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Removal Of Pharmaceuticals from Municipal Wastewater Using Malaysian *Ganoderma Lucidum* Fungal Strain

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ABSTRACT

Emerging contaminants are currently a serious issue primarily because conventional wastewater treatment plants are unable to eliminate them. Environmental pollution caused by various emerging pollutants (particularly pharmaceutical active compounds) in wastewater poses a significant threat to public health and ecological balance. In 21st century, bioremediation is regarded as the most environmental friendly and cost-effective treatment technology. In this study, we investigated the removal efficiency of 19 popular pharmaceutical active compounds (PhACs) from municipal wastewater using Malaysian Ganoderma lucidum fungal strain (G. lucidum). The initial and final concentrations of each compound were determined using liquid chromatography time of flight mass spectrometry (LC-TOF/MS), and the percentage of removal was calculated. In this study, experimental results revealed diverse removal efficiencies for the investigated PhACs in municipal wastewater. The PhACs Azithromycin, doxycycline, clindamycin, ciprofloxacin, sulfamethoxazole, loratadine, and citalopram, showed remarkable removal rates of 90% and above in municipal wastewater, indicating their potential for effective treatment. Conversely, trimethoprim, ketoprofen, diclofenac, venlafaxine, dexamethasone, atenolol, propranolol, losartan, valsartan, metoprolol, fluconazole, and carbamazepine, demonstrated varying removal efficiencies, suggesting the need for further optimization in wastewater treatment processes for these compounds. This study highlights the importance of understanding the biodegradation of different PhACs in municipal wastewater to develop efficient and targeted bioremediation treatment strategies using local fungal strain of G. lucidum. Overall, this study provides promising suggestions for the bioremediation of pharmaceuticals from wastewater.

Keywords: Municipal wastewater; emerging pollutants; pharmaceuticals; bioremediation; Ganoderma lucidum

INTRODUCTION

Environmental pollution caused by pharmaceutical active compounds (PhACs) in wastewater has become an urgent concern due to its potential adverse impacts on both public health and ecological balance (antibiotic resistance genes) (Hou et al. 2019; Sayed et al. 2024; Tahiri et al. 2023). The widespread use of pharmaceutical drugs in human and veterinary medicines, coupled with inadequate removal during conventional wastewater treatment processes, has led to the presence of these compounds in wastewater effluents and subsequently in receiving water bodies (final disposal) (Hanafiah et al. 2023; Hanafiah et al. 2022). Today's increased pharmaceutical drug intake has contributed to an increase in the frequency of occurrence and substantial concentration of pharmaceuticals in domestic wastewater (Hanafiah et al. 2022). Several categories of PhACs, including antibiotics, antivirals, analgesics, antidepressants, cardiovascular and hormones, can persist in the environment and potentially disrupt aquatic ecosystems and enter the food chain, raising significant health and ecological concerns (Bankole et al. 2023; Hanafiah et al. 2023; Kumar et al. 2022). Superbugs (horizontal genes transfer) can be induced even by low concentrations of locally detected PhACs (Hanafiah et al. 2023; Hou et al. 2019; Lu et al. 2020). As such, some prior studies have been published, however the complete removal of these PhACs cannot be accomplished with a single approach (Bhuyan and Ahmaruzzaman 2023; Ghosh et al. 2023; Nasrollahi et al. 2022).

To address this problem, there is a growing interest in exploring sustainable municipal wastewater treatment approaches that can completely remove PhACs before final disposal (Bhuyan and Ahmaruzzaman 2023; Ghosh et al. 2023). Among these approaches, bioremediation using fungi has garnered attention due to its potential to harness the natural metabolic capabilities to biodegrade and remove various xenobiotic and biorecalcitrant compounds from wastewaters (Akerman-Sanchez and Rojas-Jimenez, 2021; de Wilt et al. 2018; Ghosh et al. 2023). Several fungal species have shown promising results in removal of PhACs, making them a potential technology for wastewater treatment applications (Brigita Dalecka, Caroline Oskarsson, 2020; Kang et al. 2023; Naghdi et al. 2018). Fungi have been reported to use mycoremediation technology to remove pollutants from wastewater (Ghosh et al. 2023; Mooralitharan et al. 2023). Further research is needed to understand the mechanisms behind these removal processes, optimize the conditions, and assess the potential environmental impacts of these removal techniques (Liu et al. 2023; Mir-Tutusaus et al. 2018).

In this context, the present study focuses on investigating the removal efficiency of 19 commonly used PhACs from municipal wastewater using the Malaysian *G. lucidum* fungal strain. The fungal strain *G. lucidum*, commonly known as Lingzhi or Reishi mushroom, is recognized for its bioactive compounds and enzymatic capabilities that enable it to remove a range of biorecalcitrant and xenobiotic pollutants. However, the removal efficiency of investigated PhACs using *G. lucidum* optimal inoculum dose is yet to be evaluated and reported. By assessing the removal efficiencies of various PhACs using *G. lucidum*, this study aims to provide insights into the potential of fungal-based bioremediation for tackling emerging pharmaceutical pollution in municipal wastewater. These findings can contribute to the design and optimization of municipal wastewater treatment processes to mitigate environmental pollution and protect human and ecological health. Further research is necessary to explore the mechanisms behind the observed removal efficiencies and develop innovative and sustainable solutions for wastewater treatment.

METHODOLOGY

SAMPLING OF WASTEWATER

The influent oMalaysian urban domestic wastewaterrom a local sewage treatment plant (STP) was studied with a population equivalent (PE) of 60000. The wastewater was collected using the ISCO 3700 autosampler. Correspondingly, the sample bottles were initially preserved with 0.3 mL of 50% sulphuric acid (H_2SO_4) and were immediately sent to the laboratory after sampling. The samples were kept in a refrigerator (at 4 °C) until the analysis.

CHEMICALS AND REAGENTS

All chemicals and reagents used in the experiments were of analytical grade quality. Accordingly, individual stock standard solutions were made by dissolving a suitable amount of each PhAC powder separately in methanol (MeOH, HPLC grade, Fisher Scientific, Korea) to a final concentration of 1000 mg/L. The working standards were made by diluting the stock standard. Similarly, acetone and acetonitrile (HPLC grade from Merck, Germany); hydrochloric acid (HCl, Fisher Scientific, Malaysia); ormic acid (FA, analytical reagent grade Fisher Scientific, UK); and ultrapure water (UPW) were generated from the Smart N system (Heal Force, China). The Oasis HLB cartridge (6 mL, 200 mg) from (Waters, USA) was used for solid phase extraction (SPE).

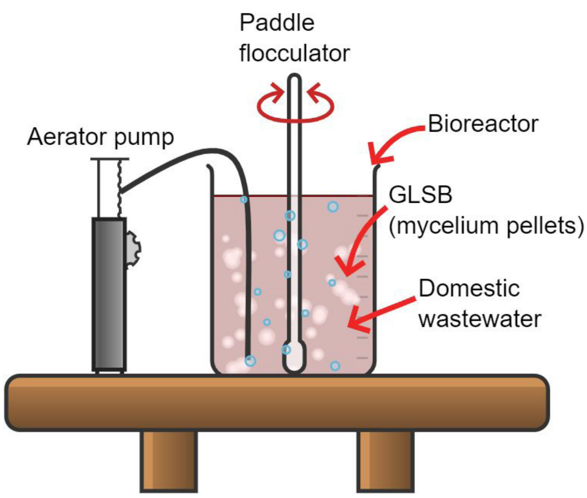

FIGURE 1. Bioreactor experimental setup

Popular and commonly determined PhACs were chosen as the study's target compounds, because they are requently detected in the influent and effluent o municipal wastewater treatment plants (WWTPs) in several countries. The 19 PhACs (Azithromycin, Doxycycline, Clindamycin, Ciprofloxacin, Sulfamethoxazole, Trimethoprim, Fluconazole, Venlafaxine, Citalopram, Atenolol, Propranolol, Dexamethasone, Losartan, Ketoprofen, Valsartan, Diclofenac, Metoprolol, Loratadine, Carbamazepine) investigated were supplied by Sigma (USA), Sigma-Aldrich (Germany), Alfa Aesar (Thermo-Fisher) and Merck (Germany). All stock solutions of these PhACs and external standards were prepared in methanol and kept in a refrigerator at -20 °C.

INOCULUM

The chosen Malaysian *Ganoderma lucidum* strain QR5120 was acquired from the University Malaya, (Kuala Lumpur, Malaysia). To get the mycelium, a tissue culture of *G. lucidum* was conducted according to (Z. M. Hanafiah et al. 2022; Mooralitharan et al. 2023) and culture preservation in a potato dextrose agar (PDA, Sigma-Aldrich, Dorset, UK) slant was performed, incubated at 28 °C for seven days, and stored at 4 °C. The *G. lucidum* mycelium pellets were prepared in accordance with (Abdullah et al. 2022; Supramani et al. 2023, 2019), which included a two-stage seed culture in growth media.

BIOREACTOR EXPERIMENTS

As shown in Figure 1, 1200-mL reactors in duplicate were filled with 1000 mL o municipal wastewater. A 100-µg/L concentration of each PhAC compound was maintained in reactors. The pre-grown *G. lucidum* pellets of volume 1% were inoculated into reactor (filled with domestic wastewater) at room temperature (about 25 °C) using a sterile pipette. To avoid fungal cementation at the reactor wall, the reactor was constantly fed with 3 L/min of air, generating an aerobic state and homogenization (Supramani et al. 2023). At the same time, the reactor was stirred at 100 rpm using a pad flocculator (Figure 1). The treatment period was set to 48 hours.

LIQUID CHROMATOGRAPHY SAMPLE PREPARATION

A 200 mL sample was obtained from each reactor and the SPE method was followed. In the reactors, SPE separated all investigated PhAC compounds from the wastewater sampled from reactors. As previously done by (Hanafiah et al. 2022), the sorbent medium used was Oasis HLB cartridges (6 mL, 200 mg). Initially, the cartridge was conditioned at a 1 mL/min flow rate with 3 mL o acetone, 3 mL of MeOH, and 3 mL of UPW (adjusted to a pH of 3.0). The cartridge was then loaded with a 200 mL filtered wastewater sample at a flow rate of 10 mL/min. To eliminate the interfering chemicals, the cartridge was thoroughly washed twice with 5 mL UPW-MeOH (95:5, v/v), followed by vacuum drying for about 20 minutes. The collecting glass tube was then placed in the SPE apparatus, and the residual investigated PhAC compounds were eluted with 2 x 3 mL of MeOH-30% FA (50:50, v/v). The extracted samples were subsequently dried under a mild stream of pure nitrogen before being reconstituted in 1 mL of UPW-acetonitrile (90:10, v/v). After elution the samples were stored at -20 °C, they were placed into a 1.5 mL amber septic vials.

PHARMACEUTICALS ANALYSIS

The investigated PhACs were determined and quantified using liquid chromatography time of flight mass spectrometry (LC-TOF/MS). Analytes were separated using a Thermo Scientific C18 column (AcclaimTM Polar Advantage II, 3 x 150 mm i.d., particle size 3.5 µm) on a Dionex Ultimate 3000 UHPLC system. Gradient elution was carried out at a flow rate o 0.4 mL/min at a column temperature of 40 °C using mobile phases A (H_2O and 0.1% FA) and B (100% MeOH) for a total run time of 22 minutes. The mobile phase gradient began with 5% B for 3 minutes, then increased to 80% B for 7 minutes before returning to 5% B with an equilibrium duration of 12 minutes. The sample injection volume was 3 µL. Using high-resolution mass spectrometry of MicroTOF QIII Bruker Daltonic, a mass spectrometer was employed in positive electrospray ionization mode. The positive ion modes were tested at 300 °C gas temperature, 8 L/min gas flow rate, 4500V capillary voltage, and 2.0 bar nebuliser pressure. The mass ranged from 50 to 1000 m/z. Compass Data Analysis software (Bruker Daltonics GmbH) was used to process the accurate mass data of the molecular ions provided by the TOF analyzer. To calculate the concentrations of the PhACs in the wastewater, a 5-point calibration (graph) of each PhAC was prepared.

RESULTS AND DISCUSSION

PHARMACEUTICAL ACTIVE COMPOUNDS REMOVAL

The characteristics of the wastewater is published elsewhere (Hanafiah et al. 2021, 2019; Mooralitharan et al. 2023). The PhACs are categorized into different classes:

antibiotics, analgesics and NSAIDs, antidepressants, cardiovascular drugs, and others. The findings of the current bioreactor experiment provide valuable insights into the potential of *G. lucidum* fungal species for the removal of PhACs from domestic wastewater. The observed removal rates of various PhACs align with previous research in the field. Figure 2 shows the categories of PhACs and their respective removal efficiency.

Interestingly, antibiotics (azithromycin, doxycycline, clindamycin, ciprofloxacin) showed 100% removal, indicating the robust effectiveness of *G. lucidum* in biodegrading them. For instance, *G. lucidum* appears to lead in breaking down these compounds commonly used in infection treatment. The complete removal (100%) of antibiotics such as azithromycin, doxycycline, clindamycin, and ciprofloxacin by *G. lucidum* is consistent with the findings of Álamo et al. (2021), they reported similar efficient biodegradation of some of these antibiotics using fungal-based bioremediation methods. This highlights the broad-spectrum antibiotic-degrading capability of *G. lucidum*, which can be attributed to its enzymatic pathways (Akerman-Sanchez and Rojas-Jimenez, 2021; Kang et al. 2019; Manasfi et al. 2020). Accordingly, sulfamethoxazole is a recalcitrant compound with lower degradation rates in prior studies (Alharbi et al. 2019; Martínez-Costa et al. 2020). In fact, sulfamethoxazole with a 95% removal rate, *G. lucidum* demonstrated significant success in breaking down this antibiotic, suggesting its potential in addressing antibiotic pollution in wastewater. In contrast, trimethoprim achieved 51.66% removal rate suggests that *G. lucidum* might have been moderate efficient in breaking down trimethoprim, highlighting a potential area for improvement. The limited removal rates of compound like trimethoprim are in line with the results observed in previous studies (Alharbi et al. 2019; Martínez-Costa et al. 2020; Ooi et al. 2018). Similarly, fluconazole (antifungal) had 33.48% removal rate suggests that *G. lucidum* had low success in biodegrading fluconazole. The recalcitrant nature of such compounds to fungal biodegradation has been attributed to their complex molecular structures and biorecalcitrant (Mir-Tutusaus et al. 2018).

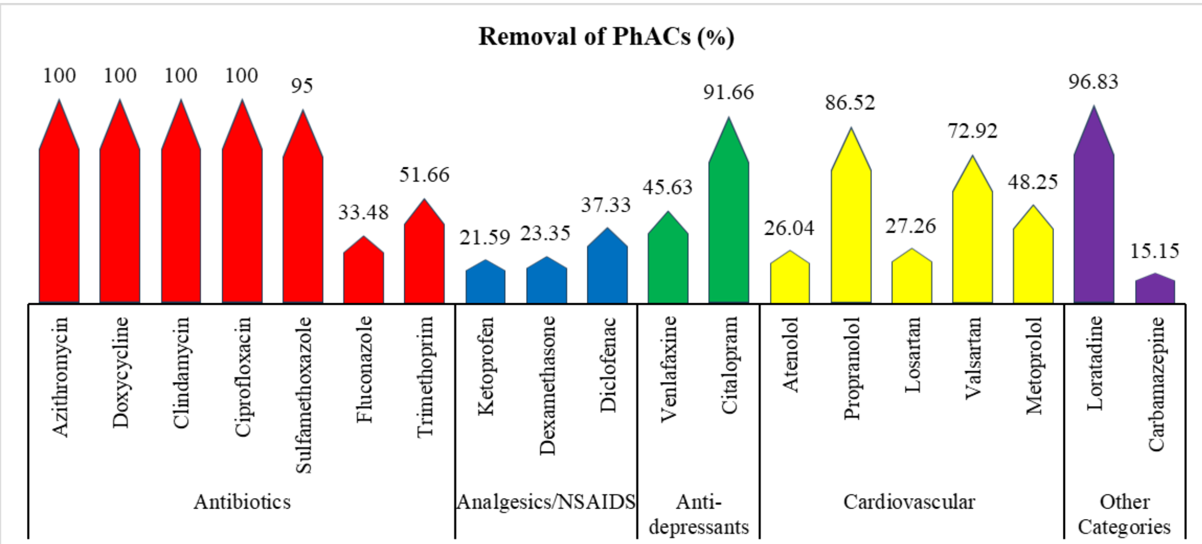

FIGURE 2. Removal efficiencies of various PhACs using GLSB bioreactor

Comparatively, diclofenac (a nonsteroidal anti-inflammatory drug) had removal rate of 37.33% suggests that *G. lucidum* had low success in breaking down diclofenac. Likewise, ketoprofen attained a removal rate of 21.59%, *G. lucidum* appears to have low success in breaking down ketoprofen, suggesting some challenges in the biodegradation of analgesics. The low removal of ketoprofen are in agreement with Cruz-Morató et al. (2014) and Arca-Ramos et al. (2016). In contrast, diclofenac removal was more than 80% in the same study by Cruz-Morató et al. (2014). Similarly, Sánchez et al. (2022) observed more than 99.5% of diclofenac and ketoprofen removal using 3-step hybrid treatment. Further, dexamethasone often used as an anti-inflammatory attained removal rate of 23.35% suggests that *G. lucidum* might not be highly effective in biodegrading dexamethasone.

Following this, a known recalcitrant antidepressant drug citalopram achieved a high removal rate of 91.66%, exhibiting *G. lucidum* potential in breaking down antidepressant compounds. However, commonly used venlafaxine (as antidepressant) had removal rate of 45.63%, suggests moderate success in biodegrading venlafaxine. The removal rates observed for certain antidepressants, such as venlafaxine and citalopram, correspond well with the results of Cruz-Morató et al. (2014), authors demonstrated comparable biodegradation efficiencies for these compounds in a different fungal-based bioreactor setup. These results collectively underscore the potential of fungal species in tackling the presence of antidepressants in wastewater.

In addition to, cardiovascular drugs, propranolol with an 86.52% removal rate, *G. lucidum* exhibited good efficacy in breaking down propranolol. Similarly, valsartan (used to treat hypertension and heart failure) attained a substantial 72.92% removal rate. The removal of valsartan is with agreement with prior study by Auvinen et al. (2017), wherein authors employed a sub-surface flow constructed wetland for wastewater treatment and achieved complete removal. Whereas metoprolol (a beta-blocker used for heart conditions) had a removal rate of 48.25%, *G. lucidum* showed moderate success in breaking down metoprolol. Subsequently, moderate removal of metoprolol was observed by de Wilt et al. (2018), using 3-step bio-ozone-bio treatment. Nevertheless, atenolol achieved a relatively low removal rate of 26.04% suggests that *G. lucidum* might face challenges in breaking down atenolol (a common cardiovascular). Furthermore, the varying removal rates of cardiovascular medications, such as propranolol and atenolol, are in agreement with studies by Cruz-Morató et al. (2013; 2014), they found that fungal-mediated biodegradation rates for such compounds can be influenced by factors such as fungal species and environmental conditions. Although losartan (commonly used for hypertension) with a removal rate of 27.26%, *G. lucidum* again indicated low success in biodegrading losartan. The low removal of losartan aligns with findings by Lucas et al. (2016), highlighting the optimization of fungal species in breaking down angiotensin II receptor antagonists (Siegl et al. 1995).

Other categories, loratadine (antihistamine) shows up a high removal rate of 96.83%, signifying G. lucidum effectiveness in biodegrading this type of antihistamine. Similarly, carbamazepine (anticonvulsant and mood-stabilizing) attained a low removal rate of 15.15% suggests that *G. lucidum* might struggle with carbamazepine biodegradation. Similar observations were reported in prior studies (Ba et al. 2014; Naghdi et al. 2017). In contrast, researchers Alharbi et al. (2019) observed higher removal of carbamazepine in prior studies.

The pharmaceutical category-based analysis reveals that *G. lucidum* is highly effective in breaking down antibiotics like azithromycin, doxycycline, clindamycin, and ciprofloxacin. It also demonstrates potential in biodegrading some cardiovascular medications, notably propranolol and metoprolol. While *G. lucidum* shows promise in breaking down certain analgesics and antidepressants, it faces challenges in biodegrading compounds like trimethoprim, atenolol, and dexamethasone. These findings suggest that the effectiveness o *G. lucidum* varies across different categories o PhACs due to varying chemical structures and properties.

GANODERMA LUCIDUM VS OTHER FUNGI

The *G. lucidum* bioreactor treatment investigated for PhACs removal was effective in removing various classes of PhACs. Specially, *G. lucidum* demonstrated high removal efficiency (100%) or a variety o antibiotics such as azithromycin, doxycycline, clindamycin, and ciprofloxacin. Although, the removal efficiency for analgesics and NSAIDs varied. While loratadine showed higher removal (above 90%), ketoprofen and diclofenac showed lower removal efficiency. Similarly, *G. lucidum* exhibited high removal efficiency or antidepressants, with citalopram being the most effectively removed (91.66%). In addition, *G. lucidum* showed varying removal efficiency for cardiovascular drugs. For instance, propranolol and valsartan demonstrated relatively higher removal rates compared to atenolol, losartan, and metoprolol. Further, the removal efficiency or other PhACs, such as fluconazole and carbamazepine, was relatively lower.

Several fungi species like *Trametes versicolor, Isochrysis galbana, Aspergillus oryzae, Echinodontium taxodii, Pleurotus florid, Myceliophthora thermophila etc* have been reported for removal of PhACs in prior studies (Table 1). As reported in literature, *Trametes versicolor* fungal species demonstrated a wide range of capabilities in removing various PhACs from different types of wastewaters. It was particularly effective in removing antibiotics such as ciprofloxacin, sulamethoxazole, and trimethoprim from synthetic and hospital wastewater samples (Table 1). Accordingly, it also showed effectiveness in removing cardiovascular drugs like atenolol and propranolol from synthetic and urban wastewater samples (Table 1). Comparatively, Table 1 focuses on the effectiveness of different fungal species in removing PhACs from various types of wastewaters.

Similarly, *Isochrysis galbana* and *Aspergillus oryzae* ungal species showed substantial removal efficiency or specific removal o PhACs. Particularly, *Isochrysis galbana* was effective in removing azithromycin and ofloxacin rom hospital wastewater (Table 1), while *Aspergillus oryzae* demonstrated significant removal o sulamethoxazole and diclofenac from spiked water (Table 1). Interestingly, *Echinodontium taxodii* species showed complete removal

TABLE 1. Comparison of PhACs removal efficiencies (%) with available literature

Category/Type	Fungi(samples) PhACs	Present study (Urban Wastewater)	Trametes versicolor (Synthetic Wastewater)	Trametes versicolor (Hospital Wastewater)	Trametes versicolor (Urban Wastewater)	Trametes versicolor (Wastewater)	Isochrysis galbana (Hospital Wastewater)	Aspergillus oryzae (Spiked Water)	Echinodontium taxodii (Spiked Water)
			(Tormo-Budowski et al. 2021)	(Cruz-Morató et al. 2014)	(Cruz-Morató et al. 2013)	(Kumar && Cabana 2016)	(Álamo et al. 2021)	(Nguyen et al. 2016)	(Shi et al. 2014)
Antibiotics	Azithromycin	100	41.3	26.00	100	-	88.00	-	-
	Doxycycline	100	-	-	-	-	-	-	-
	Ciprofloxacin	100	97.4	99.00	35.00	-	-	-	-
	Sulfamethoxazole	95	95.8	100	-	-	-	59.00	100
	Fluconazole	33.48	-	-	-	-	-	-	-
	Trimethoprim	51.66	98.2	-	-	60	-	-	-
	Clindamycin	100	-	-	-	-	-	-	-
Analgesics and NSAIDs	Ketoprofen	21.59	99.4	95.00	100	48	-	-	-
	Dexamethasone	23.35	-	-	-	-	-	-	-
	Diclofenac	37.33	-	100	-	95	-	60.00	-
Antidepressants	Venlafaxine	45.63	-	-	-	-	-	-	-
	Citalopram	91.66	-	-	-	-	-	-	-
Cardiovascular	Atenolol	26.04	60.9	-	-	90	-	-	-
	Propranolol	86.52	-	-	100	-	-	-	-
	Losartan	27.26	-	-	-	-	-	-	-
	Valsartan	72.92	-	-	-	-	-	-	-
	Metoprolol	48.25	-	-	-	-	-	-	-
Others	Loratadine	96.83	-	-	-	-	-	-	-
	Carbamazepine	15.15	-	0.00	-	-	-	40.00	-

of specific sulonamide group antibioticsrom spiked water samples (Table 1).

The comparison of G. lucidum with several other prior studies shows that the fungal species Trametes versicolor appears to be highly versatile in removing a wide range of PhACs rom various wastewater sources. Its efficiency varies significantly depending on the specific PhAC and the type of wastewater. The G. lucidum, on the other hand, shows remarkable removal efficiency or many PhACs across different classes. It demonstrates a high removal efficiency or antibiotics and reasonable efficiency or other categories, although the effectiveness varies. The comparison among the fungal species contributes valuable insights into addressing the challenges of PhACs pollution in wastewater and provides potential strategies for improving water quality. In comparison with the available literature the removal efficiencies of some PhACs investigated in this study are in agreement with prior studies (Table 1), Indeed the removal efficiencies or each PhAC can vary significantly depending on the ungal species, type of wastewater, and experimental conditions (Akerman-Sanchez and Rojas-Jimenez, 2021; Ghosh et al. 2023). In fact, the fungal bioreactor treatments show potential solutions for PhACs removal from municipal wastewater. Certainly, fungal species provide a biological means of degradation using various enzymes and adsorption (Kang et al. 2023).

CONCLUSION

Present study sheds light on the significant potential of fungal-based bioremediation, specifically employing *G. lucidum*, as a viable strategy for mitigating emerging pharmaceutical pollution in municipal wastewater. The experimental results highlight a diverse range of removal efficiencies or different PhACs, with some exhibiting impressive rates of removal, indicating their suitability for effective bioremediation treatment. The results highlight its effectiveness in biodegrading antibiotics, cardiovascular, analgesics and antidepressants compounds. Notably, azithromycin, doxycycline, sulfamethoxazole, clindamycin, ciprofloxacin, and loratadine demonstrated substantial removal rates of 90% and above in the municipal wastewater sample, emphasizing their removal by *G. lucidum* for efficient wastewater treatment.

However, the varying removal efficiencies observed or other compounds, such as trimethoprim, fluconazole, and others, emphasize the complexity of PhACs biodegradation in wastewater and suggest the need for further optimization and investigation. This investigation could guide further research to optimize *G. lucidum* performance in biodegrading compounds that show lower removal rates. Hence underscores the necessity of continued research to elucidate the mechanisms underlying removal variability and to develop innovative, sustainable, and targeted solutions for municipal wastewater treatment.

By categorizing PhACs, this study contributes to a more targeted approach to wastewater treatment and emphasizes the need for tailored strategies in PhACs bioremediation. The findings othis study contribute to the broader understanding of potential of fungal-based bioremediation strategies and provide valuable insights for design and optimization of wastewater treatment processes to combat pharmaceutical pollution. By advancing our knowledge of PhACs biodegradation, we move closer to safeguarding environmental integrity, human health, and ecological equilibrium. Further research attempts are required to solve the intricate pathways of PhACs biodegradation and to pave the way for comprehensive and effective solutions or the mitigation o pharmaceutical pollution in wastewater.

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DECLARATION OF COMPETING INTEREST

None

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