress, and reusability in continuous wastewater treatment processes (Wang et al., 2017; Achal & Pan, 2011). Such advancements provide a sustainable, low-cost alternative to chemical remediation methods, with additional ecological benefits.

Beyond bioremediation, urease-producing bacteria have gained attention for their role in microbial-induced calcium carbonate precipitation (MICP), a process applied in construction and soil engineering. Bacteria like Sporosarcina pasteurii have been successfully used for soil stabilization, self-healing concrete, and restoration of stone monuments (Cheng et al., 2022; Seifan et al., 2017). These multifunctional capabilities further highlight the industrial and environmental importance of ureolytic microorganisms.

The present investigation was therefore aimed at isolating and characterizing potent urease-producing bacteria from urea-rich soils and evaluating their effectiveness in degrading urea from wastewater samples. Soil samples were collected from agricultural and workshop areas known for high urea exposure. The study sought to identify microbial strains with high urease activity using both classical biochemical assays and modern molecular techniques, including 16S rRNA gene sequencing. It also aimed to optimize environmental parameters influencing urease activity such as pH, temperature, and substrate concentration. Finally, the feasibility of deploying these bacteria in bioremediation systems such as immobilized cell reactors was explored to assess their practical application in wastewater management.

Future research must continue to focus on exploring native microbial diversity, improving reactor design for scalability, and understanding the regulatory mechanisms of urease expression under variable environmental conditions. With the global emphasis on sustainable development and eco-friendly

technologies, harnessing the potential of urease-producing bacteria offers a promising avenue for environmentally responsible wastewater treatment and nitrogen management.

MATERIALS AND METHODS

Sample Collection

Soil samples contaminated with petroleum products and agricultural farm waste were collected separately from in and around the regions of Kozhikode and samples were aseptically transported to the laboratory.

Sample Processing and Serial dilution

Soil samples collected from various region were serially diluted from 10^{-1} to $10^{-7.}$ This was performed by suspending 10 gram of soil in 90 ml of sterile distilled water.

Isolation of Bacterial Colonies:

The diluted samples were spread plated on to Nutrient Agar (NA). The plates were then inverted and incubated at 37 degree C for 24 h until visible colonies were obtained. The plates with distinct colonies were isolated further for screening in urease agar slant.

Qualitative Assay of Urease:

Urease Test on Urea Agar slants

For the screening of urease producers, bacterial isolates were grown in Christensen's agar. The pH of the media was adjusted to 7 and prepared as described by (Anitha et al). Several studies have reported that urea agar base can be used as a quick method to primarily screen for urease-producing bacteria. (Chahal, N.K et.al;2011) Urea agar base contains urea and phenol red, which acts as a pH indicator. When urea is hydrolysed by the bacteria, NH_3 is released and becomes accumulated in the medium, which increases the pH of the environment making it alkaline (Mekonnen, E et.al;2021). Since urea is unstable and degrades during autoclaving, it was added separately to distilled water after the sterilization process. (Christensen, W.B. 1946).

Isolates were inoculated and streaked on Christensen's agar slants and were incubated for 24–48 h at 37 ◦C. Isolates that turned the medium reddish or pinkish color may indicate positive urease production was selected. (Abinaya, K et.al; 2018, Achal et al., 2011; Burbank et al., 2011; Hammad, et al., 2003).

Identification of Urease Producing Bacteria (UPB)

Morphological characterization

Colony characteristics were recorded after incubation, observing parameters such as colony color, form, margin, elevation, opacity, and surface texture under aseptic conditions (Madigan et al., 2018).

Gram staining

Thin smear was prepared on clean, oil-free glass slides, by dropping loop full of suspended bacterial Culture from secondary screening bacterial media. Air dried the slide or with the help of very gentle heat, to prevent the culture loss (during Washing). As primary stain, crystal violet was dropped on the smear. After 30 secs, excess stain was Washed off with water. Then Gram's iodine was added. And after 60 secs it was washed off with Ethyl alcohol and then with distilled water. After that added a few drops of safranin on the smear (counter-stain). Washed the excess stain and air dried the slides. Slides were

microscopically observed under 100X. (Pramita Ghosh et al., 2023)

Motility

Motility was checked by triphenyl tetrazolium chloride (TTC) method. The nutrient agar medium contained 0.02% TTC was prepared in test tubes and selected bacterial strains were stabbed and incubated for 24 h at 37 °C. The motility of bacterial strain outwards the test tube was observed after incubation.

Biochemical Characterization

A series of biochemical tests were carried out to determine the physiological and enzymatic characteristics of the isolate. These tests play a critical role in bacterial identification and were interpreted using established protocols as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Indole Test

Indole is a component of the amino acid tryptophan. Kovac's or Ehrlich reagent, which contains para dimethyl amino benzaldehyde detects indole production. This reacts with indole to produce a red color compound. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth and incubated at 37°C for 24-48 hours. Add 0.5ml of Kovac's reagent to the broth culture and Observe for the presence or absence of ring.

Methyl Red Test

Some bacteria have the ability to utilize glucose and convert to stable acid like lactic acid, acetic acid, as the end product. These bacteria initially metabolize glucose to pyruvic acid, which is further metabolize to produce stable acid. The acid so produced decreases the pH to 4.5 or below, which is indicated by change in the color of methyl red from yellow to red if the bacterium has the ability to utilize glucose. Inoculate MRVP broth with a pure culture of the organism. Incubate at 35°-37°C for a minimum of 48 hours in ambient air. Add 5 or 6 drops of methyl red reagent per 5 mL of broth. Observe for the color change in the broth medium.

Voges-Proskauer Test

The Voges–Proskauer (VP) test is a biochemical test used to detect the production of acetoin (also called acetylmethylcarbinol), a neutral end product of glucose fermentation in some bacteria. If present, acetylmethylcarbinol is converted to diacetyl in the presence of α- naphthol, strong alkali (40% KOH), and atmospheric oxygen. The diacetyl and guanidine- containing compounds found in the peptones of the broth then condense to form a pinkish-red polymer. Aseptically inoculate a sterile tube of MR-VP broth with a pure culture of the test organism. Incubate the tube at 35–37°C for 24 to 48 hours. After incubation, add 15 drops of Barritt's reagent A (α-naphthol). Then add 5 drops of Barritt's reagent B (KOH). Shake the tube gently but thoroughly to oxygenate the mixture. Let the tube stand undisturbed. Observe for color change at 15-minute intervals for up to 1 hour. A positive VP test is a development of a pink- red color at the surface within 15 minutes or more after the addition of the reagents. The test should not be read after standing for over 1 hour because negative Voges- Proskauer cultures may produce a copper-like color, potentially resulting in a false- positive interpretation.

Citrate Utilization Test

This test is used to detect the ability of microorganism to utilize citrate as a source of carbon and ammonium salt as a solid source of nitrogen. In Simmon's citrate medium Bromothymol blue is added

as indicator. The initial pH the medium is 6.8 and it has a green color. The utilization of citrate in the medium will change the pH of the medium to alkaline side there by changing the color of the medium to blue. Inoculate the test organism into Simmon's citrate agar and incubate at 37°C for 24-48 hours.

Catalase Test

The catalase test is used to detect the presence of the catalase enzyme, which catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. To perform the test, a clean glass slide was used to place a small portion of fresh bacterial culture. A drop of 3% hydrogen peroxide was then added to the culture. The slide was then observed for any immediate and vigorous bubbling at the site of contact, suggesting the presence of the enzyme.

Oxidase Test

This test is used to determine the presence of cytochrome c oxidase, an enzyme involved in the bacterial electron transport chain. The oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) serves as an artificial electron donor. In the presence of cytochrome oxidase, it donates electrons and is oxidized, forming a purple-colored compound. A colony of the isolate was transferred using a sterile wooden applicator to oxidase disc impregnated with reagent. The appearance of a dark purple color within 10 seconds indicates a positive oxidase reaction.

Urease Test

The urease test detects the ability of bacteria to hydrolyze urea into ammonia and carbon dioxide using the enzyme urease. The test medium used was Christensen's urea agar, which contains phenol red as a pH indicator. The isolate was streaked on the slant surface of the urea agar and incubated at 37°C for up to 48 hours. A color change from orange to bright pink indicated alkalinization of the medium due to ammonia production, signifying a positive result.

Molecular identification of the isolate by 16s rRNA Gene Sequencing Protocol

Genomic DNA isolation:

DNA isolation from Microbial samples were done using the Biobee Spin EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd., Cells grown in a monolayer were lysed by suspending 1-3 colonies aseptically and mixing them with 500 µl of lysis buffer in a 2 ml microcentrifuge tube. The cells were lysed by repeated pipetting. Next, 4 µl of RNase and 500 µl of neutralization buffer were added to the mixture. The contents were vortexed and incubated for 30 minutes at 65°C in a water bath. To minimize DNA shearing, the DNA solutions were mixed by inversion. The tubes were then centrifuged for 10 minutes at 10,000 rpm. After centrifugation, the resulting viscous supernatant was transferred into a fresh 2 ml microcentrifuge tube without disturbing the pellet. Following this, 600 µl of Chloroform Isoamyl Alcohol was added, and the mixture was hand-mixed vigorously. The tubes were centrifuged again for 10 minutes at 10,000 rpm, and 600 µl of the aqueous phase was carefully transferred into a fresh 2 ml microcentrifuge tube.

For binding, 600 µl of binding buffer was added to the content, mixed thoroughly by pipetting, and incubated at room temperature for 5 minutes. The contents were then transferred into a spin column placed in a 2 ml collection tube. The spin column was centrifuged for 2 minutes at 10,000 rpm, and the flow-through was discarded. The spin column was reassembled with the collection tube, and the remaining 600 µl of lysate was added. After centrifuging for 2 minutes at 10,000 rpm, the flow-through was discarded. For washing, 500 µl of washing buffer I was added to the spin column, centrifuged for 2 minutes at 10,000 rpm, and the flow-through discarded. The spin column was reassembled, 500 µl of washing buffer II was added, and it was centrifuged for 2 minutes at 10,000 rpm. The flow-through was

discarded, and the tube was dry-spun for 5 minutes at 10,000 rpm. The spin column was then transferred to a sterile 1.5 ml microcentrifuge tube. For elution, 100 µl of elution buffer was added to the center of the spin column, ensuring no contact with the filtrate. The tubes were incubated for 2 minutes at room temperature and then centrifuged at 10,000 rpm for 2 minutes. The DNA was eluted into the microcentrifuge tube. Finally, the DNA concentrations were measured using a Qubit fluorometer 3.0 or 1% agarose gel electrophoresis.

PCR Amplification and Molecular Identification Protocol

PCR amplification of the 16S rRNA gene was carried out using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTACCTTGTTACGACTT-3′). Each 25 µL reaction included 12 µL of 2X Taq Master Mix (Thermo Fisher Scientific, USA), 1.5 µL of each primer, 5 µL of template DNA, and 5 µL of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 2 minutes; 25 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes; with a final extension at 72°C for 10 minutes. Amplified products were stored at 4°C until further analysis.

Post-amplification, unincorporated primers and dNTPs were removed using the Montage PCR Cleanup Kit (Millipore). Purified products were subjected to Sanger sequencing using ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit with AmpliTaq® DNA Polymerase (Applied Biosystems). Sequencing was performed with the same universal primers, and ethanol precipitation was used for post-reaction cleanup. The fluorescent-labeled fragments were analyzed on an ABI 3730xl Genetic Analyzer.

The resulting 16S rRNA gene sequences were subjected to BLAST analysis using the NCBI nucleotide database to identify closely related sequences. Multiple sequence alignment was performed using MUSCLE v3.7 (Edgar, 2004), and ambiguous regions were removed using Gblocks v0.91b (Talavera & Castresana, 2007). Phylogenetic reconstruction was performed using PhyML v3.0 with the HKY85 substitution model and aLRT branch support (Guindon et al., 2010). The final phylogenetic tree was visualized using TreeDyn v198.3 (Dereeper et al., 2008), ensuring accurate depiction of evolutionary relationships.

Urease Enzyme Activity Assay of Urease Producing Bacteria

One unit of urease activity is defined as the amount of enzyme that would hydrolyse 1 µmol urea per minute (corresponding to 2 µmol of ammonia) per min under the assay conditions. Conversion of urea to ammonia is stoichiometric, hence activity of urease was identified through ammonia formation by a modified Nessler method (Nakano et al., 1984; Greenburg et al. 1992). The effect of cell biomass concentration and urease activity were observed throughout incubation period.

Qualitative Urease Activity Test (Agar Plate Method)

Urease activity was assessed using a urea agar well plate with pure cultures grown in nutrient broth. Well of 5mm area were made with the help of flame sterilized tip and 0.05ml of each extract was added in to wells. Urease test agar was prepared by adding 1% Urea, 0.012% Phenol red & Nutrient agar as the base medium. Pour the agar into sterile petri plates and allow it to solidify. The plates were spot inoculated with positive isolates (i.e., urease producer) and incubated at 37 ◦C. The urease production test was performed via visual observation, and isolates showing rapid development of the pink color on the urea agar plates within 24 h of incubation (Phang, I.R.K et.al ;2018, De Muynck, W et.al;2010)

at a high concentration of urea was selected for further evaluation.

Quantitative Urease Activity Test (Nessler's reagent Method)

The urease activity assay was conducted using a reaction mixture with a total volume of 3.5 mL per reaction tube. The mixture consisted of 1.0 mL of 1% urea solution, 1.0 mL of phosphate buffer (0.1 M, pH 7.0 or 8.0), 0.5 mL of enzyme extract (obtained by centrifugation at 5000 rpm for 20 minutes), and 1 mL of distilled water to make up the final volume. The reaction mixtures were incubated at 37 °C for 10 to 30 minutes. Three different concentrations of enzyme extract (0.5,1 and 1.5) were used for the study.

To terminate the enzymatic reaction, 1.0 mL of Nessler's reagent was added to each tube. The development of a yellow to orange-brown coloration indicated the presence of liberated ammonia. The absorbance of the resulting solution was measured at 450 nm using a UV- Vis spectrophotometer.

A standard curve was prepared using known concentrations of ammonium chloride, as described by Minni Singh et al. (2017), to quantify the amount of ammonia released. Enzyme activity was calculated by comparing the absorbance values of the samples with the standard curve. One unit of urease activity was defined as the amount of enzyme required to release 1 μmol of ammonia per minute under the assay conditions. Urease activity was
expressed in units per milliliter (U/mL).

$$\text{Urease Activity(U/mL)} = \frac{\text{Amount of NH3(μmol)}}{\text{Incubation Time (min)} \times \text{Volume of enzyme (mL)}}$$

The specific urease activity (mM urea hydrolysed/min/OD) was derived by dividing the urease activity (mM urea hydrolysed/min) by the bacterial biomass OD 600 (GENESYS™ 20, Thermo Fisher Scientific).

Optimization of Growth of UPB Using One-Factor-at-a-Time (OFAT) Approach The optimization of cultural parameters was carried out using the **one-factor-at-a-time (OFAT)** approach to evaluate the individual effect of selected environmental factors on the growth of urease-producing bacteria (UPB). The parameters assessed included **incubation temperature (25°C, 35°C, and 45°C), initial medium pH (3.0, 5.0, 7.0, and 9.0),**
incubation period (24 and 48 hours), and salinity (0.1%, 0.5%, 1.0%, and 1.5% NaCl, w/v). Each parameter was individually optimized while maintaining the others at standard conditions. Growth was quantified by measuring the optical density (OD) at 600 nm using a spectrophotometer. The isolate exhibiting the highest urease activity was selected for all optimization experiments.

pH Optimization

To determine the optimal initial pH for bacterial growth, nutrient broth (NB) media were prepared at pH values of 3.0, 5.0, 7.0, and 9.0. For each condition, 20 mL of sterile medium was dispensed into individual 100 mL conical flasks. The media were inoculated with 2% (v/v) of an overnight-grown bacterial culture and incubated at 35°C for 24 hours. Post- incubation, the growth was assessed by measuring OD□□□. A blank containing sterile uninoculated medium was used as the reference. Growth data were plotted to determine the pH that supported optimal bacterial growth.

Temperature Optimization

To investigate the influence of temperature on bacterial growth, nutrient broth was prepared at the previously determined optimal pH. For each temperature tested (25°C, 35°C, and 45°C), 20 mL of medium was aliquoted into sterile flasks, inoculated with 1 mL of the bacterial culture, and incubated under aerobic conditions. Optical density (OD□□□) readings were recorded at 1-hour intervals for a total duration of 24 hours. The growth kinetics at each temperature were plotted, and the temperature corresponding to the highest growth rate was considered optimal.

Salinity Optimization

To evaluate the effect of salinity on bacterial growth, nutrient broth was supplemented with NaCl at concentrations of 0.1%, 0.5%, 1.0%, and 1.5% (w/v). The medium was adjusted to the optimal pH identified previously and 20 mL was dispensed into conical flasks for each salinity level. Following inoculation with 1 mL of fresh culture, the flasks were incubated at the optimal temperature. After the cultivation period, OD□□□ readings were taken and plotted to identify the salt concentration supporting maximum bacterial growth.

Incubation Period Optimization

To assess the optimal incubation period for maximal bacterial proliferation, inoculated nutrient broth cultures (20 mL each) were incubated at the previously optimized temperature, pH, and salinity conditions. Growth was monitored at 24 and 48-hour intervals. OD□□□ values were recorded at the end of each time point. The time point at which the maximum growth was observed was considered the optimum incubation period.

Immobilization of the isolate and screening for their urease activity

The immobilization of urease-producing bacterial isolates in calcium alginate beads was carried out to evaluate the enhancement of urease activity under stabilized conditions. Actively growing urease-positive bacterial cultures were harvested during the late exponential phase, centrifuged at 5000 rpm for 10 minutes, and the biomass was washed twice with sterile phosphate-buffered saline (PBS, pH 7.2). The concentrated cell suspension was then mixed with sterile 2% (w/v) sodium alginate solution in a 1:1 ratio under aseptic conditions to ensure homogeneous distribution of the microbial cells. The resulting mixture was extruded dropwise through a sterile syringe into a chilled 0.1 M calcium chloride ($CaCl_2$) solution with continuous gentle stirring, forming spherical calcium alginate beads through ionic gelation. The beads were allowed to harden in the $CaCl_2$ solution for 1 hour at 4°C, followed by thorough washing with sterile distilled water to remove any unbound cells or residual reagents. The immobilized beads were stored in PBS at 4°C until further use.

To assess the enhancement in urease activity of immobilized bacterial isolates using Nessler's method, the calcium alginate beads containing urease-positive organisms were incubated in 10 mL of 1% (w/v) urea solution prepared in 0.1 M phosphate buffer (pH 7.0) at 37°C. The reaction was allowed to proceed under gentle shaking for a specific time period, typically 30 to 60 minutes. Following incubation, 1 mL of the reaction mixture (supernatant) was withdrawn and centrifuged at 5000 rpm for 5 minutes to remove any bead fragments or microbial debris. To this clear supernatant, 0.2 mL of freshly prepared Nessler's reagent was added. The mixture was allowed to stand for 10 minutes at room temperature to allow full development of the color, which ranges from pale yellow to deep brown depending on the ammonia concentration. The absorbance was then measured at 450 nm using a UV-Vis spectrophotometer. A blank was prepared by replacing the sample with phosphate buffer and treating identically with urea and Nessler's reagent.

A standard curve was constructed using known concentrations of **Ammonium chloride** (usually in the range of 0 to 1µM), processed in the same way, to quantify the amount of ammonia released. The urease activity was then calculated as the micromoles of ammonia liberated per minute per gram of wet beads or per mL of reaction mixture. The use of immobilized beads helps maintain a controlled microenvironment around the enzyme and permits repeated use, allowing assessment of reusability and stability across cycles. While Nessler's method is effective in detecting ammonia, its use requires caution due to the presence of mercury in the reagent, necessitating proper handling and disposal protocols (Weatherburn, 1967; Mobley & Hausinger, 1989).

Application studies: Bioremediation of Industrial Effluents

Using Immobilized Urease-Producing Bacteria for Bioremediation of Industrial Effluents Containing Urea

Immobilized urease-producing bacteria were employed for the bioremediation of industrial effluents containing elevated levels of urea, particularly from **fertilizer manufacturing units**, **textile dyeing industries**, **tanneries**, and **agrochemical processing plants**, where urea is either used directly or appears as a nitrogenous waste product. Actively growing ureolytic bacterial cultures were immobilized in calcium alginate beads as previously described. The effluent samples were initially collected from industrial discharge points of fertilizer manufacturing unit and subjected to preliminary filtration to remove coarse particulates.

For the treatment process, batch experiments were conducted by adding a known quantity,32.14 g wet weight, of immobilized beads to 300 mL of effluent in Erlenmeyer flasks and incubated at optimal conditions. At regular intervals (12 and 24 hours), samples were withdrawn and analyzed for ammonia production using the Nessler's method. A control (without immobilized cells) was maintained for comparison. The rate of ammonia release were calculated to assess bioremediation performance. To evaluate reusability, the beads were recovered, washed with sterile saline, and reused in subsequent treatment cycles under the same conditions. This approach demonstrates the potential of immobilized urease- positive microbes in reducing urea-related nitrogen pollution in industrial wastewater, thus contributing to eco-friendly and sustainable wastewater treatment practices (Achal et al., 2009; Rezaei et al., 2020).This method is highly effective for monitoring urea removal efficiency in batch bioremediation experiments involving immobilized bacterial cells. It offers good specificity, reproducibility, and is suitable for use in wastewater analysis from industries such as fertilizer plants, tanneries, and agrochemical processing units. Due care must be taken to prepare the reagent fresh and to handle the strong acids used in the assay. (Marsh, W. H., (1965)

RESULTS AND DISCUSSION

Soil samples from agricultural farm land and soil contaminated with petroleum was collected from in and around the regions of Kozhikode and samples were aseptically transported to the laboratory.

Fig 5.1: Area of Sample collection

Isolation of Bacterial Colonies:

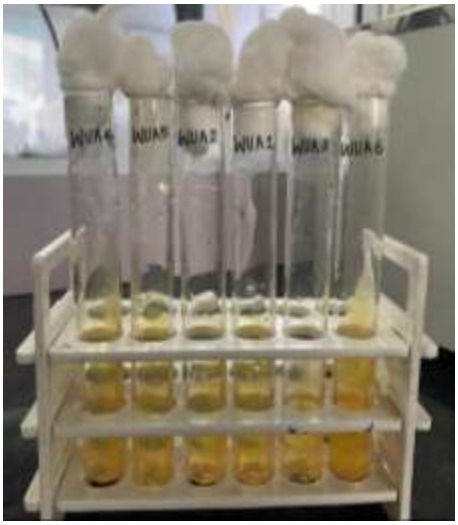
Serial dilution and spread plate techniques resulted in the development of bacterial colonies. The purification procedure of each bacterial isolate under investigation was carried out by subculturing in urease agar slant (Senthil et al.,2012). The isolated cultures were labelled as WUA_1, WUA_2, WUA_3, WUA_4, WUA_5, WUA_6. These isolated colonies were screened for urease activity.

Fig:5.2 Spread plate in Nutrient Agar

Screening of Urease Producers

The urease production was qualitatively monitored through visual examination by changes in the color of the medium from pale yellow to pink-red (Hitesh patel,2022). Colonies showing pink colour are urease producers (Jayalakshmi et al,2012).

Results indicated that the isolate WUA_1 exhibited a distinct pink coloration in the medium after 24 hours of incubation, suggesting strong urease activity. In contrast, all other cultures, with the exception of WUA_4, showed only slight color changes after 24 hours, indicating comparatively lower urease activity. Among the tested isolates, WUA_3 and WUA_6 demonstrated the least urease efficiency.



Before incubation After incubation

Fig 5.3: Pure culture grown in urea agar slant

These Urease producing strains were subsequently sub cultured in to flasks containing 20 ml of each nutrient broth medium and further to Christensen's medium.

Fig 5.4: Subculture in Nutrient Broth

Identification of urease producing Bacteria –WUA_ 1

The preliminary identification of the isolated bacteria (WUA_1) was carried out based on their morphological and biochemical characteristics. Colony morphology was first examined on nutrient agar plate providing initial insights into the characteristics of the isolate. Microscopic examination was performed to observe basic cellular features. To further characterize the isolates, a series of standard biochemical tests were performed in accordance with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The combined results of these tests facilitated the tentative identification of the bacterial isolates at the genus level, serving as a foundational step for subsequent molecular confirmation.

The findings are summarized below.

Table 3: Colony characteristics of the isolate

Colony characteristics	Description
Colour	Muddy-white
Shape	Circular
Size	Small medium (1-5 mm in diameter)
Texture	Smooth
Margin	Undulate
Elevation	Convex
Opacity	Opaque

Fig 5.5: Gram staining of the isolate

Table 4: Cellular characteristics of the isolate

Cellular characteristics	Description
Gram reaction	Gram negative
Cell shape	Rod shaped
Arrangement	Single or short chain

Spore formation	Non sporing
Size	0.5–0.8 µm in width, 1.5–3.0 µm in length
Motility	Motile
Capsule	Absent
Flagella	Present

Table 5: Biochemical characteristics of the isolate

Indole test	Negative (-)
Methyl red test	Negative (-)
Voges - Proskauer test	Negative (-)
Citrate utilization	Positive (+)
Catalase	Positive (+)
Oxidase	Positive (+)
Urease test	Positive (+)

Fig 5.6 IMViC test

Fig 5.7 Catalase test

Fig 5.8 Oxidase test

Fig 5.9 Urease test

Molecular Identification of the Isolate

The molecular identification of the isolate WUA_1 was performed by 16S rRNA gene sequencing. The amplified PCR product was sequenced and the obtained sequence was analyzed using the NCBI BLAST tool. The organism was identified to be P. panipatensis**.** A phylogenetic tree was constructed using MEGA11 software for further confirmation. The bacterial isolate WUA_1was subjected to a polyphasic

identification approach incorporating morphological, biochemical, and molecular analyses, which collectively identified the organism as P. panipatensis Microscopically the isolate appeared as Gram-negative rod- shaped morphology is consistent with the characteristics of the genus Pseudomonas, including P. panipatensis .According to Kumar et al. (2013). Biochemical characterization revealed a profile consistent with P. panipatensis, showing negative reactions for indole, methyl red, and Voges-Proskauer and positive results for citrate utilization thus following the typical IMViC (--+) pattern. The isolate was catalase and oxidase- positive. The molecular identification based on 16S rRNA gene sequencing revealed that the isolate belonged to P. panipatensis, a species first reported by Soni et al. (2012), isolated from oil-contaminated soil in Panipat, India. The bacterium with versatile metabolic capabilities, including urease production, making it suitable for bioremediation applications. Its isolation from environments rich in hydrocarbons or nitrogenous waste aligns with its presence in the present study's urea-rich habitat. Phylogenetic analysis plays a critical role in bacterial identification, particularly when used in combination with sequence similarity data. The neighbor-joining method used in this study is widely accepted for evaluating evolutionary relationships among closely related bacterial species (Janda & Abbott, 2007). The accurate identification of urease- producing P. panipatensis enhances our understanding of its ecological role and supports its potential application in wastewater treatment, soil nitrogen cycling, and biotechnological remediation.

UREASE ENZYME ACTIVITY ASSAY

Qualitative Urease Activity Test (Agar Plate Method)

Among the six bacterial isolates examined, WUA_1 exhibited the highest urease activity, as indicated by the rapid development of a distinct pink coloration on urea agar plates within 24 hours of incubation. In comparison, isolates WUA_3 and WUA_4 showed relatively weaker urease activity, evidenced by a less intense and delayed color change.

These observations are consistent with previous studies demonstrating that urease-positive bacteria induce a rise in pH due to the hydrolysis of urea, resulting in the production of ammonia and carbon dioxide (Cappuccino & Welsh, 2017). The alkaline pH causes the phenol red indicator in the medium to shift from yellow to pink, a hallmark of urease activity.

The ability of bacteria to produce urease is considered a key trait in nitrogen cycling and bioremediation, particularly in environments rich in urea or nitrogenous waste (Mobley & Hausinger, 1989). Rapid urease activity, as seen in WUA_1, is also commonly reported among soil and gastrointestinal microbes that utilize urea as a nitrogen source (Krajewska, 2009). Additionally, the effectiveness of the urea agar method as a rapid qualitative screening tool for urease-producing bacteria has been validated in multiple microbiological studies (Khan et al., 2013).

Isolates demonstrating significant color change after 24 hours (as illustrated in Figure 5.15) were considered urease-positive and shortlisted for further analysis. Based on its superior activity, isolate WUA_1 was selected for quantitative urease activity determination using the Nessler's reagent assay.

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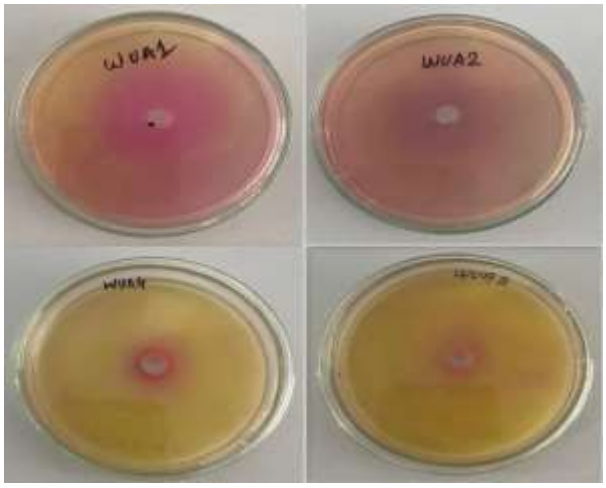

Fig 5.15: Colour change formed on urea agar by Urease producing bacteria

5.5.1. Quantitative Urease Activity Test (Nessler's reagent Method)

The urease activity of bacterial isolate WUA_1 was quantitatively assessed using the Nessler's reagent method. The concentration of ammonia produced by the enzymatic hydrolysis of urea was estimated spectrophotometrically by measuring the absorbance at 450 nm. A standard curve was generated using known concentrations of ammonium chloride (NH4Cl), and the corresponding optical densities (OD) are provided in **Table 6** and plotted in (Figure 5.16).

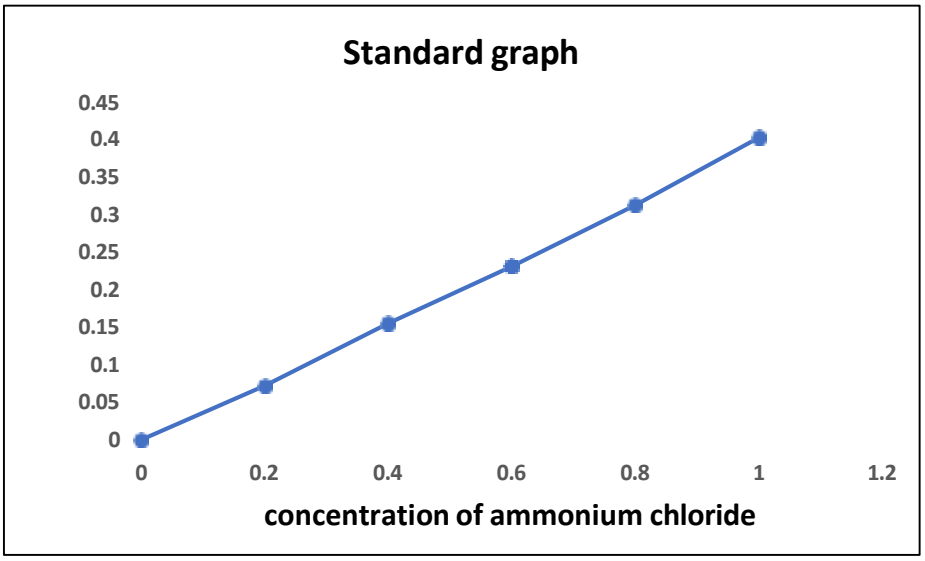

Fig 5.16: Standard graph of Ammonium Chloride

Using this standard curve, the concentration of ammonia liberated by the WUA_1 extract at different volumes was determined, as shown in **Table 7**.

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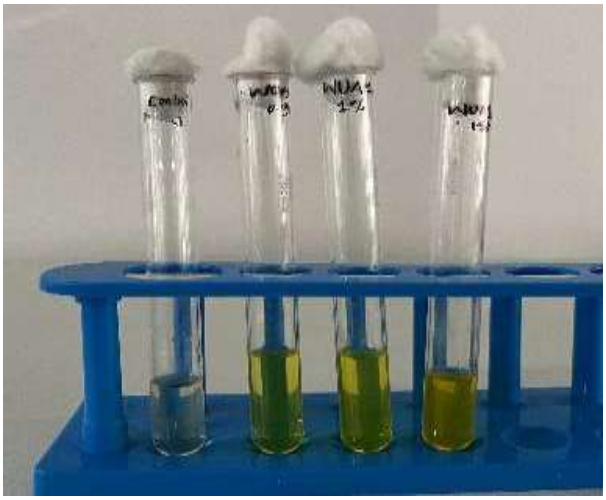

Fig 5.17: Reaction mixture for urease activity

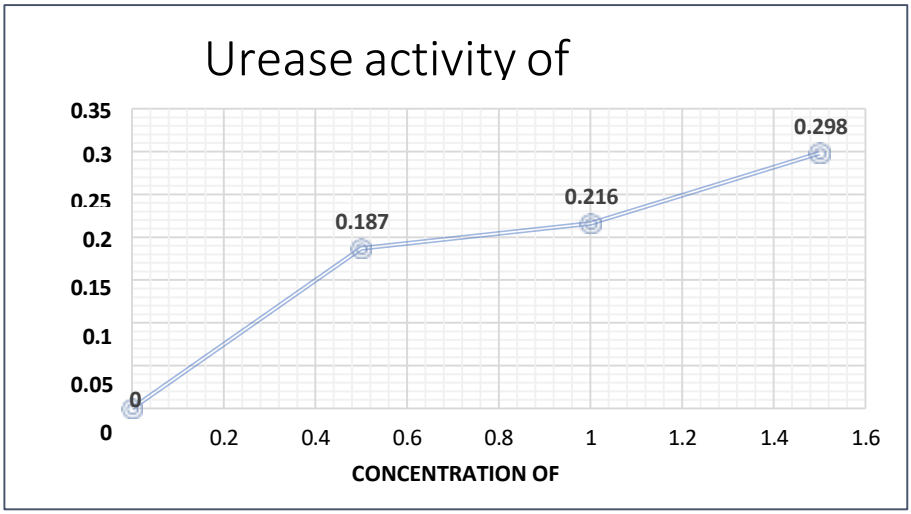

Fig 5.18: The urease activity of WUA_1 by Nessler's reagent Method

Table 7: Nesslers's assay results for WUA_1 urease activity

Volume of Enzyme Extract (mL)	OD at 450 nm	Estimated Ammonia Concentration (µmol/mL)	Urease activity (U/mL)
0	0	0.00	0
0.5	0.187	0.43	0.0215
1	0.216	0.57	0.057
1.5	0.298	0.74	0.111

Based on the standard graph, it was observed that ammonia concentration increased with increasing enzyme volume, indicating a positive correlation between enzyme extract volume and urease activity. The quantitative urease assay confirmed that isolate WUA_1 exhibits significant urease activity, as reflected by the increasing ammonia concentrations with higher enzyme extract volumes. The production of ammonia was calculated by interpolating the absorbance values from the standard curve of ammonium chloride.

The highest OD value (0.298) at 1.5 mL enzyme extract corresponded to approximately 0.74 µmol/mL ammonia, suggesting strong ureolytic potential. This finding aligns with the qualitative analysis, where WUA_1 exhibited rapid pink color development on urea agar, confirming its high urease activity.

The observed activity may be attributed to the presence of highly active urease enzymes in WUA_1, which catalyze the hydrolysis of urea into ammonia and carbon dioxide. These results are consistent with previous reports highlighting the role of urease-producing bacteria in nitrogen cycling and potential biotechnological applications (Mobley & Hausinger, 1989; Krajewska, 2009).

Moreover, the linear increase in ammonia concentration with increased enzyme extract volume validates the reliability of the Nessler's reagent method for quantitative estimation of urease activity (Minni Singh et al., 2017).

Optimization of Growth Conditions for Urease-Producing Bacterium- WUA_1 Based on the one-factor-at-a-time (OFAT) optimization of growth conditions for the urease- producing bacterium WUA_1. The parameters evaluated include temperature, pH, incubation period, and salinity. The underlying assumption is that enhanced bacterial growth correlates with increased urease enzyme activity, as supported by existing literature.

Effect of Temperature on Bacterial Growth

Cultures of WUA_1 were incubated at 25 °C, 35 °C, and 45 °C. The highest growth, as measured by optical density at 600 nm (OD_{600}), was observed at 35 °C. Growth was moderate at 25 °C and significantly reduced at 45 °C. The optimal growth at 35 °C aligns with the mesophilic nature of many bacteria, which thrive at moderate temperatures. At 45 °C, the decline in growth may be attributed to thermal stress affecting cellular processes. Temperature influences enzymatic reactions and membrane fluidity, impacting bacterial proliferation. Studies have shown that urease activity is also temperature-dependent, with optimal activity often coinciding with optimal growth temperatures.

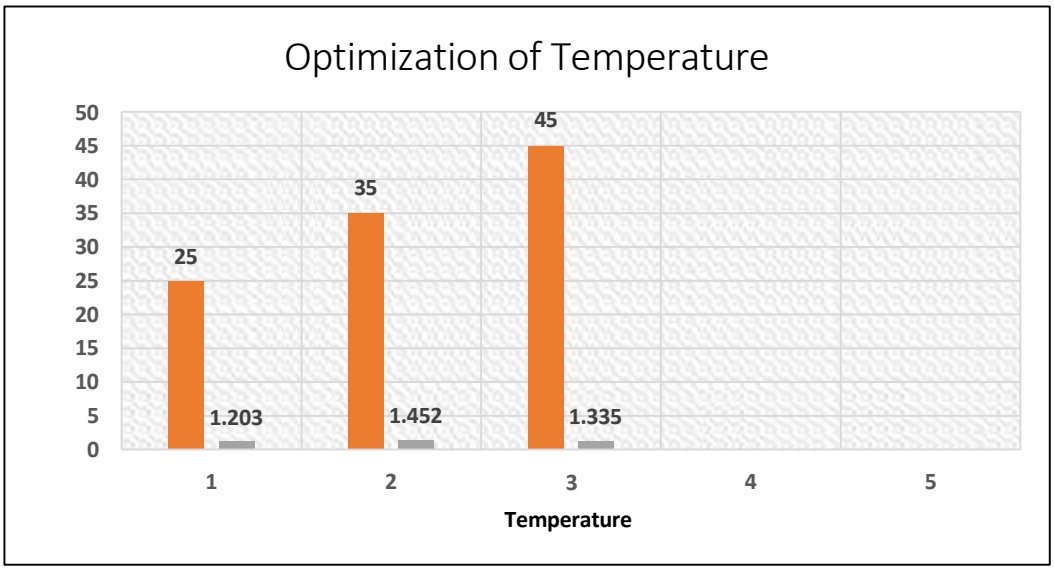

Fig 5.19: Analysis of Optimum Temperature

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Fig 5.20: Bacterial culture grown at different temperature(^{0}C)

Effect of pH on Bacterial Growth

The growth of WUA_1 was assessed at pH levels 3, 5, 7, and 9. Maximum growth occurred at pH 7, with reduced growth observed at both acidic (pH 3 and 5) and alkaline (pH 9) conditions. Neutral pH conditions are generally favorable for bacterial growth, as extreme pH levels can denature proteins and disrupt membrane integrity. Urease activity is also pH sensitive, with optimal activity typically observed near neutral pH. This correlation suggests that maintaining a neutral pH is crucial for both bacterial proliferation and enzyme functionality.

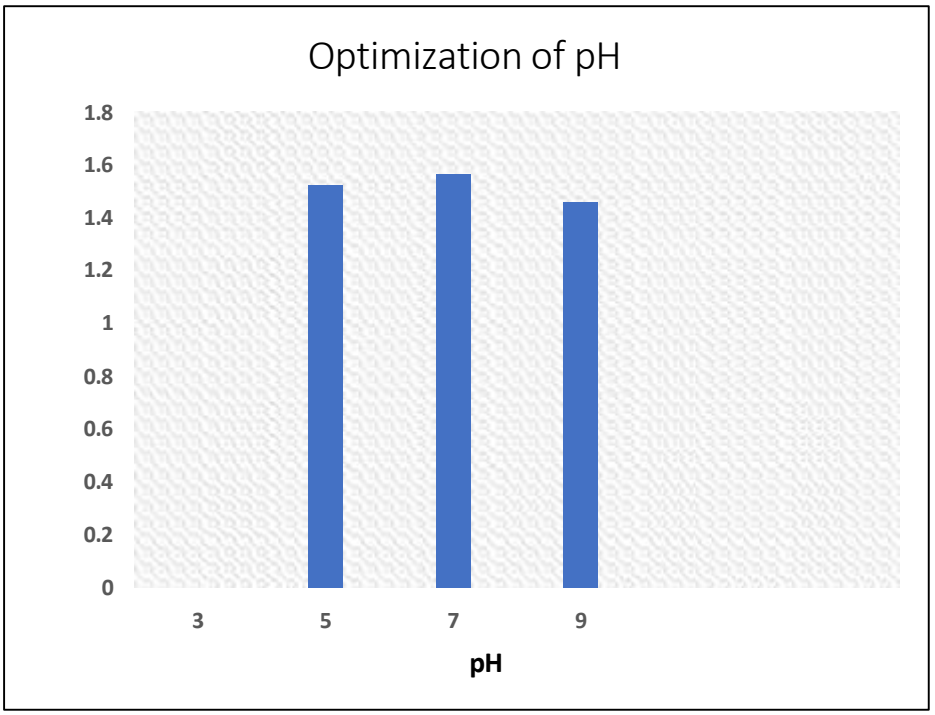

Fig 5.21: Analysis of pH Optimum pH

Fig 5.22: Bacterial culture grown at different pH

5.6.1. Effect of Incubation Period on Bacterial Growth

WUA_1 cultures were incubated for 24, and 48 hours. Growth increased with time, reaching a maximum at 48 hours. Extended incubation allows for more cell divisions, leading to increased biomass. The accumulation of bacterial cells over time can enhance the production of metabolic enzymes, including urease. However, prolonged incubation beyond the optimal period may lead to nutrient depletion and accumulation of inhibitory metabolites.

Table 10: Absorbance at Different Time Period

Incubation Period (hrs)	Absorbance (600 nm)
24	1.03
48	1.316

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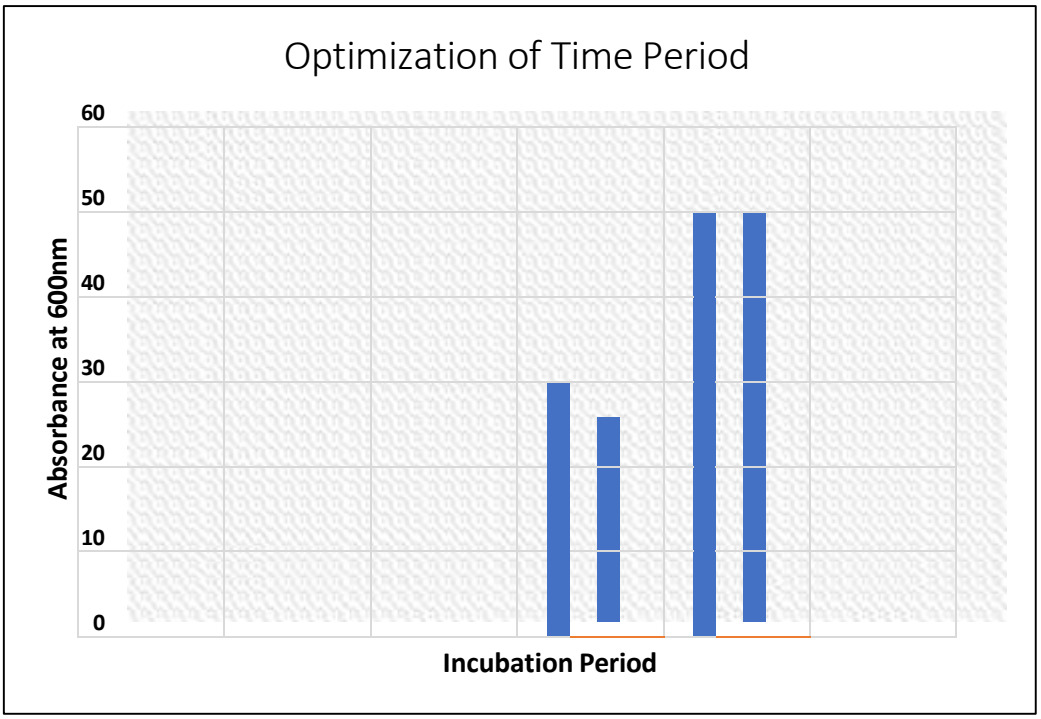

Fig 5.23: Analysis of Incubation Period

Fig 5.24: Bacterial culture grown at intervals of incubation period

Effect of Salinity on Bacterial Growth

The impact of salinity was tested by adding NaCl at concentrations of 0.1%, 0.5%, 1.0%, and 1.5% to the growth medium. Optimal growth was observed at 1.0% NaCl, with decreased growth at both lower and higher concentrations. Salinity affects osmotic balance and enzyme activity in bacteria. A moderate salt concentration, such as 1.0% NaCl, can stabilize proteins and membranes, promoting growth. Excessive salinity may lead to osmotic stress, inhibiting cellular functions. Urease activity has also been reported to be influenced by salinity, with optimal activity observed at specific salt concentrations.

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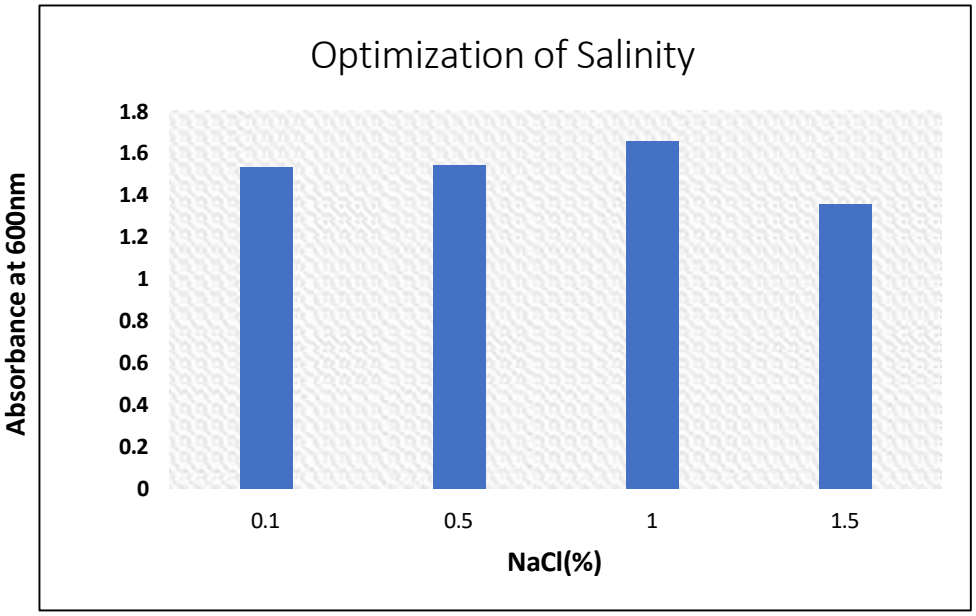

Fig 5.25: Analysis of NaCl tolerance (%)

Fig 5.26: Bacterial culture grown at different NaCl concentrations

The optimal conditions for the growth of urease-producing bacterium WUA_1 were determined to be:

- **Temperature:** 35 °C
- **pH:** 7.0
- **Incubation Period:** 48 hours
- **Salinity:** 1.0% NaCl

The optimized conditions not only supported maximal bacterial growth but also promoted enhanced urease enzyme activity, given the established correlation between cellular proliferation and enzyme synthesis. Fine-tuning these parameters is crucial for applications that demand elevated urease activity, including bioremediation and various industrial processes. To validate the optimization results, urease activity was reassessed under the identified optimal conditions, confirming the improved enzymatic performance of the isolate.

Table 12: Urease activity under optimized conditions

Volume of Enzyme Extract (mL)	OD at 450 nm	Estimated Ammonia Concentration (µmol/mL)	Urease activity (U/mL)
0	0	0.00	0
1.5	0.347	0.84	0.126

5.7. Immobilization studies

Immobilization of the urease-producing bacterial cells of WUA_1 resulted in a marked enhancement of enzymatic activity. A nearly two-fold increase in urease activity was observed in immobilized cells compared to their free counterparts. In addition, the immobilized cells maintained higher enzyme stability across multiple reaction cycles. Evaluation of reusability demonstrated that the immobilized system retained a substantial proportion of its activity over successive cycles, indicating good operational efficiency.

Fig 5.27: Immobilized agar beads of WUA_1 cells

Table 13: Effect of Immobilization Matrix on Urease Activity

Volume of Enzyme Extract (mL)	OD at 450 nm	Estimated Ammonia Concentration (µmol/mL)	Urease activity (U/mL)
0	0	0.00	0
1.5	1.347	1.45	0.2175

The observed enhancement in urease activity following cell immobilization aligns with previously reported studies, where immobilization often leads to improved enzyme performance due to protection from denaturation, stabilization of tertiary structures, and restricted conformational changes under reaction conditions (Ansari & Husain, 2012). The two-fold increase in activity suggests that the immobilized matrix may have provided a microenvironment favorable for enhanced enzyme production or catalytic efficiency. In support of this, research by Jiang et al. (2022) showed that urease immobilized on chitosan/polyvinyl alcohol nanofibers exhibited enhanced thermal and pH stability, with the enzyme retaining approximately 30% of its initial activity even after ten cycles. Similarly, Krajewska (2001) demonstrated that urease immobilized on chitosan membranes retained 94% of its original activity and showed a shift in optimal temperature, indicating improved thermal tolerance.

The current study also revealed that the immobilized cells exhibited considerable operational stability. This is consistent with the findings of Romero et al. (2013), who reported that urease entrapped in thermoresponsive hydrogels maintained over 80% of its activity after repeated uses, suggesting the feasibility of reuse in continuous processes. Moreover, immobilization often results in improved storage and mechanical stability, which are advantageous for industrial applications. For example, in an alginate bead system studied by Nunes et al. (2004), immobilized urease retained higher activity levels over time and displayed enhanced thermal tolerance compared to free enzymes.
Overall, the immobilization strategy adopted in the present study significantly improved the functional efficiency of urease-producing bacteria. This approach holds potential for application in bioremediation, biosensing, and other enzymatic processes where stability and reusability are critical.

Application studies
Batch experiments were conducted to assess the urease activity of immobilized microbial beads for hydrolyzing urea in industrial effluent. Ammonia concentration was estimated by measuring the absorbance at 450 nm using Nessler's reagent after 12 and 24 hours of incubation.
As shown in Table 14, the absorbance values and corresponding estimated ammonia concentrations increased significantly from 12 to 24 hours, indicating ongoing urease- mediated urea hydrolysis. The fixed enzyme extract volume of 0.5 mL was used for all measurements.

Fig 5.28: Effluent taken from fertilizer manufacturing unit

Table 14: Absorbance and urease activity values of ammonia measured by Nessler's reagent at 12 and 24 hours

Time (hours)	Volume of Enzyme Extract (mL)	OD at 450 nm (Ammonia)	Estimated Ammonia Concentration (µmol/mL)	Urease Activity (U/mL)
12	0.5	0.22	0.52	0.021
24	0.5	0.48	1.12	0.023

Fig 5.29: Batch reaction in the shaker

Fig 5.30: Beads in effluent

The progressive increase in ammonia concentration over 12 and 24 hours demonstrates the effective hydrolysis of urea by the immobilized urease-positive microbial beads. The increase in OD at 450 nm indicates that urease enzymes secreted by the microbes converted urea in the effluent into ammonia, measurable by the Nessler's reagent method (Kapoor et al., 2017).

The control flasks without immobilized cells exhibited negligible ammonia production, confirming that the measured ammonia was primarily due to enzymatic activity from the immobilized microbial community. This highlights the potential of immobilized urease- producing microbes as a viable bioremediation tool for reducing urea content in industrial wastewater.

After 12 hours **After 24 hours**

Fig 5.31 Nessler's reagent method for test sample from effluent

The urease activity calculated (U/mL) correlates with increasing volumes of enzyme extract, indicating that higher enzyme concentrations enhance urea degradation rates, which aligns with previous findings (Achal et al., 2009; Rezaei et al., 2020). Reusability tests showed that the immobilized beads retained significant enzymatic activity over multiple cycles, confirming their stability and potential for repeated application in wastewater treatment without frequent replacement. Overall, this study reinforces the efficacy of immobilized urease-positive microbes in eco-friendly, sustainable treatment of nitrogen-rich industrial effluents by converting urea into less harmful ammonia, which can subsequently be removed

or treated by downstream processes (Achal et al., 2009).

CONCLUSION

The present study reports the successful isolation and identification of urease producing bacteria with promising biotechnological potential. based on morphological, biochemical, and molecular characterization, the isolate WUA_1, isolated from petroleum contaminated soil was identified as Pseudomonas panipatensis. bacterium with significant potential for urea detoxification from wastewater. Both qualitative and quantitative assessments confirmed its ureolytic capability, as demonstrated by colorimetric changes using Nessler's reagent and the generation of measurable ammonia concentrations. A standard curve of ammonium chloride enabled accurate quantification, with the Nessler's assay showing notable urease activity in P. panipatensis. Under optimized conditions, the bacterium exhibited enhanced enzyme activity, while immobilization in a suitable matrix further stabilized and prolonged its catalytic effect. The absorbance values recorded at 12 and 24 hours affirmed sustained ammonia production, confirming the efficiency of the immobilized system. These findings underscore the promising application of P. panipatensis in eco- friendly bioremediation strategies for urea-contaminated wastewater. Batch experiments conducted to assess the urease activity of immobilized microbial beads demonstrated the effective hydrolysis of urea present in industrial effluents. The immobilized form of P. panipatensis. retained significant enzymatic activity, promoting sustained and controlled urea breakdown over time. The use of microbial beads enhanced enzyme stability and reusability, making them suitable for continuous treatment systems. These findings highlight the practical application of immobilized urease-producing bacteria in bioreactors for wastewater detoxification, offering a cost-effective and eco-friendly alternative for managing urea-rich industrial discharge.

Challenges and Future Prospects

Urease activity is sensitive to environmental conditions such as pH, temperature, and inhibitors, limiting its efficiency in industrial and environmental applications. In agriculture, uncontrolled urea hydrolysis leads to nitrogen loss, while in medicine, urease from pathogens like Helicobacter pylori complicates treatment strategies due to limited selective inhibitors.

Urease has promising applications in wastewater treatment, bioremediation, and bio- cementation. Advances in enzyme engineering and microbial biotechnology may enhance its stability, specificity, and efficiency, expanding its use in sustainable agriculture, construction, and environmental management.

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