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Laboratory-Scale Evaluation of Indigenous Extracellular Enzyme-Producing Bacteria for Wastewater Quality Improvement

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Abstract: This study evaluated indigenous bacteria isolated from sludge for extracellular enzyme production and assessed their potential at the laboratory scale to improve wastewater quality. Forty sludge samples were diluted in a series and spread onto nutrient agar plates. Single bacterial colonies were subsequently screened on wastewater agar medium, and seven growing bacterial isolates were identified by *16S rRNA* gene sequencing; their sequences were deposited in GenBank. Amylase, lipase, and protease activities were assessed, and five representative isolates were tested in non-sterile wastewater batch cultures for 72 h. Wastewater quality parameters were analyzed using one-way analysis of variance and Tukey's test. The isolates showed variation in enzymatic activity and treatment efficiency. *Enterococcus faecalis* DAQS7 achieved the highest overall removals of chemical oxygen demand, biochemical oxygen demand, phosphate, and nitrate, at 86.57%, 90.00%, 31.25%, and 68.66%, respectively. *Staphylococcus aureus* DAQSE3 and *Enterobacter cloacae* DAQSE5 achieved the highest total suspended solids removal with 90.12% and 88.07%, respectively.

Keywords: bioaugmentation, *16S rRNA*, extracellular enzymes, indigenous bacteria, municipal wastewater

1. Introduction

Municipal wastewater is a complex mixture containing organic matter, nutrients, suspended and dissolved solids, heavy metals, and various microbial contaminants (Du et al., 2020). Treatment of municipal wastewater remains a major environmental and public health challenge, particularly under rapid population growth and increasing pressure on freshwater resources (Todd, 2024). In Iraq, deterioration of water quality has been aggravated by inefficient water resource management, misuse of water resources, and the continued discharge of untreated or inadequately treated wastewater into natural water bodies (Auied et al., 2025). Bioaugmentation involves introducing selected microbial strains with desirable degradation capacities into the treatment environment to enhance pollutant removal. Extracellular hydrolytic enzymes may contribute to this process by degrading large and complex organic molecules into simpler compounds that can enter bacterial cells and be further metabolized (Sonune & Garode, 2018). In some cases, organic pollutants may be completely mineralized into inorganic products, such as carbon dioxide, reducing the toxicity of the original compound (Kulzhanova et al., 2024). Accordingly, extracellular enzyme-producing bacteria may improve municipal wastewater biodegradability by catalyzing the breakdown of proteins, lipids, starches, and other complex organic fractions (Sonune & Garode, 2018; Nyika & Dinka, 2022). Indicators such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), and total suspended solids (TSS) are commonly used to evaluate overall biodegradation and treatment efficiency (Gupta & Thakur, 2015). Studies have shown that the microbial community in activated sludge produces abundant hydrolytic enzymes, such as proteases, lipases, amylases, and cellulases, which cleave sensitive bonds in large organic molecules to generate monomers available for microbial uptake (Liu & Smith, 2021). Alarjani et al. (2021) reported that indigenous bacteria from municipal sludge, such as *Pseudomonas aeruginosa* NU1 and *Acinetobacter calcoaceticus* K12, exhibited high enzymatic activity in hydroxylamine oxidase and nitrite- and nitrate-reducing enzymes, resulting in nitrogen and phosphorus removal rates exceeding 80% when applied in moving-bed bioreactors (MBBRs) under optimal temperature and pH conditions.

Despite increasing interest in microbial bioaugmentation, limited information is available on indigenous extracellular enzyme- producing bacteria from municipal wastewater environments in southern Iraq, particularly in the city of Diwaniyah, where the water quality of a branch of the Euphrates River, which serves as a major water source for drinking, agriculture, and industry, is increasingly threatened by untreated sewage and industrial discharges (Al-Khuzaie et al., 2024; Al-Khuzaie et al., 2025). Growing attention has therefore been directed towards sustainable, cost-effective biological approaches to improve wastewater treatment. Accordingly,

this study aimed to isolate and characterize indigenous bacteria capable of producing hydrolytic enzymes, including proteases, amylases, and lipases, from the municipal wastewater treatment plant in Diwaniyah, and to evaluate their laboratory-scale potential to improve wastewater quality.

2. Materials and Methods

2.1. Sample Collection

Forty sludge samples were collected from July 2024 to April 2025 from the municipal wastewater treatment plant in Diwaniyah city, Iraq, in sterile containers, placed in plastic bags, and transported to the laboratory under cooled conditions in accordance with recommended procedures for water and wastewater examination (APHA, 2005; ISO 19458, 2006).

2.2. Isolation and Primary Screening of Bacterial Isolates for Biodegradation Study

Sludge samples were diluted in series, and 0.1 mL of each diluted sample was inoculated onto nutrient agar plates. Following incubation at 37°C for 24-72 h, single bacterial colonies were inoculated onto sterile wastewater agar (WWA), made by adding 2% agar to 100 mL of wastewater. All plates were incubated at 37°C for 72 h (Sonune & Garode, 2015). Bacterial isolates that showed growth on WWA plates were selected for further study.

2.3. Molecular Identification of Bacterial Isolates

Following the manufacturer's guidelines for the Genomic DNA Purification Kit (Geneaid Biotech Ltd., Taiwan), genomic DNA was extracted from bacterial isolates. The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) from Macrogen Inc. (Korea) were used to amplify the *16S rRNA* gene (Weisburg et al., 1991). A PCR reaction mixture contained 25 µL of GoTaq® G2 Green Master Mix, 2X (Promega, USA), 3 µL of forward primer, 3 µL of reverse primer, 14 µL of nuclease-free water, and 5µL of DNA template. PCR amplification was carried out under the following conditions: an initial denaturation at 95°C/120 s, followed by 30 cycles of denaturation at 95°C/30 s, annealing at 53°C/30 s, and extension at 72°C/150 s, then a final extension at 72°C/300 s. Amplicons of approximately 1498 bp were verified by 1.5% ethidium bromide (0.5 µg/mL) stained agarose gel electrophoresis (Sambrook & Russell, 2006). PCR products were sent to Macrogen Inc. for sequencing. BioEdit 7.2 was used to examine *16S rRNA* gene sequences. Sequence similarity was determined by comparison with reference sequences deposited in NCBI GenBank using BLAST (Hall, 1999).

2.4. Detection of Hydrolytic Extracellular Enzymes

The ability of selected bacterial isolates to produce extracellular enzymes was assessed using a method described by Sonune and Garode (2018). Media containing specific substrates were used to detect amylase, lipase, and protease enzymes; the appearance of a hydrolysis zone around the colony indicated extracellular enzyme secretion. The zone diameter was measured in millimeters at 24, 48, and 72 h of incubation, and the zone diameter was used as a semi-qualitative indicator of the isolate's ability to produce the enzyme.

2.4.1. Detection of Amylase Activity

Bacterial isolates were spotted on starch agar. After incubation at 37°C for 72 h, the plates were immersed in iodine solution for 30 seconds. The appearance of a clear zone around the colony indicated amylase activity.

2.4.2. Detection of Protease Activity

Bacterial isolates were spotted on milk agar. The appearance of a clear zone around the colony after incubating the plates at 37°C for 72 h indicated proteolytic activity.

2.4.3. Detection of Lipase Activity

Bacterial isolates were spotted on egg yolk agar. The appearance of a clear zone around the colony after incubating the plates at 37°C for 72 h indicated lipolytic activity.

2.5. Detection of Biodegradation Potential of Bacterial Isolates

2.5.1. Inoculum Preparation

Preparation of the bacterial inoculum was adapted from the method of Sonune and Garode (2015) with some modifications, including cell harvesting and washing before turbidity standardization as follows: each

bacterial isolate selected for the batch experiment was inoculated into a 100 mL flask containing 50 mL of sterilized wastewater broth supplemented with peptone (0.5%). Flasks were shaken at 120 rpm for 48 h at 37°C. After incubation, the bacterial cultures were centrifuged at 6,000 rpm for 10 min under sterile conditions. The bacterial pellets were collected and washed twice with sterile saline solution. The washed cells were then suspended in sterile saline, and the turbidity of each suspension was adjusted to a 0.5 McFarland standard before use in the biodegradation experiment.

2.5.2. Biodegradation Study

A batch experiment was conducted by adding 10 mL of bacterial inoculum to 90 mL of non-sterile wastewater in a 250 mL flask, and the control contained only 100 mL of non-sterile wastewater. All flasks were incubated on a shaker at 120 rpm for 72 h at 37°C. After incubation, the samples were centrifuged at 10,000 rpm for 20 min. The supernatant was used to measure COD, BOD, nitrate, ammonia, phosphate, TDS, and TSS in accordance with recommended procedures for water and wastewater analysis (APHA 2005). Removal efficiencies were determined using the following equation (Sonune & Garode, 2018):

$$Removal\ efficiency\ (RE\%) = \frac{C0-CR}{C0} \times 100 \quad (1)$$

where:
C0 – the concentration of the control,
CR – the concentration after bacterial treatment.

2.6. Statistical Analysis

All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation. The data were statistically analyzed using GraphPad Prism software with one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for multiple comparisons at $P \leq 0.05$. Statistical comparisons were performed only among the bacterial treatments to determine significant differences in their effects on wastewater physicochemical properties. The control was used as a reference value for calculating removal rates and was not included in the statistical comparison among isolates. Removal efficiencies were presented descriptively, based only on control and treatment values, and were not included in the statistical analysis.

3. Results and Discussion

3.1. Primary Screening of Bacterial Isolates

In this study, seven bacterial isolates showed growth on WWA medium. The colonies were white to creamy white in appearance, with noticeable variation in growth density among the isolates, ranging from relatively dense growth to more discrete colonies (Fig. 1). This growth indicated that these isolates could utilize wastewater components as sources of carbon and energy. Their ability to metabolize organic compounds and tolerate or transform certain inorganic components may have facilitated their growth (Sonune & Garode, 2018). Microorganisms capable of growing in wastewater typically exhibit marked metabolic flexibility, enabling them to utilize pollutants or available nutrients under varying environmental conditions. Furthermore, the presence of biodegradable organic matter, hydrocarbons, and other nutrients can support microbial proliferation and enzymatic activity, providing a biological basis for wastewater treatment applications (Nyika & Dinka, 2022).

Fig. 1. Growth of bacterial isolates on wastewater agar

3.2. Molecular Identification of Bacterial Isolates

The results of molecular identification based on the closest BLAST match are presented in Table 1, together with the GenBank accession number for each isolate. As depicted in Fig. 2, multiple sequence alignment of *16S rRNA* gene sequences revealed high conservation among the bacterial isolates and reference sequences. Conserved nucleotide regions were observed across most alignment positions, with limited differences at some sites. These similarities support the molecular identification of the isolates and indicate their taxonomic proximity to the reference bacterial sequences.

Table 1. BLAST analysis of *16S rRNA* gene sequences

Bacterial name in NCBI	Accession number	Closest GenBank match	Identity (%)
Escherichia coli DAQSE1	PV449165	PQ288717	99
Escherichia coli DAQSE2	PV449166	PQ288717	98
Staphylococcus aureus DAQSE3	PV449167	ON627809	99
Pseudomonas aeruginosa DAQSE4	PV449168	LT628101	99
Enterobacter cloacae DAQSE5	PV449169	PV056046	99
Escherichia coli DAQSE6	PV449170	PQ288717	98
Enterococcus faecalis DAQS7	PV686788	MG694670.1	97

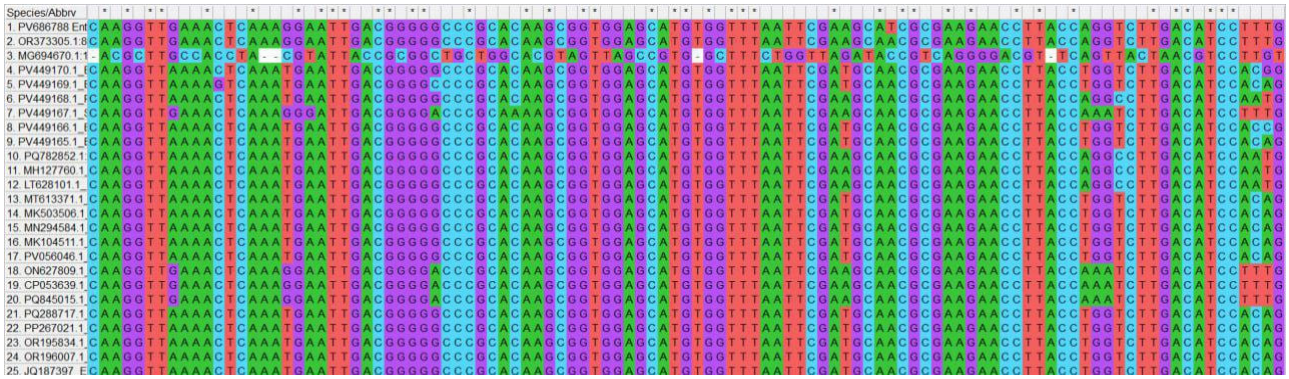

Fig. 2. Multiple alignment of the *16S rRNA* gene sequences of bacterial isolates recovered from municipal wastewater

3.3. Detection of Hydrolytic Extracellular Enzymes

The appearance of hydrolysis zones around bacterial colonies, as shown in Figs. 3-5, indicated that some isolates were able to produce extracellular enzymes capable of degrading complex organic compounds. This finding is consistent with the study by Sonune and Garode (2018), who reported that wastewater bacterial isolates producing amylase, lipase, and protease may play an important role in biodegradation processes. As shown in Table 2, the bacterial isolates exhibited clear differences in extracellular enzymatic activity across different incubation periods. *Escherichia coli* DAQSE1 and DAQSE6 showed clear amylase activity with weak or delayed lipase activity, and no protease activity was detected.

In contrast, *E. coli* DAQSE2 demonstrated a broader enzymatic profile, producing amylase, lipase, and protease, particularly after 48 and 72 h of incubation. *Staphylococcus aureus* DAQSE3 recorded the highest amylase activity after 48 h, and moderate lipase and protease activity at 24 and 48 h. *Pseudomonas aeruginosa* DAQSE4 produced amylase, lipase, and protease throughout all incubation periods, and its amylase activity increased gradually, reaching its highest value after 72 h, which is comparable with the observation of Masi et al. (2023). *Enterobacter cloacae* DAQSE5 exhibited moderate lipase and protease activity, while amylase activity was detected only after 48 h. This pattern aligns with the findings of Kulzhanova et al. (2024). *Enterococcus faecalis* DAQS7 recorded variable and weak enzymatic activity. It produced low amylase activity at 24 and 48 h, weak lipase activity at 48 and 72 h, and protease activity only after 72 h. This indicates that this isolate produced fewer extracellular enzymes than the other tested bacteria.

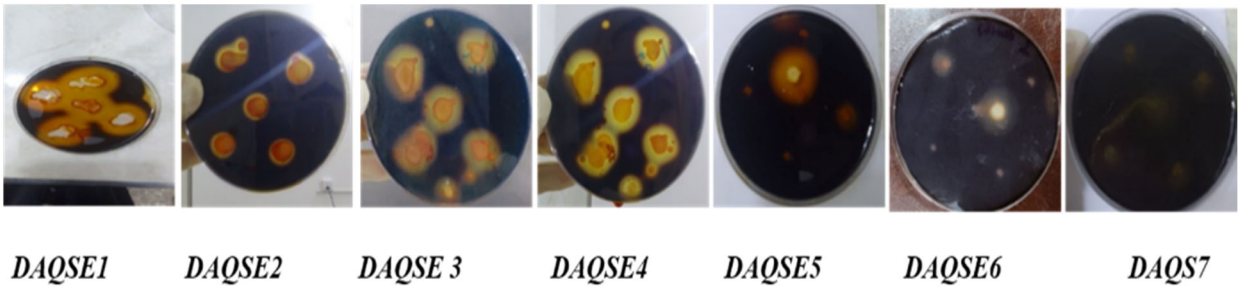

Fig. 3. Amylase activity of bacterial isolates

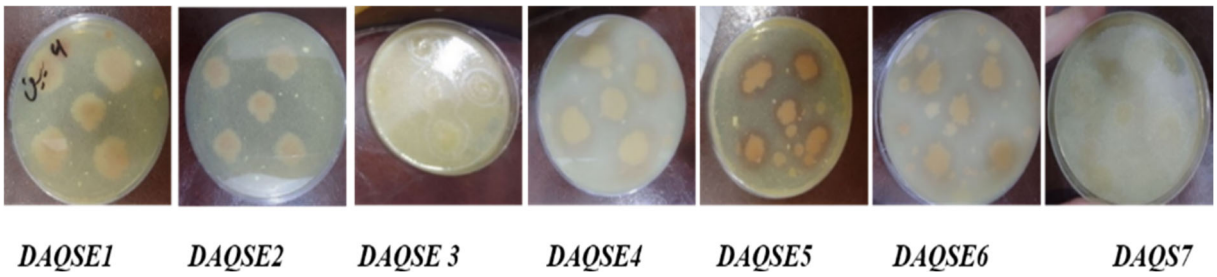

Fig. 4. Lipase activity of bacterial isolates

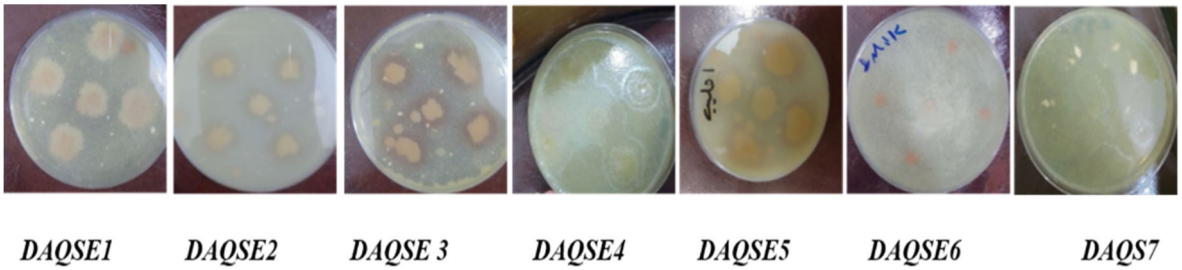

Fig. 5. Protease activity of bacterial isolates

Table 2. Comparative enzymatic activity of bacterial isolates

Enzymes	Clearance zone (mm)								
	Amylase			Lipase			Protease		
Time (h)	24	48	72	24	48	72	24	48	72
Escherichia coli DAQSE1	1	3	6	-	1	2	-	-	-
Escherichia coli DAQSE2	4	6	5	-	4	3	-	2	4
Staphylococcus aureus DAQSE3	-	8	4	4	4	-	1	4	-
Pseudomonas aeruginosa DAQSE4	4	5	8	1	2	3	1	1	3
Enterobacter cloacae DAQSE5	-	5	-	4	3	4	2	4	4
Escherichia coli DAQSE6	3	4	5	-	-	3	-	-	-
Enterococcus faecalis DAQS7	2	1	-	-	2	1	-	-	2

- indicates no detectable zone

3.4. Detection of Biodegradation Potential of Bacterial Isolates

The results in Table 3 showed clear statistically significant differences among the tested bacterial isolates in their effects on the physicochemical properties of municipal wastewater. This variation may be related to differences in the metabolic characteristics of each isolate, the nature of the target pollutants in the wastewater, and the experimental conditions. This finding is consistent with the observation by Nyika and Dinka (2022) that biological treatment efficiency depends on the type of microorganism, wastewater characteristics, and treatment conditions. Mishra et al. (2025) noted that microorganisms, particularly bacteria, possess a high capacity to transform and degrade a wide range of pollutants in wastewater, making them an effective and sustainable tool in biological treatment.

Staphylococcus aureus DAQSE3, *Enterobacter cloacae* DAQSE5, and *Enterococcus faecalis* DAQS7 recorded the highest pH mean, while *Pseudomonas aeruginosa* DAQSE4 recorded the lowest statistically significant mean. The increase in pH in some treatments may be related to the degradation of nitrogenous compounds and the release of alkaline products. At the same time, the relative decrease may be associated with the production of organic acids during biodegradation. These changes are significant because pH affects microbial enzyme activity and the efficiency of ammonia and phosphate removal (Sharma et al., 2023).

TDS results showed significant differences among the isolates. *Staphylococcus aureus* DAQSE3 recorded the lowest relative mean and achieved the greatest TDS reduction (16.49%), followed by *Enterobacter cloacae* DAQSE5 (14.74%). By contrast, *Enterococcus faecalis* DAQS7 showed a slight increase in TDS. The limited reduction in TDS can be explained by the release of salts and dissolved ions into the medium during organic matter mineralization processes, which may mask a reduction or cause a slight increase in some treatments.

Table 3. Comparison of physicochemical indicators of municipal wastewater after bacterial treatment

Parameter	Control	*Staphylococcus aureus* DAQSE3	*Pseudomonas aeruginosa* DAQSE4	*Enterobacter cloacae* DAQSE5	*Escherichia coli* DAQSE6	*Enterococcus faecalis* DAQS7	P-value
pH	7.27±0.161	8.85±0.135^{a}	7.08±0.221^{c}	8.72±0.125^{a}	7.66±0.108^{b}	8.62±0.240^{a}	<0.001
TDS (mg/L)	1852.5±85.975	1547.0±41.725^{c}	1670.5±113.279^{abc}	1579.5±93.171^{bc}	1813.5±55.847^{ab}	1878±113.277^{a}	0.004
TSS (mg/L)	243.0±9.060	24±1.769^{d}	57±1.873^{c}	29±1.758^{d}	68.2±1.473^{b}	83±7.550^{a}	<0.001
PO_4 (mg/L)	5.140±0.105	4.926±0.179^{b}	6.135±0.134^{a}	5.355±0.178^{b}	6.570±0.215^{a}	3.534±0.215^{c}	<0.001
NO_3 (mg/L)	2.045±0.285	20.918±0.189^{a}	20.491±1.386^{a}	21.277±0.145^{a}	9.437±0.666^{b}	0.641±0.109^{c}	<0.001
NH_3 (mg/L)	9.27±0.225	0.024±0.004^{b}	0.033±0.005^{b}	0.024±0.002^{b}	11.72±0.795^{a}	0.034±0.004^{b}	<0.001
BOD (mg/L)	240±22.513	40±3.606^{b}	60±2.706^{a}	35±1.480^{bc}	30±2.646^{cd}	24±3.000^{d}	<0.001
COD (mg/L)	633±26.599	134±14.436^{b}	198±10.513^{a}	139±3.606^{b}	112±11.555^{bc}	85±8.839^{c}	<0.001

- Different superscript letters within the same row in the tables indicate statistically significant differences among bacterial treatments at $P \leq 0.05$.

Table 4. Removal efficiencies (%) of physicochemical indicators of municipal wastewater after bacterial treatment

Parameter	*Staphylococcus aureus* DAQSE3	*Pseudomonas aeruginosa* DAQSE4	*Enterobacter cloacae* DAQSE5	*Escherichia coli* DAQSE6	*Enterococcus faecalis* DAQS7
pH	-21.73	2.61	-19.94	-5.36	-18.57
TDS	16.49	9.82	14.74	2.11	-1.38
TSS	90.12	76.54	88.07	71.93	65.84
PO_4	4.16	-19.36	-4.18	-27.82	31.25
NO_3	-922.89	-902.00	-940.44	-361.47	68.66
NH_3	99.74	99.64	99.74	-26.43	99.63
BOD	83.33	75.00	85.42	87.50	90.00
COD	78.83	68.72	78.04	82.31	86.57

- Negative removal efficiency indicates an increase in the measured parameter following bacterial treatment relative to control.

For TSS, *Staphylococcus aureus* DAQSE3 and *Enterobacter cloacae* DAQSE5 recorded the statistically lowest mean values, with removal efficiencies of 90.12% and 88.07%, respectively. This indicates that the two isolates can reduce suspended solids with high efficiency, possibly by breaking down suspended organic matter or forming microbial aggregates that promote particle settling. This is supported by the findings of Sharma et al. (2023), who reported that microorganism-based biological systems contribute to the removal of

organic and particulate matter from wastewater, and that TSS reduction is an important indicator of improved water quality.

Nitrate (NO_3) results showed a different pattern from ammonia (NH_3). *Staphylococcus aureus* DAQSE3, *Pseudomonas aeruginosa* DAQSE4, and *Enterobacter cloacae* DAQSE5 recorded the highest NO_3 means, indicating a marked increase in nitrate after treatment. In contrast, *Enterococcus faecalis* DAQS7 was the only isolate to clearly reduce nitrate, with the lowest significant mean and a removal efficiency of 68.66%. The increase in nitrate in most treatments may be explained by incomplete transformation of nitrogen compounds under the tested aerobic batch conditions. This is consistent with Song et al. (2021), who explained that nitrification oxidizes ammonium to nitrite and nitrate, whereas final nitrate removal requires suitable conditions for denitrification.

Regarding phosphate (PO_4), *Enterococcus faecalis* DAQS7 recorded the lowest significant mean and achieved the highest removal efficiency of 31.25%. In contrast, *Pseudomonas aeruginosa* DAQSE4 and *Escherichia coli* DAQSE6 recorded higher means, indicating phosphate release or accumulation after treatment. This discrepancy may reflect differences in phosphate uptake and incorporation into biomass, or release from organic phosphorus compounds during degradation. This result is supported by reviews by Zhang et al. (2024) and Ruiz-Haddad et al. (2024), which demonstrated that biological phosphorus removal depends on phosphate-accumulating organisms and on operational conditions such as alternating anaerobic and aerobic phases, the availability of a carbon source, pH, and dissolved oxygen.

BOD and COD results showed that *Enterococcus faecalis* DAQS7 recorded the lowest statistically significant means and achieved removal efficiencies of 90.00% for BOD and 86.57% for COD. *Escherichia coli* DAQSE6 also demonstrated high removal efficiency for these parameters, reaching 87.50% for BOD and 82.31% for COD. These reductions indicate that these isolates can utilize biodegradable organic matter and convert it into biomass and simpler metabolic products. Although *Pseudomonas aeruginosa* is widely recognized for its metabolic versatility and biodegradation capacity, the DAQSE4 isolate achieved substantial removal efficiencies of 75.00% for BOD and 68.72% for COD. These efficiencies are comparable to those reported by Shah et al. (2025), who observed BOD and COD removal efficiencies of 73.64% and 67.98%, respectively. They also fall within the ranges reported by Nurdianti et al. (2026), who found BOD and COD removal efficiencies of 64–77% and 63–77%, respectively, using *P. aeruginosa*. The relatively lower performance of DAQSE4 compared with other isolates in the present study may be related to the strain's specific metabolic characteristics, differences in wastewater composition, and experimental conditions, rather than to inherently low biodegradation capacity (Shah et al., 2025; Nurdianti et al., 2026).

4. Conclusion

Selected indigenous bacterial isolates improved the quality of municipal wastewater under laboratory-scale batch conditions. *Enterococcus faecalis* DAQS7 showed the best overall performance in reducing COD, BOD, phosphate, and nitrate, while *Staphylococcus aureus* DAQSE3 and *Enterobacter cloacae* DAQSE5 were most effective at removing TSS. The current study does not establish extracellular enzyme production as the sole or principal mechanism responsible for pollutant removal. The findings demonstrate the laboratory-scale potential of the selected bacterial isolates to improve wastewater quality; however, biosafety and pathogenicity assessments, as well as pilot-scale validation, are required before environmental application.

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This study received no external funding.

Data Availability Statement

The data supporting the results of this study are available from the corresponding author upon reasonable request.

Ethical Approval

Ethical approval was not required for this study because it did not involve human participants or animals.

Conflicts of Interest

The authors declare no conflicts of interest.

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