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Isolation and Characterization of Bacterial Strains with Organic-Degrading Potential for Municipal Wastewater Treatment

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ABSTRACT

The increasing volume of wastewater discharged in urban areas poses a significant environmental challenge, particularly due to the potential for organic carbon overload in aquatic ecosystems. This study aimed to identify the bacterial isolates with the potential to mitigate this burden by effectively degrading organic matter and exhibiting antagonistic activity against common aquatic pathogens. Through a screening process, two bacterial strains, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, were isolated based on their high amylolytic, cellulolytic, proteolytic and lipolytic activity indices. In addition, in relation to all effective strains for these activities, *Bacillus amyloliquefaciens* differed in the cellulolytic index (4.48 ± 0.12), while *Bacillus licheniformis* had a lipolytic index of 1.73 ± 0.10. Both strains were further characterized by their strong antagonistic activity against *Aeromonas*, a prevalent pathogen in aquatic environments. These findings suggest that *B. amyloliquefaciens* and *B. licheniformis* hold promise as bioremediation agents for wastewater treatment, potentially contributing to the sustainable management of urban wastewater and the protection of aquatic ecosystems.

Keywords: wastewater treatment, biological methods, enzymatic activity, microorganisms, organic compounds.

INTRODUCTION

In the modern world, the problem of pollution of water bodies, including wastewater, is becoming increasingly relevant and pressing [1]. The population is growing rapidly, and with population growth, the volume of wastewater generated and the number of people vulnerable to the effects of severe wastewater pollution increase [2]. The level of development of the food, chemical, pharmaceutical, textile and other industries is increasing, along with the discharge of waste into treatment facilities. As a result, xenobiotics and organic substances of artificial origin [3], resistant to biodegradation, a large amount of synthetic surfactants, heavy metals, acids, alkalis and salts enter treatment plants along with wastewater [4], [5]. In addition, the wastewater containing excess amounts of nutrients causes eutrophication of water bodies, which in turn leads to hypoxia of bottom vegetation, as well as the formation of strong poisons such as phenols and hydrogen sulfide [6], [7], [8]. One of the promising areas for purifying municipal and industrial wastewater from such a number of contaminants is the biological treatment [9], [10]. This is not only an environmentally sustainable approach, but also an effective way to combat water pollution. Biological wastewater treatment methods are based on the use of the biochemical activity of microorganisms [11], [12], and always require additional research, which is primarily due to the specificity of waste depending on the source of pollution, the diversity of their chemical composition and the specific microbial flora retrieved from this pollution [13], [14], [15]. A key role in biological methods is played by living cells, which participate in competitive and energy metabolism, leading to complex chemical and

biological transformations of organic substances. The main functions of organic oxidation in a living cell largely depend on how and with the participation of which enzyme systems the utilization of organic matter is carried out [16], [17].

Microbial processes appear to be the main mechanism of organic matter degradation, although animals also play a role [18]. Biodegradation of organic compounds can occur under both aerobic and anaerobic conditions as a result of microbial communities [19], [20], [21]. Microbiological degradation of contaminants can lead to the formation of many different products. Typically, biodegradation of a large organic molecule results in the formation of smaller, less complex organic molecules that may be more or less toxic than the parent compound for aquatic plants, fish, and other living organisms in water bodies [22–24]. In some cases, the organic pollutant is completely converted into an inorganic product such as CO_2. This type of transformation is called "mineralization" and involves eliminating the toxicity of the parent compound. Although plants, animals and microorganisms can metabolize some pollutants, mineralization of organic pollutants is usually carried out only by bacteria and fungi [25]. These microorganisms have a significant impact on the total amount of organic matter in wastewater, which is expressed through the concentrations of BOD (biochemical oxygen demand), COD (chemical oxygen demand), TOC (total organic carbon), and petroleum products (oil and grease) [26], [27]. Thus, biodegradation can make a significant contribution to the natural nutrient cycle and can therefore be considered a form of recycling.

Therefore, the goal of the conducted work was to select the biological agents capable of preventing excessive organic pollution through their metabolism. For this purpose, bacterial cultures were isolated from municipal wastewater and analyzed for enzymatic activity towards organic substances. In addition, their antibacterial activity against common wastewater pathogens was studied.

MATERIALS AND METHODS

Water sampling

Wastewater samples were meticulously collected in sterile 100 ml glass bottles from an urban wastewater treatment plant in Astana city, Kazakhstan, precisely at the coordinates 51°09'16"N 71°39'13"E, following the mechanical treatment stage. This stage typically involves the removal of large solids and debris, ensuring that the collected samples primarily contained suspended and dissolved organic matter as well as microorganisms relevant for subsequent analysis. The samples were carefully sealed and transported under controlled conditions to the laboratory, where they were stored at 4 °C to preserve the viability and integrity of the microbial communities for accurate microbiological analysis. This temperature was maintained to minimize metabolic activity and prevent any significant changes in the microbial population before the cultures were isolated and identified. This rigorous sampling and storage protocol ensured that the microbial communities present in the wastewater were preserved in their near-original state, allowing for precise and reliable characterization during the subsequent microbiological analysis.

Chemical analysis of water

The analysis of wastewater samples involved measuring several physicochemical parameters, essential for assessing the quality and characteristics of the wastewater. These parameters included color, odor, pH, COD, BOD, and temperature. The chemical oxygen demand of the samples was estimated in the samples using a spectrophotometer (Hach LANGE DR 3900, Hach, Germany), set to a wavelength of 600 nm [28] The reagents for the determination of COD in wastewater were COD digestion vials, high range kit (Hach, Germany). BOD was determined using the OxiTop ®measuring system (WTW, Germany) [29]. The pH of the water was determined using a pH meter (Metrohm 827, Switzerland).

Isolation of strains and culture conditions

Pure cultures of bacteria were isolated using the serial dilution method to a titer of 10^7 [24]. The serial assay was then aliquoted in a volume of 100 µl on nutrient agar and Pseudomonas agar (Himedia, Laboratories Pvt. Ltd., Mumbai, India). The nutrient agar had the following composition (g/L): peptone – 5.0 sodium chloride – 5.0, peptone – 1.50, yeast extract 1.5, agar – 15.0. Pseudomonas agar consisted of the following composition (g/L): peptic digest of animal tissue – 20.0 magnesium chloride – 1.4, potassium sulfate – 10.0, triclosan – 0.025, agar – 13.6. Incubation was carried out in a thermostat at 37 °C for 48 hours.

Determination of proteolytic, amylolytic, cellulolytic and lipolytic activities of bacterial isolates

Determination of proteolytic activity was carried out using skim milk agar plates [30]. To study the amylolytic activity, the plaque method on the medium of the following composition (g/l): peptone – 10.0; KH_2PO_4 – 5.0; soluble starch – 2.0; agar – 15.0. medium pH 6.8–7.0 was used. Sowing was carried out by injection. The amount of amylolytic activity was determined by the diameter of the light zone. The starch hydrolysis zone is measured in millimeters from the edge of the colony stroke to the border of the light zone. To detect cellulolytic activity, bacterial cultures were plated by plaques using a loop onto a CMC (carboxymethylcellulose) agar medium. Bacterial isolates were grown on a medium containing 1% CMC and incubated at 37 °C for 48 hours. After incubation, Petri dishes were flooded with 0.1% Congo red reagent and left for 30 min. Then, 1 M NaCl solution was added to the Petri dishes and left for 5 min. Plate surfaces were discarded by NaCl solution and rinsed with distilled water. Then, were added 5% acetic acid and left for 5 min to observe and record the halo size. The presence of transparent halos resulting from the hydrolysis of cellulose and starch was measured [31], [32]. To detect lipolytic activity, the studied microorganisms were plated on a nutrient medium containing the corresponding oleic acid tween 80 (HiMedia, Mumbai, India). The composition of the nutrient medium (g/l): tween – 10, peptone – 10, NaCl – 5, $CaCl_2xH_2O$ – 0.1; agar – 20, pH of the medium – 7.4. Tween was autoclaved separately at 0.5 atmospheres for 15 minutes and added to the sterile main medium. The nutrient medium was poured into sterile Petri dishes, and after solidification of the medium, microorganisms were plated. The cultivation duration was 2–10 days at 37 °C. The presence of lipase is indicated by the formation of an opaque zone of calcium salts of fatty acids released from tween around a streak or colony [33]. The degree of hydrolase activity of enzymes secreted by bacteria was assessed by the index value. The determination of enzymatic indices (EI) by amylolytic (AI), cellulolytic (CI) and proteolytic activities (CI) was calculated using the equation proposed by Naresh [34]:

$$EI = \frac{(Halo\ diameter - Bacterial\ colony\ diameter)}{Bacterial\ colony\ diameter} \quad (1)$$

Phenotypic analysis of isolates

The pure bacterial isolates were identified microscopically using a microscope (Micros MC300X, Austria) with a gram staining method [28]. Further phenotypic identification was carried out using Vitek 2 systems (BioMérieux, USA). In addition to this analysis, bacterial colonies were collected and verified by biochemical tests using triple sugar iron (Condalab, Spain) [35].

Antimicrobial susceptibility testing

The antagonistic activity of isolated bacteria was assessed by the diameter of the zones of inhibition of opportunistic and pathogenic cultures. Isolated microorganisms were analyzed regarding the antagonistic activity against bacterial strains, such as *Enterococcus faecium* 27LB B-RKM-0612, *Staphylococcus aureus* ATCC 6538 B-RKM 0470, *Salmonella enteritidis* B-RKM 0680, *Pseudomonas aeruginosa* G13 B-RKM0427, *Pseudomonas taiwanensis* CB 2R-1B-RKM 0726, *Aeromonas punctata* G30 B-RKM0287, *Klebsiella pneumonia* B-RKM 0444, which are in the collection of industrial microorganisms of the Republican microorganism collection [36].

Genetic identification of bacteria

The identification of bacterial isolates was carried out using the method of determining the direct nucleotide sequence of the 16s rRNA gene with the subsequent comparison of nucleotide identity with sequences deposited in the international Genbank database. The PCR reaction was carried out with universal primers 8f (5' AgAgTTTgATCCTggCTCAg-3') and 806R (5'-ggACTACCAgggTATCTAAT-3'). The nucleotide sequences were analyzed and combined into a common sequence using SeqScape 2.6.0 software (Applied Biosystems)[37]. The Mega 6 software was used to construct phylogenetic trees. The Muscle algorithm was used to align nucleotide sequences, and the phylogenetic tree was constructed using the Neighbor-Joining NJ method [38].

Statistical analysis

In this work, all experiments were performed in triplicate. Means, standard errors, and standard deviations were calculated from replicates within the experiments [39]. To assess the statistical

significance of variations in the studied parameters, a one-way analysis of variance (ANOVA) was performed, followed by a post-hoc Tukey HSD Test with significance at $p < 0.05$ [40].

RESULTS

Physicochemical characteristics of wastewater samples

The collected wastewater sample was carefully analyzed to explore its potential as a reservoir for bacteria with the ability to hydrolyze organic substrates and compete with existing pathogenic microflora. The results of the physicochemical analyses, summarized in Table 1, provide an in-depth view of the wastewater characteristics, including key parameters like pH, COD, BOD, temperature, color, and odor. These measurements are critical for assessing the overall quality of the wastewater and understanding the extent of organic pollution present, which is crucial for determining the suitability of a sample for various treatment processes or biotechnological applications. The data also reveal the presence of organic substrates within the wastewater that could support the growth of specific bacteria capable of breaking down complex compounds. This information is essential for identifying the opportunities for bioremediation, where selected bacterial strains could be leveraged to enhance the degradation of organic pollutants. Additionally, understanding the physicochemical profile of wastewater can guide the development of targeted biotechnological applications, such as isolating bacteria with specialized metabolic pathways for pollutant degradation or the production of value-added products, ultimately improving the efficiency and effectiveness of wastewater treatment processes.As shown by the physicochemical parameters in Table 1, the wastewater exhibited the characteristic color and odor commonly associated with municipal wastewater, accompanied by elevated COD and BOD values, indicating significant organic pollution. The pH of the wastewater was measured at 8.11, suggesting a slightly alkaline environment. The COD concentration exceeded 300 mg/L, which falls within the typical range for municipal wastewater, generally between 100 and 500 mg/L. This elevated COD level reflects a substantial presence of organic matter, indicative of the high pollutant load of wastewater. The observed BOD levels further corroborate this, highlighting the potential impact of wastewater on receiving water bodies if not adequately treated. These physicochemical characteristics underscore the necessity for effective biological treatment processes, such as those involving the isolated strains, to reduce the organic load and improve the quality of the treated effluent.

Table 1. Physico-chemical characterization of the wastewater sample

Physico-chemical parameters	Observation
Color	Dark
Odor	Rotten smell
pH	8.11
COD, mg/L	316
BOD, mg/L	80
Temperature (°C average)	15

Screening of bacteria producing protease, amylase, cellulose and lipase

Fifty-six bacterial isolates were isolated from municipal wastewater and were screened for determination of extracellular enzymatic activity toward organic substrates. Only 11 (19.6%) isolates demonstrated proteolytic activity, indicating their ability to break down proteins, as detailed in Table 2. This suggests that a relatively small proportion of the isolates have potential applications in areas requiring protein degradation. In terms of amylolytic activity, 9 isolates (16.07%) were found to be capable of hydrolyzing starch. This indicates their potential utility in the processes involving starch breakdown. Similarly, cellulolytic activity was observed in 7 isolates (12.5%), confirming their ability to degrade cellulose, which is important for applications in waste management and biofuel production. Lastly, 8 isolates (14.28%) exhibited lipolytic activity, demonstrating their ability to hydrolyze fatty acids, with implications for industries focused on fat degradation. These results highlight the diverse enzymatic capabilities of the isolates and their potential applications across various biotechnological fields (Table 2).

On the basis of the data presented in Table 2, isolates TI05Ps, JI03Ps, TI48-6, JI03, and JI04Ps exhibited high proteolytic activity, as evidenced by the clear zones they formed on milk agar medium. This indicates their strong ability to hydrolyze proteins, which could be advantageous in applications

Table 2. Proteolytic, amylolytic, cellulolytic and lipolytic activities of isolates

Isolate	Proteolytic index	Amylolytic index	Cellulolytic index	Lipolytic index
JI01	0	1.50 ± 0.75^{d}	0	1.45 ± 0.02^{h}
JI03	1.44 ± 0.05^{a}	0.90 ± 0.04^{e}	0	1.38 ± 0.12^{h}
JI05	0.50 ± 0.0^{5ab}	0.40 ± 0.05^{b}	0	0.45 ± 0.06^{j}
JI10	0.93 ± 0.02^{ab}	0	0	0
JI02Ps	0.88 ± 0.03^{ab}	0.26 ± 0.02^{e}	0	0.89 ± 0.11^{ij}
JI03Ps	1.50 ± 0.11^{a}	0.18 ± 0.01^{e}	0	0.61 ± 0.09^{ij}
JI04Ps	1.22 ± 0.02^{a}	1.10 ± 0.04^{d}	0	1.30 ± 0.05^{h}
TI04As	0.20 ± 0.05^{b}	0	0.30 ± 0.11^{g}	0
TI48-5	0	0	1.00 ± 0.01^{g}	0
TI48-6	1.45 ± 0.0^{8a}	1.09 ± 0.02^{d}	3.57 ± 0.15^{f}	1.73 ± 0.10^{h}
TI01Ps	0.62 ± 0.02^{ab}	0.40 ± 0.05^{e}	3.80 ± 0.10^{f}	0
TI05Ps	2.41 ± 0.14^{a}	1.67 ± 0.09^{d}	4.48 ± 0.12^{f}	0.18 ± 0.02^{j}
TI06Ps	0	0	0.21 ± 0.02^{g}	0
TI01Mur	0.30 ± 0.05^{b}	0	0.25 ± 0.05^{g}	0

Note: Superscripts (letters a, b, c, d, e, f, g, h, i, j) in the table indicate significant differences between groups based on Tukey's test, $p < 0.05$. The same superscripts mean that there are no statistically significant differences between isolates. Different superscripts mean that there are statistically significant differences between these isolates.

such as waste treatment, food processing, and detergent formulation. Among these, isolate TI05Ps demonstrated the highest proteolytic activity with an enzymatic index of 2.41 ± 0.14, making it particularly noteworthy for its potent protein-degrading capabilities. In contrast, isolate TI04As showed the lowest proteolytic activity with an index of 0.20 ± 0.05. The differences in hydrolase activity among isolates JI10 (0.93 ± 0.02), JI02Ps (0.88 ± 0.03), TI01Ps (0.62 ± 0.02), JI05 (0.50 ± 0.05), TI01Mur (0.30 ± 0.05), and TI04As (0.20 ± 0.05) were not significant, indicating a relatively uniform but low level of proteolytic activity across these strains. This suggests that while some isolates are highly effective at protein hydrolysis, others have limited capabilities in this regard.

The isolates TI05Ps, JI01, JI04Ps, and TI48-6 were noted for their high amylolytic activity, with TI05Ps exhibiting the highest enzymatic index of 1.67 ± 0.09. This indicates a strong ability to hydrolyze starch, making these isolates potentially valuable in industries such as food processing, where starch degradation is critical. On the other hand, isolate JI03Ps showed the lowest amylolytic activity with an index of 0.18 ± 0.01, suggesting minimal capacity for starch breakdown compared to the other isolates. This aspect of the study provides a comprehensive overview of the capabilities of isolates to degrade cellulose, further characterizing their enzymatic profiles and potential applications. The combination of amylolytic and cellulolytic activities in isolates like TI05Ps underscores their versatility in breaking down different types of polysaccharides, which could be harnessed for biotechnological applications, including bioethanol production and waste treatment.

During the detection of cellulose hydrolysis, isolates TI05Ps, TI01Ps, and TI48-6 displayed pronounced halo zones on the medium after the addition of the indicator, indicating strong cellulolytic activity. This suggests that these isolates are capable of efficiently breaking down cellulose, a key component of plant cell walls, which could be beneficial in industries such as biofuel production and waste management [41]. The cellulolytic activity, quantified by the enzymatic index, ranged between 3.57 ± 0.15 and 4.48 ± 0.12, highlighting the substantial ability of these strains to hydrolyze cellulose. Lipase activity is crucial for the breakdown of fats, and these results further characterize the multifaceted enzymatic profiles of the tested isolates. The combination of strong cellulolytic and lipolytic activities in isolates, such as TI05Ps and TI48-6, suggests their potential for applications in the processes that require the degradation of complex organic materials, such as composting or bioconversion of agricultural residues.

Isolates TI48-6, JI01, JI03, and JI04Ps demonstrated a remarkable ability to hydrolyze lipids, with enzymatic indices ranging from 1.73 ± 0.10 to 1.30 ± 0.05. This indicates strong lipolytic activity, which is critical for breaking down fats and oils,

potentially useful in industrial applications such as bioremediation and food processing. The differences in enzymatic indices between these high-performing isolates and others with lower activity levels were statistically significant ($p < 0.05$), emphasizing the robustness of their lipolytic capacity. Notably, while 25% of the isolates were capable of producing at least two of the four enzymes tested – lipolytic, proteolytic, cellulolytic, and amylolytic – they were not classified as strong potential producers, as their enzymatic indices were less than or equal to 1.0, indicating only moderate enzyme production capabilities. Isolates TI48-6 and TI05Ps were particularly noteworthy, as they exhibited simultaneous expression of three enzymatic activities – proteolytic, cellulolytic, and amylolytic—along with significant lipolytic activity. This multifaceted enzymatic profile suggests that these isolates have versatile metabolic capabilities, making them valuable for various biotechnological applications. Among them, TI48-6 stood out with significantly higher lipolytic activity, which could make it particularly useful in the processes that require efficient lipid breakdown. Figure 1 provides a visual representation of the isolates that excelled in hydrolyzing key substrates, such as casein, starch, cellulose, and Tween 80, further highlighting their potential in the applications where comprehensive enzymatic activity is essential.

Phenotypic characterization of bacterial isolates

More than half of the isolates were identified as Gram-negative bacteria through Gram staining. This high proportion of Gram-negative bacteria is consistent with their common occurrence in wastewater environments, where they often play key roles in organic matter degradation and nutrient cycling. The identification of these bacteria provides valuable insight into the microbial community present in the wastewater sample. In addition to the Gram-negative isolates, two Gram-positive isolates were noted for their distinctive enzymatic

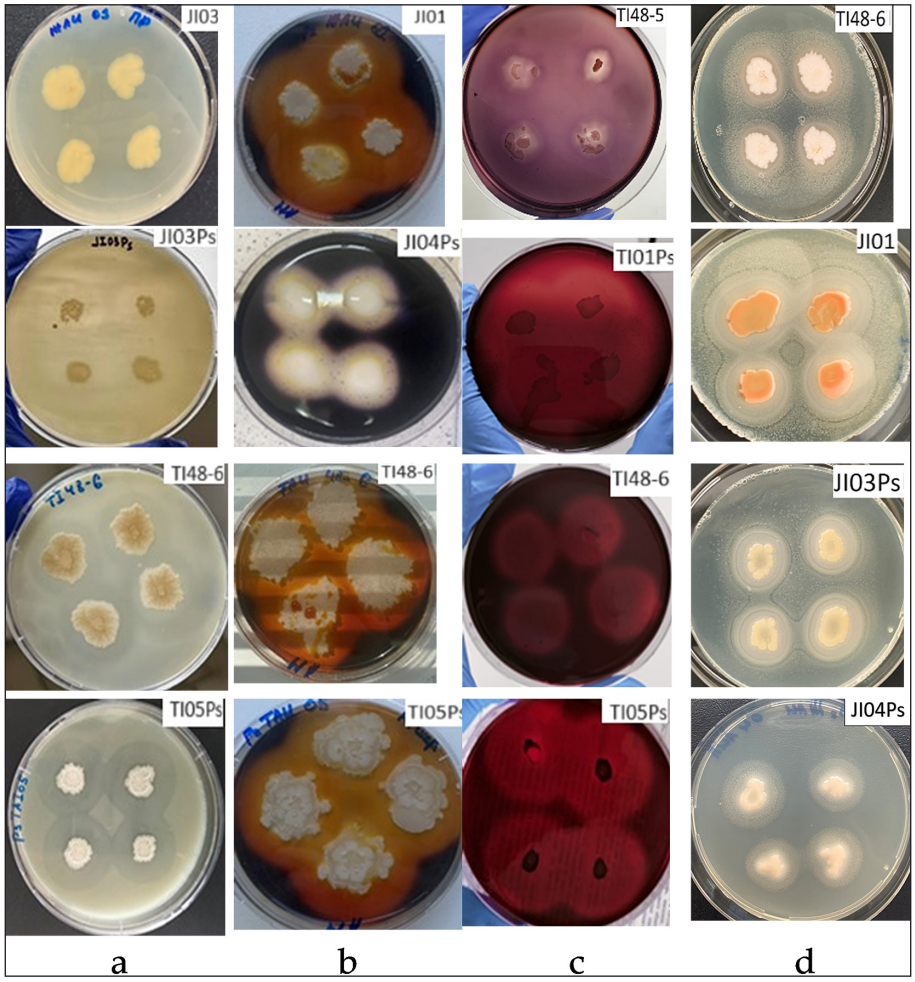

Figure 1. Enzymatic activity of isolates (a-proteolytic, b-amylolytic, c-cellulozolytic, d- lipolytic)

activity. These catalase-negative microorganisms, while less commonly detected in wastewater due to their typically lower adaptability and survival rates, were observed to persist in the sample. Their presence was attributed to their significant survival capabilities in the wastewater environment, which was further validated by their notable proteolytic and cellulolytic activities. The remaining 12 isolates were classified as catalase-positive and exhibited a diverse profile of carbohydrate assimilation, as detailed in Table 3. This broad carbohydrate assimilation capability suggests a high level of metabolic versatility among these isolates, potentially enhancing their ability to utilize various organic substrates in the wastewater. Such diversity in metabolic functions highlights the potential of these bacterial strains for various biotechnological applications, including bioremediation and wastewater treatment processes.

Identification of proteolytic, amylolytic, cellulozolytic and lipolytic isolates

Bacterial isolates demonstrating organic substrate hydrolysis capability underwent sequencing using the 16S rDNA technique. Subsequently, their sequences were compared with the NCBI database via the BLAST tool, and the findings are summarized in Table 4. This table details the molecular identification results of diverse bacterial strains isolated from wastewater samples, aligning each strain with existing sequences in the GenBank database to determine their species and subspecies identities. The percentage match indicates

Table 3. Differentiating properties of isolates

Characteristics	Isolates													
	JI01	JI03	JI05	JI10	JI02 Ps	JI03 Ps	JI04 Ps	TI01 Ps	TI05 Ps	TI06 Ps	TI48-5	TI48-6	TI04 As	TI01 Mur
Gram stain	-	-	-	+	-	-	-	-	+	-	+	+	-	-
Cell morphology	Rod	Rod	Rod	Cocci	Rod	Rod	Rod	Rod	Rod	Rod	Cocci	Rod	Rod	Rod
Catalase	+	+	+	-	+	+	+	+	+	+	-	+	+	+
Hydrogen sulfide	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Nitrate reduction	+	+	+	-	+	+	+	+	+	+	-	+	+	+
Voges - Proskauer test	-	-	-	+	-	-	-	+	+	+	-	+	+	+
Hydrolysis of														
Gelatin	-	+	+	-	-	+	+	-	+	-	-	+	-	-
Urea	-	-	-	-	-	-	-	-	-	+	-	+	+	+
Assimilation from														
D glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D fructose	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Xylose	-	+	-	-	+	+	+	+	-	+	-	-	+	+
Ribose	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Arabinose	-	+	+	-	+	+	+	+	-	+	-	-	+	+
Mannite	-	+	+	+	+	+	+	+	-	+	+	-	+	+
Inositol	-	+	-	-	-	+	-	+	-	+	-	-	+	+
D-sorbitol	-	+	+	-	+	+	+	+	-	+	-	-	+	+
D-maltose	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Cellulose	-	-	-	-	-	-	-	-	+	-	-	+	-	-
D-lactose	-	-	-	-	-	-	-	-	+	+	-	+	+	+
Salicin	+	+	+	-	+	+	+	+	-	+	+	-	+	+
D-mellibiose	-	-	-	-	-	-	-	+	+	+	-	-	+	+
Sucrose	-	+	+	+	+	+	+	+	-	+	+	-	+	+
Raffinose	-	-	-	-	-	-	-	+	+	+	-	+	+	+
Glycogen	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Glycerol	-	+	+	-	-	+	-	+	-	+	-	+	+	+
Citrate	-	+	+	-	+	+	+	+	-	+	-	+	+	+

Table 4. The results of identification by analysis of the nucleotide sequence of the 16S rRNA gene

Strains	GenBank inventory number (accession number)	Name of the strain	% match
JI01	AF002709.1	*Aeromonas salmonicida*	100
JI03	CP034967.1	*Aeromonas veronii*	100
JI05	KU725737.1	*Aeromonas hydrophila*	96.79
JI10	MT348592.1	*Leuconostoc mesenteroides*	100
JI02 Ps	MT271886.1	*Aeromonas caviae*	100
JI03 Ps	LC383908.1	*Aeromonas sobria*	100
JI04 Ps	LC505495.1	*Aeromonas allosaccharophila*	99.87
TI01 Ps	OR426325.1	*Enterobacter cloacae*	98.50
TI05 Ps	MT579842.1	*Bacillus amyloliquefaciens*	100
TI06 Ps	CP044121.1	*Raoultella planticola*	99.87
TI48-5	CP014949.1	*Enterococcus faecalis*	100
TI48-6	MT642945.1	*Bacillus licheniformis*	100
TI04 As	MH671643.1	*Raoultella ornithinolytica*	99.73
TI01 Mur	CP090222.1	*Klebsiella pneumoniae*	100

the degree of similarity between the genetic material of the isolate and the reference sequence, with higher percentages indicating a more confident identification. *Aeromonas salmonicida* (JI01) and *Aeromonas veronii* (JI03) were identified with a 100% match, indicating that the isolates were identical to these known species in the GenBank database. *Aeromonas hydrophila* (JI05), however, showed a slightly lower match of 96.79%, suggesting some genetic variation from the reference strain, which could be due to strain-specific differences or potential mutations. Other *Aeromonas* species, including *Aeromonas caviae* (JI02 Ps) and *Aeromonas sobria* (JI03 Ps), were also identified with 100% matches, confirming their presence in the wastewater samples.

In addition to the Aeromonas species, other bacteria were identified with high confidence. *Leuconostoc mesenteroides* (JI10) and *Enterococcus faecalis* (TI48-5) both had 100% match to nucleotide sequences in the NCBI database. *Raoultella ornithinolytica* (TI04 As) and *Raoultella planticola* (TI06 Ps) were identified with very high matches of 99.73% and 99.87%, respectively, suggesting close similarity to their reference strains. *Enterobacter cloacae* (TI01 Ps) and *Klebsiella pneumoniae* (TI01 Mur) also showed strong matches, though with slightly lower percentages of 98.50% and 100%. The two strains of particular interest in this study, *Bacillus amyloliquefaciens* (TI05 Ps) and *Bacillus licheniformis* (TI48-6) were identified with 100% matches, indicating that these isolates are very likely to be accurately classified within these species. This strong match supports the reliability of their identification and underpins their potential use in bioremediation and wastewater treatment due to their known antimicrobial and enzymatic activities. The high level of genetic similarity found in these isolates suggests they may be effectively used in the targeted treatment of wastewater, reducing microbial contaminants and improving water quality.

The sequencing results identified a diverse range of bacterial isolates from the wastewater sample. Specifically, 6 isolates were classified under the genus *Aeromonas*, 2 under *Bacillus*, 2 under *Raoultella*, 1 under *Leuconostoc*, 1 under *Enterobacter*, and 1 under *Enterococcus*. During the isolation phase, colonies from the genera *Aeromonas* and *Bacillus,* both of which are facultative anaerobes, were the most frequently observed. *Aeromonas* bacteria are Gram-negative, short rods with rounded ends and are widely distributed in various aquatic and terrestrial environments, establishing them as a prevalent genus in such settings. On nutrient agar, *Aeromonas* colonies appeared as shiny, translucent with a whitish-yellow tint. When grown in nutrient broth, these bacteria produced uniform turbidity with a film on the surface and a flocculent white sediment at an optimal growth temperature of 37 °C. These growth characteristics underscore their adaptability and prevalence in wastewater environments. Two specific isolates, TI05 Ps and TI48-6, were further examined and are illustrated in the phylogenetic tree (Fig. 2). Notably, sample 1 (TI48-6) aligns closely with *Bacillus licheniformis* on the phylogenetic tree. Given the high similarity of their 16S rRNA genes, additional nucleotide sequence analysis of protein-coding genes or

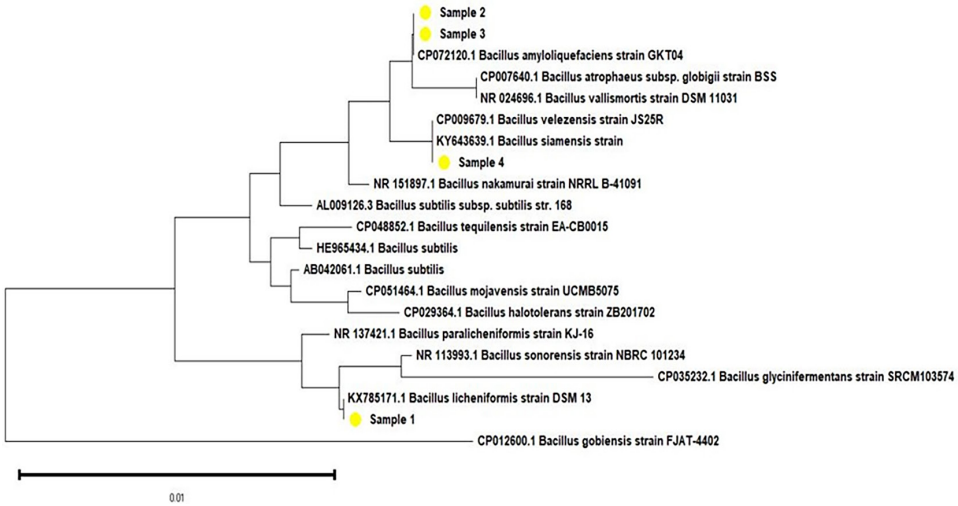

Figure 2. Phylogenetic tree built by algorithm Neighbor Joining (NJ).
Sample 1 – *Bacillus licheniformis*, sample 2 and 3 – *Bacillus amyloliquefaciens*

phenotypic characterization is required for precise identification. Meanwhile, samples 2 and 3 (TI05 Ps) cluster with *Bacillus amyloliquefaciens*, indicating a relatedness within this bacterial group.

Antibacterial activity

Further investigation was conducted on the antagonistic activity of two selected isolates, TI48-6 and TI05Ps, which had previously shown the most effective enzymatic activity in breaking down organic substrates. Molecular characterization identified these isolates as *Bacillus amyloliquefaciens* (TI05Ps) and *Bacillus licheniformis* (TI48-6), both of which are recognized for their biotechnological potential, including their roles in enzyme production and antimicrobial properties. The focus of the study was to assess their ability to inhibit various bacterial pathogens and contaminants, which is essential for applications in bioremediation and wastewater treatment. The antagonistic activity of these isolates was rigorously analyzed against a diverse set of test strains from RCM LLC, as illustrated in Figure 3. This selection included

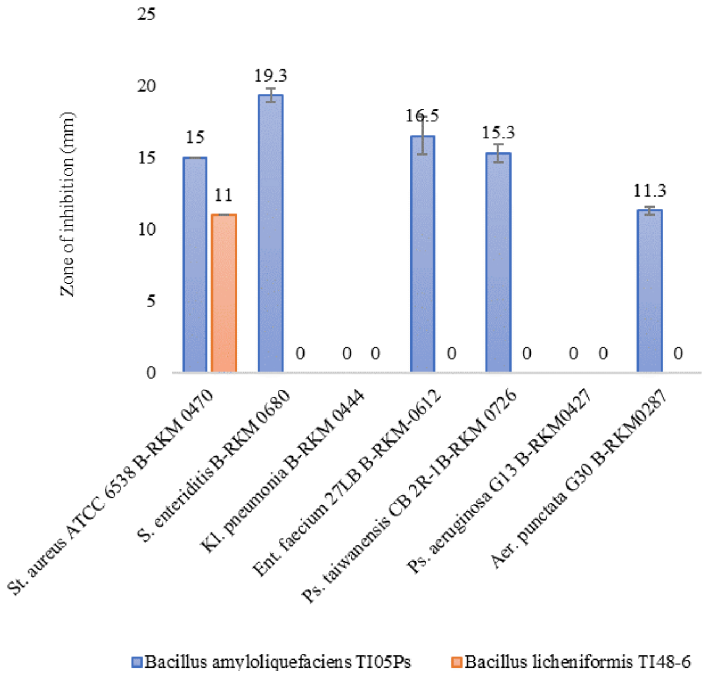

Figure 3. The antagonistic spectrum of *B. amyloliquefaciens* TI05Ps and *B. licheniformis* TI 48-6 against 7 pathogenic collection strains (b)

a range of common bacterial pathogens, offering a comprehensive assessment of the antimicrobial spectrum of the isolates. The study also included testing against bacteria from the genus *Aeromonas*, which are prevalent in the studied wastewater and are known to cause infections in both humans and animals. This approach aimed to determine the effectiveness of these *Bacillus* strains in reducing the microbial load in wastewater. The results from these analyses were intended to evaluate whether *Bacillus amyloliquefaciens* and *Bacillus licheniformis* could effectively diminish pathogenic microorganisms in wastewater, thereby improving its quality and safety. Successful reduction of microbial contaminants could enhance the feasibility of reusing treated wastewater in agricultural or industrial applications, contributing to more sustainable waste management practices. The study findings have the potential to develop informed strategies for leveraging these bacterial strains in environmental and industrial biotechnological processes.

The results of the antagonistic activity tests revealed notable differences in the effectiveness of the bacterial strains studied. The *Bacillus licheniformis* strain TI48-6 exhibited activity exclusively against *Staphylococcus aureus*, highlighting its potential for targeted inhibition of this specific pathogen. This targeted effect suggests that TI48-6 may produce particular compounds or enzymes that are highly effective against *S. aureus*. However, this strain did not demonstrate antagonistic activity against other test strains, such as *Salmonella enteritidis*, *Enterococcus faecium*, *Pseudomonas taiwanensis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, indicating a narrow spectrum of activity. In contrast, isolate TI05Ps displayed a broader range of antagonistic activity, as shown in Figure 4. It demonstrated moderate to high effectiveness against *Salmonella enteritidis*, *Enterococcus faecium*, and *Pseudomonas taiwanensis*. This broader spectrum suggests that TI05Ps may produce a variety of antimicrobial compounds or possess a more versatile mode of action, making it potentially useful against a wider array of bacterial pathogens. Despite its broad activity, TI05Ps, like TI48-6, was ineffective against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The resistance of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* to both TI48-6 and TI05Ps underscores the complexity of microbial resistance and the limitations of the current bacterial strains in addressing all targeted pathogens. This finding highlights the need for further research into the mechanisms behind this resistance and the development of more effective antimicrobial agents. Understanding these mechanisms could lead to the identification of novel strategies or compounds that can enhance the effectiveness of bacterial antagonists in controlling a broader range of pathogens.

Figure 4 illustrates that both selected isolates, TI05Ps and TI48-6, demonstrated antagonistic activity against bacteria of the genus *Aeromonas*,

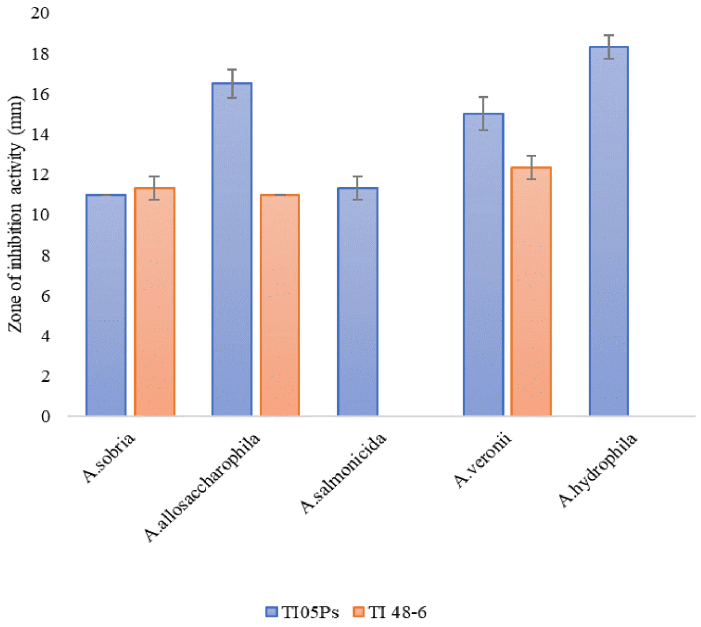

Figure 4. The antagonistic spectrum of TI05Ps and TI 48-6 isolates against 5 *Aeromonas* strains

which are common representatives of the pathogenic microflora found in municipal wastewater. Specifically, the TI05Ps isolate exhibited broad-spectrum antagonistic activity, effectively inhibiting all studied *Aeromonas* species. This suggests a strong potential for TI05Ps in combating a range of pathogenic *Aeromonas* strains present in wastewater environments. In contrast, the TI48-6 isolate showed a more targeted antagonistic effect, successfully inhibiting only three *Aeromonas* species: *A. sobria*, *A. allosaccharophila*, and *A. veronii*. This selective inhibition indicates that TI48-6 may have a more specialized role in suppressing certain pathogenic strains of *Aeromonas*. The differing profiles of antagonistic activity between TI05Ps and TI48-6 highlight their potential utility in targeted bioremediation strategies or as components of microbial consortia aimed at managing specific pathogenic bacteria in wastewater systems.

DISCUSSION

This study focused on the bacteria isolated from municipal wastewater, which was characterized by a distinct putrid odor and dark coloration. The wastewater analyzed had a BOD concentration of 80 mg/L and a COD concentration of 316 mg/L, the levels that fall within the range for biological treatment according to indicator rankings. Literature data indicate that the maximum allowable BOD concentration is 10 mg/L for aquaculture treatment, 100 mg/L for microbiological and phytotreatment, and 50.000 mg/L for natural treatment methods. The maximum concentration of COD is 50 mg/L for aquaculture treatment, 500 mg/L for microbiological and phytotreatment, 100.000 mg/L for natural treatment. Among the 56 isolates, bacteria of the *Aeromonas* genus predominated, accounting for 50% and being the dominant genus. In addition to the genus *Aeromonas*, the following species were found: *Routella*, *Klebsiella*, *Leuconostoc*, *Bacillus*, *Enterococcus*, *Enterobacter*. The safest strains when isolated from the treatment facility settling tanks were gram-positive bacteria of the *Bacillus* and *Leuconostoc* genus.

The study was aimed at finding the isolates capable of decomposing organic matter and suppressing pathogenic microorganisms. Most of the isolates that hydrolyzed casein belonged to the genera *Aeromonas* and *Bacillus*. The protease-producing TI05Ps and TI 48-6 isolates in this study were genetically identified as *B. amyloliquefaciens* and *B. licheniformis*. *B. amyloliquefaciens* was considered to be the most active proteolytic bacterium with significant proteolytic activity among 56 bacterial strains ($p < 0.05$). Among the isolates, *B. amyloliquefaciens* had the highest amylolytic index (1.67 ± 0.09). Thus, the broadest profile of enzymatic activity of these organic substances was demonstrated by *B. amyloliquefaciens* and *B. licheniformis*. Isolates TI05Ps, TI01Ps, TI48-6 showed pronounced halo zones, where the index of cellulolytic activity varied within the range of 3.57 ± 0.15 and 4.48 ± 0.12. Elimination of industrial by-products from cellulose will ensure proper waste disposal, thereby reducing the organic load on the environment [31]. It is important to note that the bacteria with high enzymatic activity are potential agents for biological treatment, the purpose of which is to improve the quality of wastewater, namely the reduction or elimination of organic pollutants [42], [31].

According to the literature, one of the key advantages of using *Bacillus* bacteria in wastewater treatment processes is their ability to decompose organic matter [23], [43]. They have high enzyme activity that promotes depolymerization and assimilation of organic compounds. This is important for effective biological treatment since *Bacillus* bacteria can convert organic pollutants into simpler and less harmful forms. It is also important to keep in mind that *Bacillus* are aerobic bacteria, which means that they function effectively in the presence of oxygen, which ensures more effective wastewater treatment. *Bacillus* bacteria are an interesting and promising group of microorganisms for use in wastewater treatment systems [44], [45].

To make soluble and insoluble biopolymers – primarily carbohydrates, proteins, and lipids – accessible for bacterial respiration, these macromolecules must be hydrolyzed by exoenzymes. These exoenzymes are typically produced and released by bacteria only after contact with specific inducers. Once adsorbed onto the biopolymers, the exoenzymes break them down into monomers or, at the very least, oligomers. Only soluble, low molecular weight compounds, such as sugars, disaccharides, amino acids, oligopeptides, glycerol, and fatty acids, can be absorbed by microorganisms and metabolized to generate energy and support cell growth. In general, the process of assimilation of organic macromolecules in aerobic and facultative-anaerobic bacteria occurs completely, up to carbon dioxide and water, whereas in anaerobes assimilation of substrates is less efficient,

decomposition of organic macromolecules occurs with the formation of organic acids, alcohols and gases without access to oxygen, for example, in *Clostridium* [46]. After absorption into cells, degradation occurs through glycolysis (sugars, disaccharides, glycerol), hydrolysis and deamination (amino acids, oligopeptides) or hydrolysis and oxidation (phospholipids, long-chain fatty acids). The metabolism of almost all organic compounds results in the formation of acetyl-CoA as a central intermediate, which is used for biosynthesis, released as acetate, or oxidized to CO_2 and reducing equivalents in the tricarboxylic acid (TCA) cycle. Reducing equivalents are inhaled with molecular oxygen in the respiratory chain.

During the respiration of carbohydrates by aerobic bacteria, about one-third of the initial energy content is lost as heat, and two-thirds are stored biochemically in the 38 phosphoanhydride bonds of ATP. In activated sludge reactors or wastewater treatment ponds not loaded with highly concentrated wastewater, irradiation of the walls and heat loss through the flow of aeration waste gases to the atmosphere prevent self-heating. However, in the activated sludge reactors for treating highly concentrated wastewater, self-heating to thermophilic temperatures can occur if the wastewater is initially warm, the hydraulic retention time for biological treatment is short (short aeration time), and the air or oxygen flow for aeration is limited to ensure sufficient oxygen for complete oxidation of pollutants (low volume of aeration).

The study by Shin and Song [43] revealed that the breakdown of certain organic materials, including glucose, starch, and food waste, proceeded faster through hydrolysis (initial breakdown) than methanogenesis (methane production) under anaerobic conditions. However, for materials like newspapers and leaves, hydrolysis was the slowest step, limiting the overall degradation process. The breakdown of organic matter in waste treatment can occur through different pathways: aerobic respiration (with oxygen), anaerobic denitrification (using nitrates), or anaerobic methanogenesis/sulfidogenesis (producing methane or sulfide) [48]. Aerobic digestion of sewage sludge for waste treatment. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects 39, 1056–1062] Research indicates that the aerobic bacteria from wastewater treatment plants can effectively decompose cellulose-based waste after adaptation. While both aerobic and anaerobic bacteria can efficiently decompose organic pollutants in concentrated wastewater or sludge, aerobic methods often exhibit faster degradation rates. Aerobic treatment is preferred for the wastewaters with low pollutant concentrations due to its greater stability, despite being more expensive and generating more sludge. To achieve complete mineralization of sludge, aerobic treatment with low loading or extended retention times is necessary to promote the breakdown of all organic reserves. Notably, the breakdown of complex organic molecules like biopolymers can be facilitated by various microorganisms, including strict anaerobes, facultative anaerobes, and aerobes. Further, the antagonistic activity of two selected isolates TI 48-6 (*B. licheniformis*) and TI05Ps (*B. amyloliquefaciens*), which demonstrated the most effective enzymatic activity in relation to organic substrates was studied. These isolates, i.e. TI05Ps and TI48-6, identified as *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, were analyzed for antagonistic activity to the collection test strains of RCM LLC and to the genus of bacteria Aeromonas, prevalent in this wastewater.

The isolated *Bacillus* species exhibited antagonism to a broad spectrum of *Aeromonas* species, which are toxic to many organisms, especially fish, and resistant to a number of antibiotics, such as penicillin [49], [50]. *B. amyloliquefaciens* (TI05Ps) and *B. licheniformis* (TI 48-6) hydrolyze organic matter to a greater extent than the pathogenic *Aeromonas* species [51], typical municipal wastewater bacteria, thereby becoming capable of enhancing biological treatment. Numerous studies have shown that *Bacillus* species produce inhibitory substances that affect bacterial pathogens in aquaculture systems [52]. It is known that *A. hydrophila* can infect most farmed fish, causing massive mortality and economic losses to the aquaculture industry [53]. Moreover, bacteriocins such as subtilin and barnase produced by *B. amyloliquefaciens* have antibacterial effects against pathogens [54]. The combination of antagonistic and enzymatic activity of bacteria can promote their biodegradation of organic matter by regulating competitive interactions, maintaining ecosystem stability and protecting the microbial community from negative impacts. The antagonistic activity of environmentally friendly bacteria can protect the environment from pollution and block the development of microorganisms that could produce toxic substances or interfere with the biodegradation process. In addition, according to the research by Wróbel et al. [55], the genus *Bacillus*

is capable of producing the enzymes that can disintegrate heavy metals [56] and other harmful compounds. This makes them useful in the field of water purification from various contaminants, including industrial and agrochemical waste. *Bacillus amyloliquefaciens* has the capability to degrade low density polyethylene (LDPE) [44]. Research and practical application of bacteria of the genus *Bacillus* in wastewater treatment systems continue to develop.

As a result, the selected bacteria *B. amyloliquefaciens* strain TI05Ps and *B. licheniformis* strain TI 48-6 provide promising opportunities for the development of efficient, environmentally friendly and sustainable methods of biological water treatment, contributing to the conservation of natural water resources. The research focused on the further application of these strains as bioproducts to intensify and improve the efficiency of municipal wastewater treatment from toxic organic pollutants. The identity of *B. amyloliquefaciens* and *B. licheniformis* as naturally non-pathogenic microorganisms and their safety for the environment allows the development of this study [57].

CONCLUSIONS

This study successfully isolated and identified 56 microbial cultures from municipal wastewater, confirming their identity with 100% homology to known strains through 16S rRNA gene sequencing. Among them, *Bacillus amyloliquefaciens* (TI05Ps) and *Bacillus licheniformis* (TI 48-6) emerged as promising candidates for wastewater treatment due to their demonstrated ability to degrade organic matter and their significant antagonistic activity against various pathogenic microorganisms. Further investigation into their enzymatic activity revealed broad effectiveness of TI05Ps against *Salmonella enteriditis*, *Enterococcus faecium*, and *Pseudomonas taiwanensis*, while TI48-6 showed specific activity against *Staphylococcus aureus*. Despite their potential, limitations were identified for using these strains in wastewater treatment. Both strains exhibited reduced growth activity at low temperatures, which could restrict their efficacy in colder climates. Additionally, optimal pH conditions were crucial, with TI48-6 performing best at pH 7–9 and TI05Ps at pH 6–7; significant deviations from these ranges may necessitate adjustments for optimal performance. Moreover, as aerobic bacteria, adequate oxygen availability is essential for their efficient function; inadequate aeration in wastewater treatment plants could diminish bacterial activity. Overall, *B. amyloliquefaciens* (TI05Ps) and *B. licheniformis* (TI 48-6) show promise for sustainable wastewater treatment, but further research is needed to elucidate their specific mechanisms of action and to optimize their application under varying environmental conditions. These findings underscore the potential of these isolated strains and advocate for continued exploration to harness their full potential in practical wastewater treatment settings.

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