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Studies on the treatment of anaerobically digested sludge by white-rot fungi: evaluation of the effect of *Phanerochaete chrysosporium* and *Trametes versicolor*

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Abstract

Background The composition of anaerobically digested sludge is inherently complex, enriched with structurally complex organic compounds and nitrogenous constituents, which are refractory to biodegradation. These characteristics limit the subsequent rational utilization of resources from anaerobically digested sludge. White-rot fungi (WRF) have garnered significant research interest due to their exceptional capacity to degrade complex and recalcitrant organic pollutants. However, the exploration of WRF in the context of sludge treatment remains an under-investigated area within the scientific community. The present investigation explores the application of WRF in the treatment of anaerobically digested sludge, offering a novel approach for the valorization of sludge resources.

Results In this study, WRF enzymes, manganese peroxidase (MnP) and lignin peroxidase (LiP), exhibited sustained high activities of approximately 102 U/L and 26 U/L, respectively, within the anaerobically digested sludge under a controlled pH of 5.5 within the growth system. These conditions were found to significantly enhance the treatment efficacy of the anaerobic sludge. The removal of soluble chemical oxygen demand (COD) and Total COD by *Trametes versicolor* powder was better than that of *Phanerochaete chrysosporium* powder. The treatment of sludge samples with WRF, specifically *Phanerochaete chrysosporium* powder, resulted in a significant reduction of ultraviolet radiation (UV_{254}). Fourier-transform infrared spectroscopy (FTIR) analysis revealed that the application of *Trametes versicolor* powder exerted a notably pronounced impact on the functional groups present in sludge samples. Specifically, there was a significant decrease in the peak intensities corresponding to the C-O bonds, indicative of saccharide degradation, alongside an observable increase in the intensities of amide peaks, which is suggestive of protein synthesis enhancement. Microbial community analysis demonstrated that *Phanerochaete chrysosporium* was the predominant fungal species, exerting a significant regulatory role within the sludge ecosystem.

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Conclusion In conclusion, this research furnishes a robust scientific foundation for the utilization of WRF in the treatment of anaerobic digestion sludge. It elucidates the fungi's capacity to ameliorate the physicochemical attributes and microbial community composition within the sludge. Furthermore, the study offers a certain reference for the subsequent use of WRF in the treatment of other types of sludge.

Keywords White-rot fungi, Anaerobically digested sludge, Enzyme activity, Organics

Introduction

The number of wastewater treatment plants around the world has proliferated since 2010, increasing in size as a result of growing populations and rapid urban development [14]. Sludge is an inevitable by-product of the wastewater treatment process and its production is increasing day by day [25]. Due to the diversity of wastewater treatment processes, different sludge is produced, including aerobic sludge and anaerobic sludge, collectively known as residual sludge or sludge [10]. Sludge is of complex composition. During the sewage treatment process, part of the pollutants were degraded due to the metabolism of microorganisms, and about 30–60% of the contaminants were transferred to the sludge through adsorption, flocculation, and other different forms [33]. If sludge is disposed of without proper treatment, there is a risk of secondary pollution to the ecological environment [35]. Therefore, the scientific and reasonable disposal of sludge can no longer be ignored, and the sludge problem has become a significant challenge for us.

In conjunction with the increasingly stringent regulations on sludge treatment and disposal in China, enhancing sludge treatment technologies is conducive to the resource recovery and reduction of sludge. The pursuit of more efficient, economical, and eco-friendly sludge treatment methods is becoming increasingly important [37]. Among the sludge treatment technologies, anaerobic digestion has been widely studied for its various advantages, such as lower environmental impact, lower cost, better digestion and resource reuse [23]. Anaerobic digestion of sludge is the most commonly used sludge treatment method in large wastewater treatment plants, and the amount of anaerobically digested sludge is increasing day by day [30]. The treatment and disposal of anaerobically digested sludge are highly challenging due to its high water content, high refractory organic content, high ammonia and nitrogen, associated odours and pathogen content, as well as potential toxic metals and organic contaminants [13].

White-rot fungi(WRF) are widely reported for their high degradation capacity towards various substances that are difficult to biodegrade. These fungi are increasingly being applied to treat various refractory organic compounds in industrial wastewater, yet research on their application in sludge treatment remains relatively scarce [26]. WRF are known for their ability to decompose lignin, playing a crucial role in nature by breaking down lignocellulosic biomass, including lignin and cellulose biopolymers. They also possess the unique capability to effectively degrade lignin through the production of synergistic ligninolytic enzymes. WRF possess a unique oxidative and extracellular ligninolytic system that allows them to transform or degrade a variety of environmental pollutants, which has garnered increasing attention from researchers [6].

The enzymatic system of WRF is a crucial component of their biodegradation activities, comprising both extracellular and intracellular enzymes [19]. The extracellular enzyme system of WRF, which is crucial for their biodegradation activities, mainly includes laccase(Lac), lignin peroxidase(Lip) and manganese peroxidase(Mnp). The intracellular enzymes are primarily cytochrome P450. WRF may not secrete all types of enzymes simultaneously under different growth conditions, which is related to their cultivation parameters [21]. WRF and their enzyme systems have been extensively utilized for the removal of synthetic dyes, pharmaceutically active compounds (PhACs), endocrine disruptor compounds (EDCs), pesticides, polycyclic aromatic hydrocarbons(PAHs), and other environmental pollutants [4, 22].

Due to the high efficiency and broad-spectrum degradation capabilities of WRF (lignin, etc.), this study attempts to apply WRF in the treatment of anaerobically digested sludge [5, 15]. Aiming at the above problems after anaerobic digestion of sludge, the present work utilises the broad-spectrum and efficient degradation ability of WRF to treat anaerobically digested sludge, and investigates the effects of different WRF and different fungi morphology on the evolution of the organic matter, physical and chemical properties and microbial populations in both sterilised and non-sterilised anaerobically digested sludge. The aim was to explore a new way of biological treatment of anaerobically digested sludge and to provide a theoretical basis for the practical application of WRF in sludge treatment.

Previous investigations into the utilization of WRF for sludge treatment have predominantly focused on dewatered sludge as the substrate. In this study, we diverge from this approach by employing anaerobically digested sludge mixture as the medium, thereby facilitating comprehensive contact between the WRF and its enzymatic arsenal with the sludge matrix. This methodology is hypothesized to enhance the biodegradation of sludge constituents by WRF, potentially offering a more efficient

avenue for sludge treatment. The anaerobically digested sludge, predominantly hosting anaerobic fungi, was used in this aerobic experiment to minimize the influence of competing microbial growth on WRF, potentially enhancing their sludge degradation efficiency. In addition, this experiment verified the feasibility of WRF in treating anaerobically digested sludge by comparing the treatment effects of different fungi and different fungal dosing methods (fungal powder, fungal pellet and fungal immobilization) on anaerobically digested sludge. Although our study provides important insights, there are still some limitations. The poor treatment effect of WRF on actual anaerobically digested sludge is mainly related to the activity of the strain, and it is recommended that the subsequent research on the use of the strain in the actual anaerobically digested sludge environment for many times in the domestication of the strain, to get the strain that can grow stably in the actual anaerobically digested sludge, and the enzyme secretion ability of the strain is well.

In summary, the project utilized WRF for the treatment of anaerobically digested sludge, investigating the effects on key parameters such as chemical oxygen demand(COD), ultraviolet radiation (UV_{254}), and extracellular polymeric substances(EPS), and analyzing the changes in microbial populations within the actual anaerobically digested sludge following treatment with WRF. This study provides a novel approach to the biological treatment of anaerobic digestion sludge and verifies the feasibility of using WRF for anaerobically digested sludge treatment. The study not only elucidates the capacity of WRF to degrade recalcitrant organic matter in sludge but also offers novel insights into the resource recovery and environmental management of sludge.

Materials and methods

Media and reagents

Experimental strains: *Phanerochaete chrysosporium* (lyophilized powder) was purchased from Henan Provincial Industrial Microbial Strain Engineering and Technology Research Center Mall BeiNaChuangLian Biotechnology Co(BNCC); *Trametes versicolor* was purchased from Minyuan Institute of High-Tech Technology. Both strains were cultured in potato agar dextrose medium (PDA) and potato dextrose liquid-enriched medium (PDL) at an optimal temperature of 28 °C. The cultures were incubated in potato agar dextrose medium (PDA) and potato dextrose liquid-enriched medium (PDL).

Experimental sludge: The sludge used in this experiment was taken from anaerobically digested sludge of a sewage treatment plant in Jinshan District, Shanghai, and brought back to the laboratory after sampling and encapsulating in plastic bottles, the pH of the sludge was 8.31, and the other indexes were as shown in Table 1. The following sludge indicators were averaged over three measurements.

Methods of experimentation

Medium preparation

Preparation of potato extract: The fresh potatoes were peeled and cut into small pieces, and 200 g of potato pieces were weighed and put into a 2000 mL beaker. 1000 mL of pure water was added and boiled for 30 min to dissolve the nutrients in the potatoes completely, and then filtered through gauze to obtain the potato extract, which was then added with pure water to 1000 mL.

Preparation of PDL medium: In 1000 mL of potato extract, 20.0 g of glucose, 1.5 g of $MgSO_4$-$7H_2O$, 3.0 g of KH_2PO_4, and trace amount of vitamin B1 were added and stirred continuously with a clean glass rod until all the medicines were dissolved entirely, and then the extract was sealed with a high-temperature-resistant sterile gas-permeable sealing film and put into an autoclave for sterilizing (121 °C, 30 min). The above steps were carried out on the aseptic operating table.

Preparation of PDA medium: The 20 g agar was added and stirred well. Then the culture solution was divided into 15–20 mL per plate culture dish, and the rest of the operation was the same as the preparation of PDL culture medium mentioned above.

WRF culture

Preparation of fungi suspension: *Phanerochaete chrysosporium* was inoculated in solid medium and incubated at a constant temperature of 28 °C for 5–7 d. At that time the surface of the plate medium was covered with powdered white conidia of *Phanerochaete chrysosporium* (Fig. S1). The conidia was scraped with an inoculating ring and added to a test tube containing 10 mL of sterile water and shaken well. The mycelial fragments were filtered out by gauze to obtain the suspension of *Phanerochaete chrysosporium*.

Preparation of pellet solution: The PDL medium was divided into 250 mL conical flasks, 150 mL in each conical flask, then sealed with aseptic airtight sealing film and autoclaved (121 °C, 30 min), and then cooled in the aseptic table at the end of the sterilization, and then a certain

Table 1 Basic index of anaerobic digestion sludge

Moisture content(%)	TS (g/L)	VS (g/L)	Zn (mg/kg)	Cu (mg/kg)	*P* (mg/kg)	TCOD (mg/L)	SCOD (mg/L)
97.64	23.30	10.95	1282.58	513.14	31.40	21,240	720

amount of fungi suspension was inoculated into the PDL medium, and then the conical flasks were placed into the constant-temperature shaking Table (150 r/min) to incubate for 5–7 d to obtain the pellet solution of *Phanerochaete chrysosporium*.

Immobilization of WRF

WRF immobilization technology is quite widely used in the degradation of pollutants in water [31], and common immobilized materials include alginate, biochar, polyamide mesh, titanium dioxide nanoparticles, and wood chips [27, 32].In this experiment, polyurethane foam was chosen as the immobilization material for WRF.

The polyurethane foam was cut into small pieces and prepared with a quantity of PDA medium. The medium and the polyurethane foam pellets were sterilized, and after sterilization, both were transferred to a sterile bench for cooling. When the PDA medium was cooled down to about 45 °C, the polyurethane foam pellets were dipped into the PDA medium and then put into the sterilized Petri dishes for cooling, so that the surface of the polyurethane pellets was coated with a layer of PDA medium as shown in (Fig. S2a). Then inoculate the polyurethane spheres with *Phanerochaete chrysosporium* using an inoculating ring and let them grow in the plate medium for 5–7 d until the nutrients on the surface of the spheres were consumed. A layer of *Phanerochaete chrysosporium* mycelium was wrapped around the spheres, as shown in (Fig. S2b). (The cultivation methods for *Trametes versicolor* are consistent with those described previously.)

Measurement and analytical methods

Measurement of sludge biochemical indicators

Sludge solubility COD: The sludge sample to be measured was centrifuged and left to stand, the supernatant was taken and filtered through an aqueous filter with a pore size of 0.45 μm, and 2 mL of the filtrate was accurately added into a sample tube containing 3 mL of the COD preformulated dissolution solution, the cap of the tube was screwed tightly and the tube was inverted and oscillated, and then the tube was placed in the dissolution apparatus and dissolved at 150 °C for 120 min, and then the tube was taken out of the tube after the end of the dissolution, and the tube was cooled to room temperature away from light and the COD concentration was then determined. The Total COD was determined by adding 2 mL of well-mixed sludge into the digestion solution, and other operations were the same as those for the soluble COD. Each dataset was measured in triplicate and the results were averaged.

Extraction of EPS from sludge

EPS are some polymers secreted outside the body by microorganisms, under certain environmental conditions [18]. EPS in sludge can be categorized into soluble EPS (EPS-SB), loosely bound EPS (EPS-LB) and tightly bound EPS (EPS-TB)(Fernando et al., [11]. In this experiment, a modified thermal extraction method was used to extract different types of EPS [16].

Determination of polysaccharide and protein in sludge EPS

Determination of polysaccharide: Phenol-sulfuric acid spectrophotometric method [7]. We took 1 mL of the sample to be tested, added 6% phenol solution, and 2.5 mL of concentrated sulfuric acid, thoroughly shaking and mixing, and then measured the absorbance at 490 nm after standing for 30 min.

Determination of protein: Spectrophotometric method of Kaumas Blue G-250. We took 0.1 mL of the sample to be tested, added 5 mL of Kaomas Brilliant Blue G-250 standard solution, inverted several times to mix well, and stood for 2 min, then measured its absorbance.

Determination of enzyme activity of WRF

Enzyme activity refers to the amount of enzyme required to convert 1 μmol of substrate per minute under optimal conditions, with this amount being defined as one unit of enzyme activity (U). The following enzyme activity data were tested three times and averaged.

Mnp: Take 3.4 mL of sodium lactate buffer solution with a concentration of 50 mmol/L, and 0.1 mL of hydrogen peroxide solution with a concentration of 1.6 mmol/L to initiate the reaction, and measure the change in absorbance at 240 nm.

Lip: Take 2.2 mL of the test solution, add 0.1 mL of methylene blue solution at a concentration of 1.2 mmol/L, 0.6 mL of sodium tartrate buffer solution at a concentration of 0.5 mol/L, and finally add 0.1 mL of H_2O_2 solution at a concentration of 2.7 mmol/L to initiate the reaction. Measure the change in absorbance at 664 nm and calculate the amount of Lip.

Fourier-transform infrared spectroscopy analysis

The Fourier-transform infrared spectrometer(FTIR) we employed is the Nicolet iS10 model from ThermoFisher Scientific, USA. The spectral wavelength scanning range is from 4000 to 500 cm^{-1}, with a sampling rate of 80 spectra per second and a resolution of 4 cm^{-1}. In this study, FTIR was utilized to analyze the changes in organic functional groups within sludge. The sludge samples to be analyzed were subjected to solid-liquid separation, with both the supernatant and solid fractions placed into evaporation dishes, covered with plastic wrap, and frozen in a refrigerator for 24 h to ensure complete solidification of the samples. Thereafter, uniform holes were punched through the plastic wrap, and the samples were lyophilized in a freeze dryer for 16 h. Subsequently, a small amount of the freeze-dried sludge was taken out, mixed

with potassium bromide at a ratio of 1:100, and ground uniformly. The mixture was then pressed into pellets under a pressure of 2 tons for 3 min. Finally, the pellets were placed into the infrared spectrometer for infrared spectral analysis.

Analysis of microbial community structure

The microbial community structure of the sludge was analyzed using high-throughput sequencing, with 16 S rRNA gene sequencing for bacterial analysis and ITS sequencing for fungal analysis. First, total DNA was extracted using a DNA extraction kit, followed by agarose gel electrophoresis at 1% to assess the purity and concentration of the DNA. In the PCR amplification, the primers used for the 16 S rRNA gene were 338 F(5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3'), while for the ITS region, the primers used were ITS1F(5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R(5'-GCTGCGTTCTTCATCGATGC-3'). After PCR amplification, the amplified products were checked by electrophoresis on a 2% agarose gel to confirm successful amplification. The PCR products were the purified, and the purified 16 S and ITS gene fragments were sequenced. Finally, species identification and diversity analysis were conducted by comparing the sequences with known sequences in the database.

Microbial population analyses were done by Shanghai Meiji Biomedical Technology Co. Total microbial genomic DNA was extracted from sludge samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, United States) and kept at -80 ℃ prior to further use. Bioinformatic analysis of the sludge was carried out using the Majorbio Cloud platform (https://cloud.majorbio.com). Based on the ASVs information, rarefaction curves and alpha diversity indices including observed ASVs, Chao1 richness, Shannon index and Good's coverage were calculated with Mothur v1.30.1.The similarity among the microbial communities in different samples was determined by principal coordinate analysis (PCoA) based on Bray-curtis dissimilarity using Vegan v2.5-3 package. The PERMANOVA test was used to assess the percentage of variation explained by the treatment along with its statistical significance using Vegan v2.5-3 package. The linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/LEfSe) was performed to identify the significantly abundant taxa (phylum to genera) of bacteria among the different groups (LDA score > 2, $P < 0.05$). High-throughput sequencing data were analysed using Fastp software for quality control of double-ended raw sequencing sequences and FLASH software for splicing.

WRF treatment of sterilized anaerobically digested sludge pre-experimentation

In this experiment, the temperature was set to 28 °C, sterilized anaerobic sludge pH = 7.4, the pH gradient was established in the sterilized anaerobic digested sludge. The two fungi were allowed to grow in 200 mL of sterilized anaerobic digested sludge according to the approximate same inoculum amount (10 mL of fungi suspension). The incubation was carried out for 10 d. The changes in the soluble COD of the sludge were measured every day, and the related enzyme activities were measured on the 5th and 10th d.

Results and discussions

Enzymatic activities of fungi under different pH conditions

The enzyme activities were determined(experimental reality), and it was found that in sterilized anaerobically digested sludge, both enzymes remained the most active at pH = 5.5, whereas in sterilized anaerobically digested sludge with unadjusted pH, the activities of both enzymes were at the lowest level; Fig. S3 shows the enzyme activity measured at 5 d of reaction, and the enzyme activity at 10 d of reaction was not detected. The enzyme activities of the two bacteria were very low under alkaline-biased conditions, which may be due to the fact that alkaline conditions were not adapted to the growth and reproduction of the two strains, and the activity of the fungi was reduced, thus inhibiting the secretion of the relevant enzymes [28].

Removal of sludge soluble COD by fungi under different pH conditions

In the determination of soluble COD in the experimental sludge samples at different pH, it was found that the highest removal efficiency of soluble COD from sludge was achieved by *Phanerochaete chrysosporium*, at pH = 5.5, reaching about 43%(Fig. 1a&b). In addition, the soluble COD of each group of sludge samples stabilized from 8 d of the reaction. At 10 d of reaction, the soluble COD of each group of sludge samples was close to the soluble COD value at 8 d and no longer changed. The removal trend of soluble COD from sludge by *Trametes versicolor* was like that of *Phanerochaete chrysosporium*, with the highest removal of soluble COD at pH = 5.5, with a removal rate of 36% (Fig. 1c&d). In contrast to *Phanerochaete chrysosporium*, the soluble COD removal was lower in the sludge samples spiked with *Trametes versicolor*, which could be attributed to the fact that *Trametes versicolor* was less tolerant to alkaline conditions.

Figure 1 (a&b) Removal of the soluble COD from sludge of *Phanerochaete chrysosporium* under different

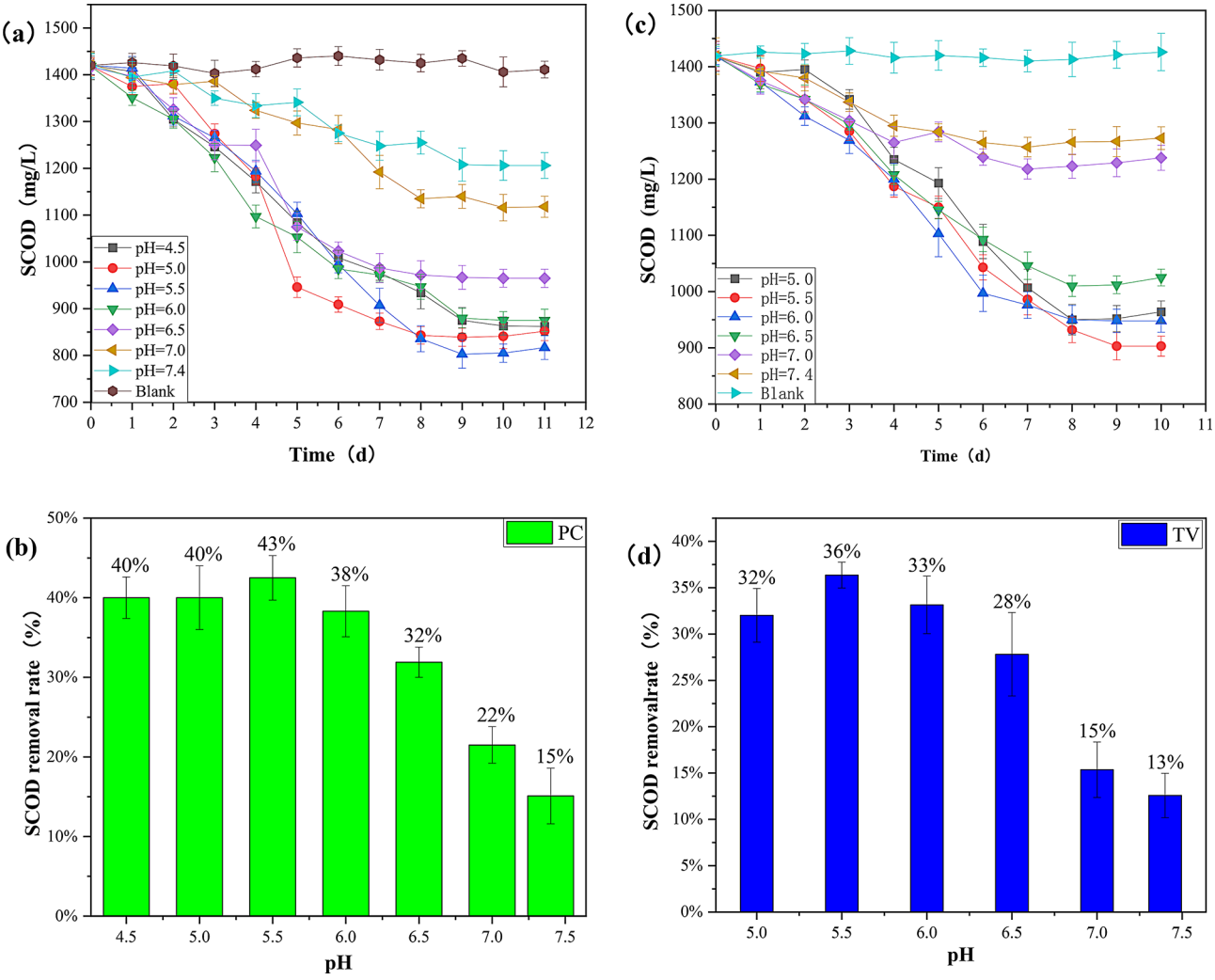

Fig. 1 Removal of the soluble COD from the sludge under different pH conditions (**a, b, c, d**)

pH conditions. (c&d) Removal of the soluble COD from sludge of *Trametes versicolor* under different pH conditions than *Phanerochaete chrysosporium*.

Effect of fungi inoculum on the soluble COD of sludge

As shown in Fig. 2, by increasing the dosage of the fungi strains, the removal of soluble COD from sludge by both *Phanerochaete chrysosporium* and *Trametes versicolor* was enhanced. *Trametes versicolor* was more effective in improving sludge soluble COD removal up to 67%, which was 1.8 times higher than that in the pre-test stage. Among the four dosages of *Phanerochaete chrysosporium*, the best removal of sludge soluble COD was achieved by *Phanerochaete chrysosporium* with a dosage of 1/4 of the medium area (the inoculum of *Phanerochaete chrysosporium* was estimated to be about 8×10^8 by plate counting), with a removal rate of 55%. In comparison, the removal rates of the other three dosages were 45% (1/16), 46% (1/8), and 50% (1/2) respectively.

The best removal of sludge soluble COD was achieved when the dosage of *Trametes versicolor* was 1/2 of the area of the medium (the inoculums of *Trametes versicolor* was estimated to be about 1×10^8 by the plate counting method), with a removal rate of 67%, while the removal rates of the other three dosages were 38% (1/16), 44% (1/8), and 58% (1/4) respectively. The best dosage of *Trametes versicolor* for soluble COD removal corresponded to 1/2 of the medium area, and the actual number of inoculums was 1×10^8, which was much less than the number of inoculums of *Phanerochaete chrysosporium*, which was related to the growth characteristics of the two fungi.

After varying the dosage of the two fungi, the removal of sludge soluble COD was improved. The removal of sludge soluble COD was enhanced by about 10% by *Phanerochaete chrysosporium* and by about 30% by *Trametes versicolor*. The removal of soluble COD was probably due to a combination of trapping by the mycelia of the fungi

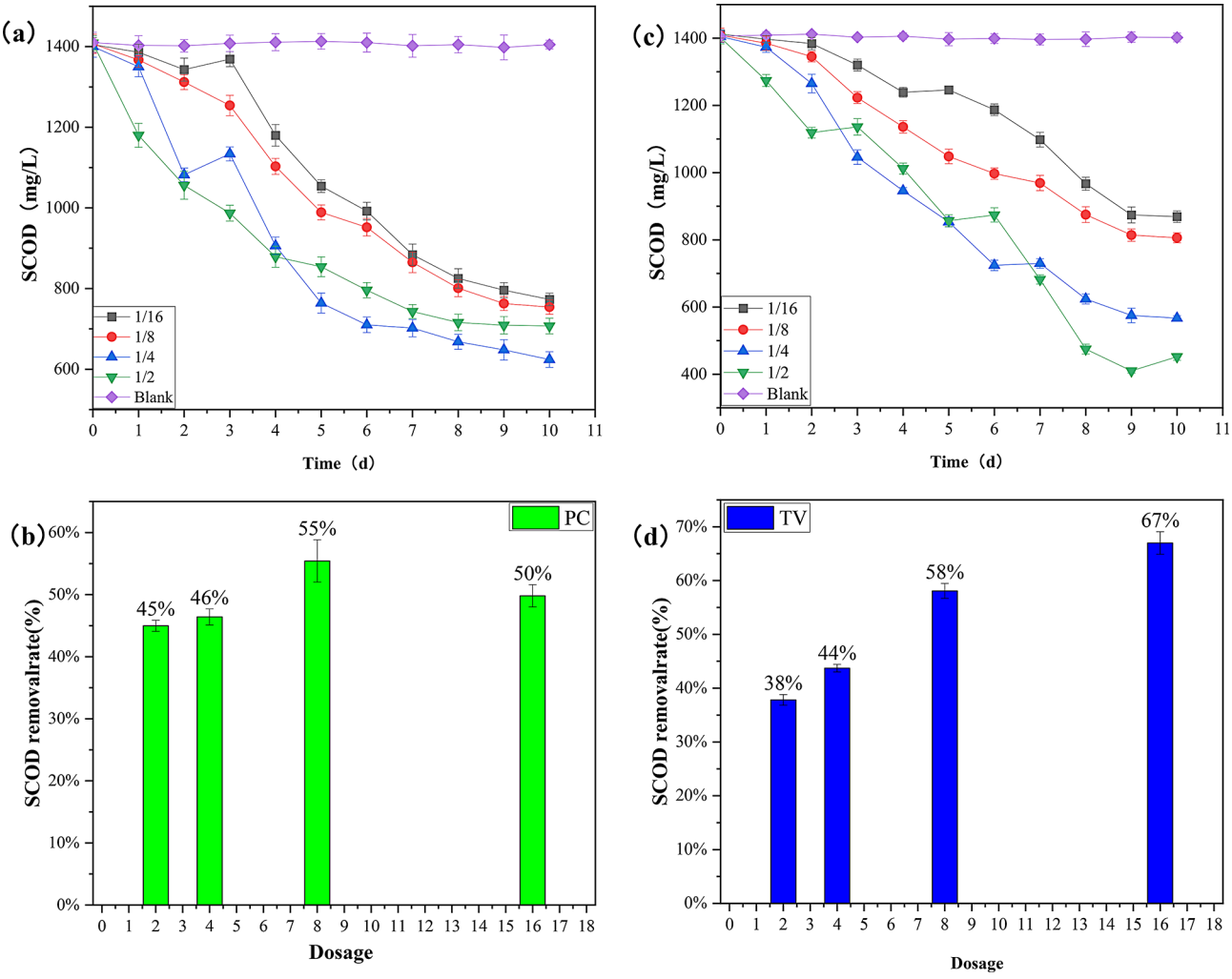

Fig. 2 (**a & b**) Removal of sludge soluble COD by different dosages of *Phanerochaete chrysosporium* (**c & d**).

and degradation by enzymes secreted by the white-rot fungi. This was a synergistic process in which substances available to the fungi were first captured by the mycelium through biosorption and physisorption, and then degraded by the fungi either through absorption and metabolism or through enzymes secreted by the fungi [3].

Variation of the sludge COD

Figure 3 shows the variation of soluble COD in each group of sludge samples during the treatment with WRF. In the sludge of the experimental group, the soluble COD basically maintained a gradually decreasing trend when *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder were added. Compared with the blank sludge soluble COD, the soluble COD was reduced by 44%, 52%, and 46% by *Phanerochaete chrysosporium* pellet, *Phanerochaete chrysosporium* powder, and *Phanerochaete chrysosporium* immobilization; The soluble COD was reduced by 51%, 71%, and 42% by *Trametes versicolor* pellet, *Trametes versicolor* powder, and *Trametes versicolor* immobilization.

The removal of soluble COD by *Trametes versicolor* was superior to that of *Phanerochaete chrysosporium* when compared to the two fungi strains. The highest soluble COD removal rate of 71% was observed in the sludge sample of *Trametes versicolor* powder. In contrast, the immobilized strains were not as effective in removing sludge soluble COD as the experimental group dosed with fungi powder.

In order to investigate whether the removal of soluble COD by pellets was adsorption or degradation, a subsequent pellet soaking experiment was carried out, in which pellets of treated sludge were fished out and put into sterile water for soaking. The COD content of the soaking solution was tested after seven days and was found to be less than 10 mg/L. It was indicated that the surface of the pellet adsorbed soluble COD, but the amount adsorbed was small, or the adsorption capacity

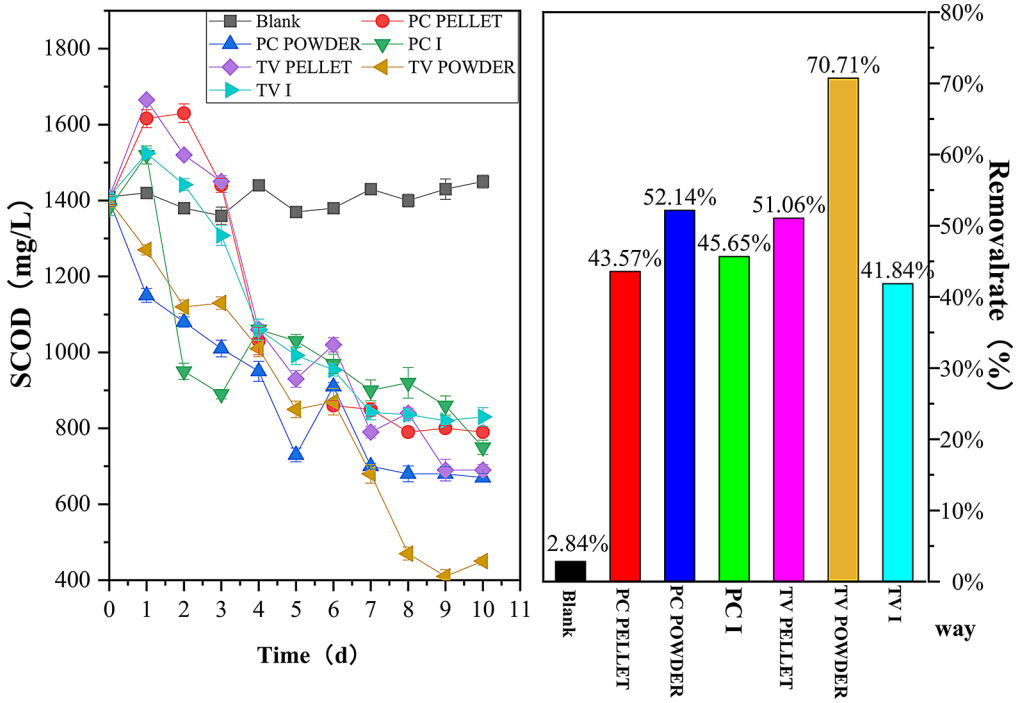

Fig. 3 Changes in the soluble COD of sludge samples from experimental groups

was strong. The amount desorbed in a short time was minimal.

The soluble COD represents only the change in dissolved COD in the sludge and represents only a tiny fraction of the sludge Total COD. In this experiment, the changes in the sludge Total COD were also determined before and after treatment with WRF, which was shown in Fig. S4. The effect of WRF on COD in sludge was measured by combining the changes in the two CODs. As seen in Fig. S4, there was a slight reduction in the sludge Total COD after the WRF treatment, and the best result was obtained from the experiment with the addition of *Trametes versicolor* powder.

The above changes in sludge COD indicate that the WRF treatment was able to remove a portion of the COD from the sludge. In terms of removal rate, the WRF removed most of the soluble COD from the sludge, but the removal efficiency for the Total COD was low [24, 40]. The removal of the soluble COD by the WRF was probably a process of adsorption followed by degradation, with the fungus first adsorbing the soluble COD in the sludge and then utilized by the growth and metabolic processes of the fungi. From the reduction of the Total COD and the soluble COD content, the decrease of the Total COD was significantly more significant than the reduction of the soluble COD. The degradation of the Total COD by the WRF treatment may be the direct degradation of a part of the insoluble material with the enzyme secreted by the fungi, such as EPS in sludge flocs, etc [2].

It was found that in the experimental sludge dosed with *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder, the fungi were mixed entirely in the sludge and could not be separated, which led to the inclusion of part of the fungal organisms in the samples taken during the measurement of the Total COD and made the value of sludge Total COD higher than the actual value. Combined with the removal of sludge soluble COD by *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder, it was speculated that the real-essential removal of sludge Total COD by the experimental groups of *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder might be better than the results shown by the experimental data. Due to the flocculent nature of the fungi powder after growth in the sludge, subsequent experiments should isolate as much of the fungi as possible from the sludge samples before measuring the actual change in the Total COD.

Change in the sludge UV_{254}

The UV_{254} reflects the amount of naturally occurring humic macromolecules and aromatic compounds with C = C and C = O double bonds in the water, and has been used as a surrogate parameter for TOC and DOC in some studies [1, 38]. Humic acid is a large molecule in anaerobically digested sludge, which is difficult to biodegrade and complicated to measure [41]. In this section of the experiment, the effect of WRF on humic acids and aromatic compounds in sludge was analyzed by UV_{254}. Figure 4 shows the variation of UV_{254} in each group of

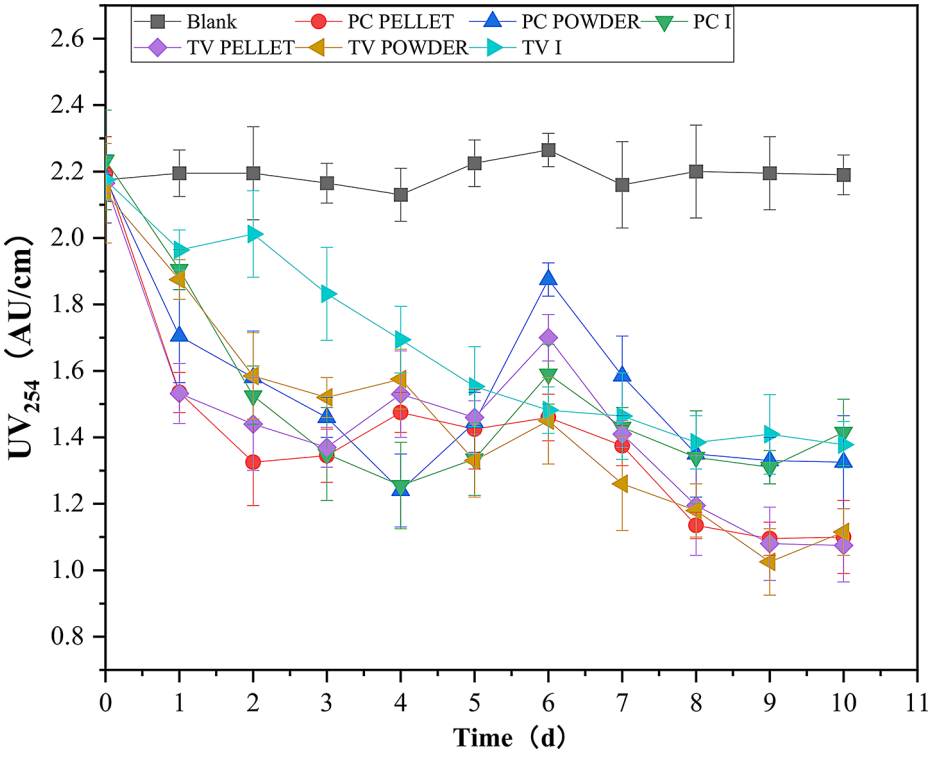

Fig. 4 UV_{254} changes in sludge samples from experimental groups

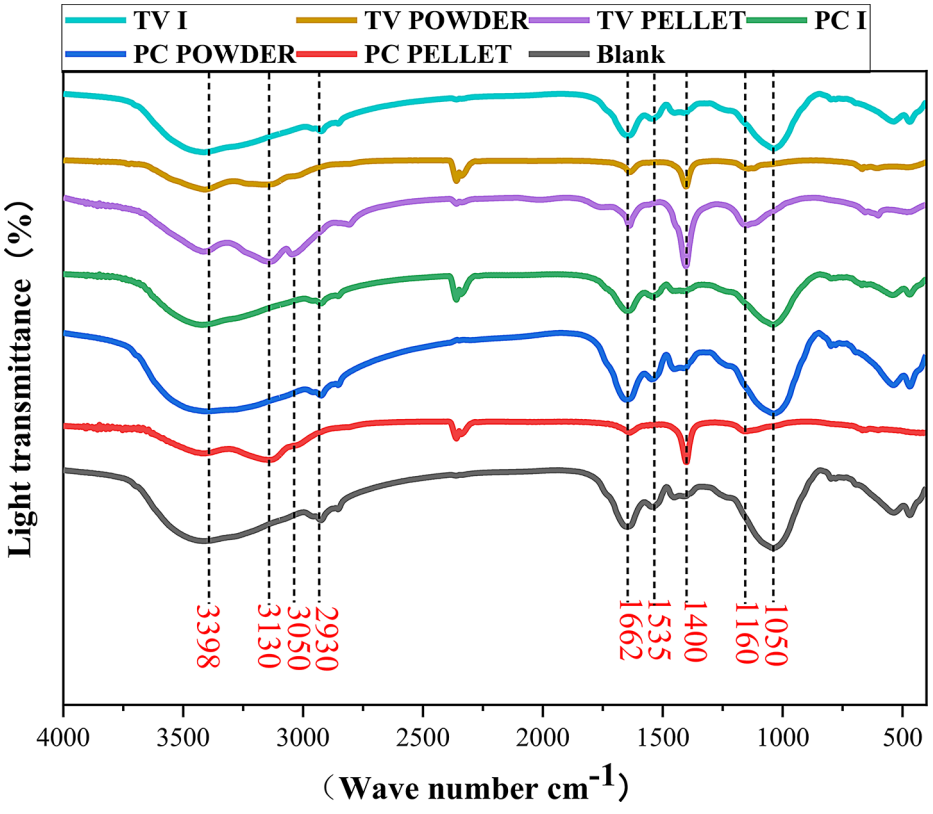

Fig. 5 Infrared mapping of the sludge from different experimental groups

sludge samples during the treatment with the WRF. The trend of UV_{254} was similar to that of COD, and generally showed a gradual decrease, with its value decreasing by about half. The reduction in UV_{254} was probably due to the degradation of humic macromolecular organic matter and aromatic compounds containing C = C and C = O double bonds in the sludge supernatant by the WRF, and LiP has been shown to eliminate a wide range of recalcitrant aromatic compounds, including polycyclic aromatic and phenolic compounds [36].

The ability of WRF to remove humic acids comes from two primary sources: biosorption and biodegradation [12]. Biosorption is mainly by the WRF mycelium and biosorption by fungi mycelium accounted for at least 20% of humic acid removal; Biodegradation by white-rot fungi can depolymerize larger humic acid molecules into smaller humic acid molecules, non-aromatic compounds, and fulvic acids. In addition, the presence of humic acid induced the activity of several enzymes [39].

Changes in protein and polysaccharide in sludge EPS

EPS is a macromolecular substance secreted on the cell surface, mainly polysaccharide and protein, which is favorable for microbial cell cohesion, and is an essential component affecting the properties of sludge adsorption, sedimentation, flocculation and dewatering [17, 34]. In this experiment, we analyzed the changes of protein and polysaccharide in the sludge EPS before and after treatment with different WRF.

As shown in (Fig. S5&S6), the polysaccharide content in EPS of blank sludge pieces was higher and the sludge polysaccharide content of the other six groups of samples was reduced. The EPS-SB polysaccharide content of the blank sludge sample was about 70 mg/L. The EPS-SB polysaccharide content of the other six groups of experimental sludge was reduced to about 50 mg/L, indicating that some of the dissolved polysaccharides were utilized or degraded by the WRF. The EPS-LB polysaccharide content of the six groups of sludge samples did not change much, and the EPS-TB polysaccharide decreased, which was probably because the WRF treatment converted a part of the sludge EPS-TB to EPS-LB, and that the WRF played a complex role in the sludge EPS, with different degradation pathways. The EPS-TB polysaccharide content of the sludge samples of *Phanerochaete chrysosporium* pellet, *Phanerochaete chrysosporium* powder, and *Trametes versicolor* powder decreased significantly, and the EPS-TB polysaccharide content of the sludge samples of *Phanerochaete chrysosporium* immobilization, *Trametes versicolor* powder, and *Trametes versicolor* immobilization did not change much, which showed that different forms of strains were added with other varying effects on the EPS-TB polysaccharide. The EPS-TB polysaccharide content of the sludge samples from *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder was reduced more, which could be because the growth of mycelium could enter into the interior of sludge flocs, which provided better degradation and transformation of EPS-TB polysaccharide. The EPS-TB polysaccharide content of the sludge samples from the pellet and immobilization, there was a slight decrease in the EPS-TB polysaccharide content of the pellet of *Phanerochaete chrysosporium* samples. The EPS-TB polysaccharide content of the other three groups of sludge samples didn't change much, which indicated that the immobilization of the strain of the pellet of immobilization had no significant effect on the EPS-TB polysaccharide of the sludge.

From the variation of protein content, the protein content of the six experimental sludge samples EPS-SB was lower than that of the blank sludge samples, which might be due to the degradation and metabolism of some proteins in the sludge during the growth process of the WRF. The protein content in EPS-LB didn't change much, the protein content in EPS-LB of *Phanerochaete chrysosporium* immobilization and *Trametes versicolor* powder sludge increased, and the protein content in EPS-TB decreased, which might be due to the action of the WRF converting a part of proteins tightly adhering to sludge flocs into loosely adhering protein. The protein contents in EPS-TB all decreased slightly, probably due to the enzymes secreted by the WRF and mycelium releasing proteins inside the sludge flocs. From the changes of polysaccharide and protein in sludge EPS, the effect of white-rot fungi treatment on sludge EPS-SB, EPS-LB and EPS-TB varied greatly, the degradation paths of the three were different, and the content and nature of the organic matter in EPS-SB changed considerably. In contrast, the range of the organic matter in EPS-LB didn't change much but the nature of the matter changed markedly.

Changes in functional groups of the sludge fractions

The effects of different fungi and their other various forms of treatment on sludge properties varied [20], and the changes in functional groups in the sludge were determined by Fourier transform infra-red spectrometry [8].

The infrared spectra of multiple experimental sample groups revealed that the positions of the absorption peaks for each sample were similar. Still, the peak vibration intensity was different, indicating that the content of substances with corresponding functional groups was different in each sample. Seven groups of samples showed an absorption peak with an apparent width at 3398 cm^{-1}, which was mainly formed by the stretching vibration of the O-H bond in the hydroxyl functional group, and the main reason for this strong absorption peak was the moisture in the samples.

Compared with the blank sludge, a new absorption peak appeared at 1400 cm^{-1}, which was formed by the amide in the protein structure, probably the protease secreted by *Phanerochaete chrysosporium* pellet, and the absorption peak caused by the C-O bond in the saccharides disappeared at 1050 cm^{-1}, which indicated that the saccharides were degraded. The intensity of the absorption peaks at 1050 cm^{-1} and 1662 cm^{-1} of *Phanerochaete chrysosporium* immobilized samples was weakened, indicating the reduction of saccharide and protein in *Phanerochaete chrysosporium* immobilized samples. The new absorption peaks at 1400 cm^{-1} and 1160 cm^{-1} in the *Trametes versicolor* pellet samples were associated with the secretion of enzymes by *Trametes versicolor*, and the absorption peak at 1160 cm^{-1} was probably due to the shift of the absorption peak formed by the vibration of C-O bonds. The *Trametes versicolor* powder sample showed a new absorption peak at 1400 cm^{-1}. The *Trametes versicolor* immobilization sample and the blank sample appeared at similar peak positions with differences in peak intensities. The changes of peak positions and peak intensities in the infrared spectra indicated that the treatment of saprophytic fungi influenced the functional groups of sludge components.

Experiments on the treatment of actual anaerobically digested sludge by WRF

Sludge sterilization technology can be energy and time intensive and is difficult to apply in large-scale systems, making it an important consideration in biological treatment [42]. Therefore, it was of practical significance to investigate the effect of the WRF on the treatment of unsterilized anaerobically digested sludge. Combining the experimental results of the WRF treatment of sterilized anaerobically digested sludge with practical applications, the treatment effect of the WRF on unsterilized anaerobically digested sludge was investigated, and the effect of adding WRF on the characteristics of anaerobically digested sludge microbial populations was analyzed. Based on the above experimental results, the more effective way of adding fungi powder was chosen to treat the actual sludge.

Changes in COD of anaerobically digested sludge

Figure 6 indicats the variation of actual anaerobically digested sludge soluble COD. The soluble COD removal rate of blank sludge was 10%, and the soluble COD removal rate of *Trametes versicolor* powder and *Phanerochaete chrysosporium* powder sludge was 33% and 21%, respectively. As can be seen from the figure, the sludge SCOD of the experimental groups of *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder increased in the first two days of the reaction. It was

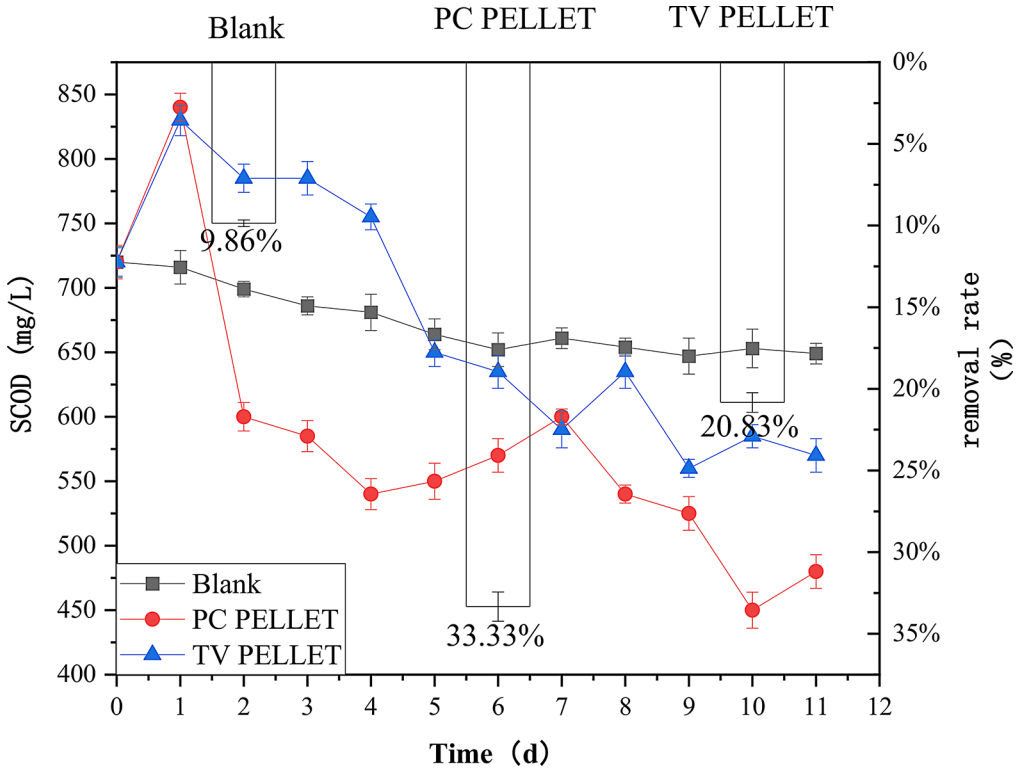

Fig. 6 Changes in the soluble COD of unsterilized anaerobically digested sludge

possible that the added fungi converted some of the Total COD to the soluble COD. The addition of white-rot fungi increased the removal of sludge soluble COD, and the experimental group with the addition of *Phanerochaete chrysosporium* showed a higher removal of soluble COD than the experimental group with the addition of *Trametes versicolor*. The results of the above sterilized sludge experiments showed that *Trametes versicolor* showed a better removal efficiency of sludge soluble COD, probably due to the different coping mechanisms of *Phanerochaete chrysosporium* and *Trametes versicolor* to different environmental conditions.

The variation of sludge Total COD was shown in Fig. S7. Due to the large total amount of sludge Total COD, the effect of the two WRF treatments on sludge Total COD was not obvious. Only 1800 mg/L of the Total COD was removed, and the removal rate was only about 6%. WRF removed Total COD mainly by converting a portion of the insoluble COD into the soluble COD, which was then utilized by the fungal growth metabolism, or by directly degrading and mineralizing a portion of the organic matter through secreted enzymes. Comparing soluble COD and Total COD reductions, the Total COD was reduced significantly more than the soluble COD. It could be WRF converting a portion of Total COD to soluble COD, which was reabsorbed and utilized, or directly degrading and mineralizing a portion of the Total COD.

In comparison with the above changes in COD of sterilized sludge, the removal of soluble COD from actual sludge by the WRF was weaker, and probably the activity of the WRF in actual sludge was lower, which was mainly due to the competition from other microbial populations and the alkaline conditions that were unfavorable for the growth and reproduction of WRF.

Changes in the enzyme activity

Figure 7 shows the changes in enzyme activity during the reaction. In this experimental determination, the actual activity changes of MnP and LiP were measured. The activities of both enzymes in *Phanerochaete chrysosporium* were found to be higher than those in *Trametes versicolor*. The patterns of enzyme activity changes correspond with the degradation trends of various indicators of anaerobic digestion sludge by WRF. Enzyme activity approximately reached its peak around the fifth day, at which point the degradation rate of organic matter in anaerobic digestion sludge was the fastest. As the WRF aged, their extracellular enzyme activities significantly decreased, leading to a slower degradation rate of organic matter and eventually reaching a saturation point where no further degradation occurred. In actual anaerobic digestion sludge, *Phanerochaete chrysosporium* showed a 33% removal rate for the soluble COD and a 7% removal rate for Total COD; *Trametes versicolor* showed a 21% removal rate for the soluble COD and a 5% removal rate for Total COD. The organic matter removal effect of *Phanerochaete chrysosporium* was superior to that of *Trametes versicolor*, which is also consistent with the measured results of enzyme activities of the two fungi.

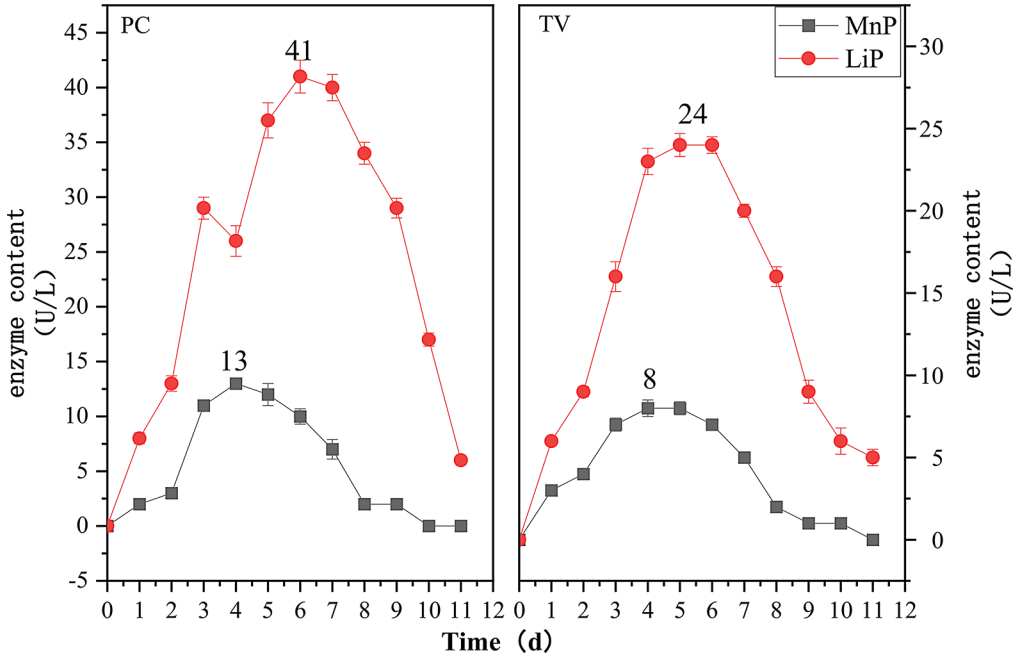

Fig. 7 Enzymatic activities of WRF in unsterilized anaerobically digested sludge

Changes in the UV254 of anaerobically digested sludge

As shown in Fig. 8, the elevated UV_{254} content in the blank sludge might be the result of the original microorganisms in the sludge condensing some simple organic matter into larger macromolecules. The changes in UV_{254} content in the sludge of *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder showed an overall decreasing trend, and the final removal rates were 66% and 53%, respectively. Adsorption and degradation by WRF reduced the amount of humic substances and aromatic compounds containing C = C double bonds and C = O double bonds in the sludge supernatant, and LiP was shown to eliminate a wide range of recalcitrant aromatic compounds, including polycyclic aromatic and phenolic compounds [36]. The increase in the UV_{254} content in the blank samples was due to the humification of some organic matter by the original microorganisms in the sludge samples.

Effect of WRF on microbial populations of actual anaerobically digested sludge

Samples were obtained from both blank sludge and sludge amended with *Phanerochaete chrysosporium* powder, demonstrating promising outcomes in our previous experiments.

Effect of WRF treatment on fungal communities in actual anaerobically digested sludge

Figure 9(a) demonstrats the variations at the level of fungal population clades in the two sets of sludge samples. From the relative abundance of each phylum in the graph, it was evident that four main phyla of fungi, namely *Basidiomycota*, *Ascomycota*, *Rozellomycota*, and *Chytridiomycota*, were present in the blank sludge. In contrast, the highest abundance of the *Basidiomycota* phylum was found in the *Phanerochaete chrysosporium* powdered sludge sample, and the abundance of the other phyla almost disappeared. It showed that the addition of the *Phanerochaete chrysosporium* powder changed the composition of the sludge fungi greatly, and the *Phanerochaete chrysosporium* became the dominant phylum in the sludge, competing with other phyla for the growth environment and nutrients, which led to the gradual extinction of the other phyla.

Figure 9(b) shows the variations of abundance at the level of fungal genera in the two sets of sludge samples. From the abundance of genus level in terms of genus level abundance, the *Phanerochaete chrysosporium* powder samples were dominated by *Phanerochaete* and *Xeromyces*, with other genera showing relatively low abundance. The reason for the change in the abundance of other genera was partly due to the fact that *Phanerochaete chrysosporium*, as a dominant genus, inhibited the growth of other genera, leading to their gradual extinction. On the other hand, it was the rapid multiplication of *Phanerochaete chrysosporium* that increased the base of fungal abundance in the samples, resulting in relatively low abundance of the other genera.

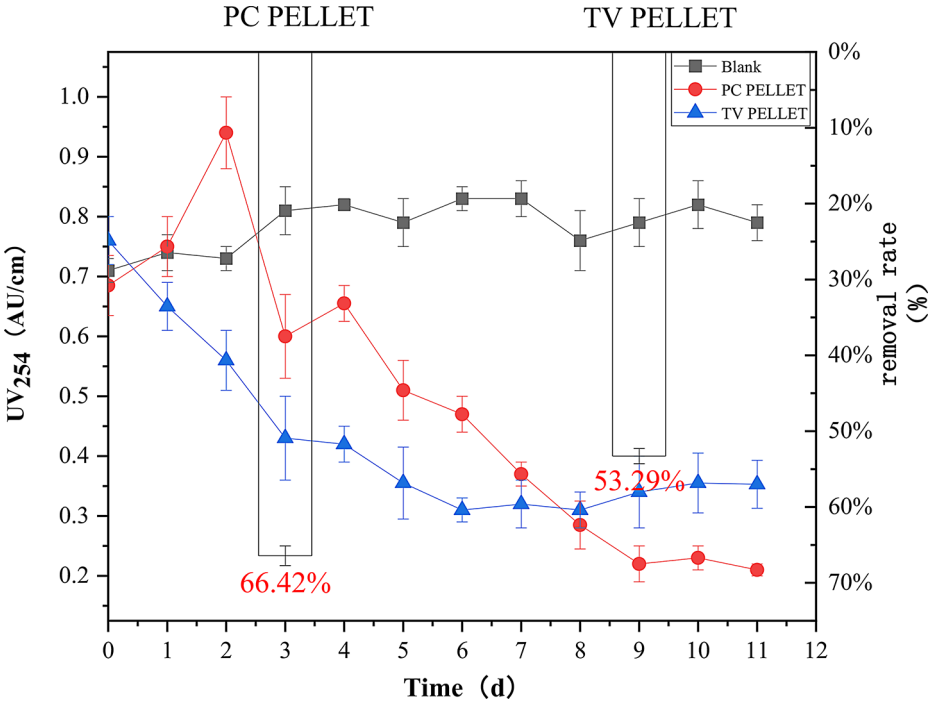

Fig. 8 Changes in the UV_{254} of unsterilized anaerobically digested sludge

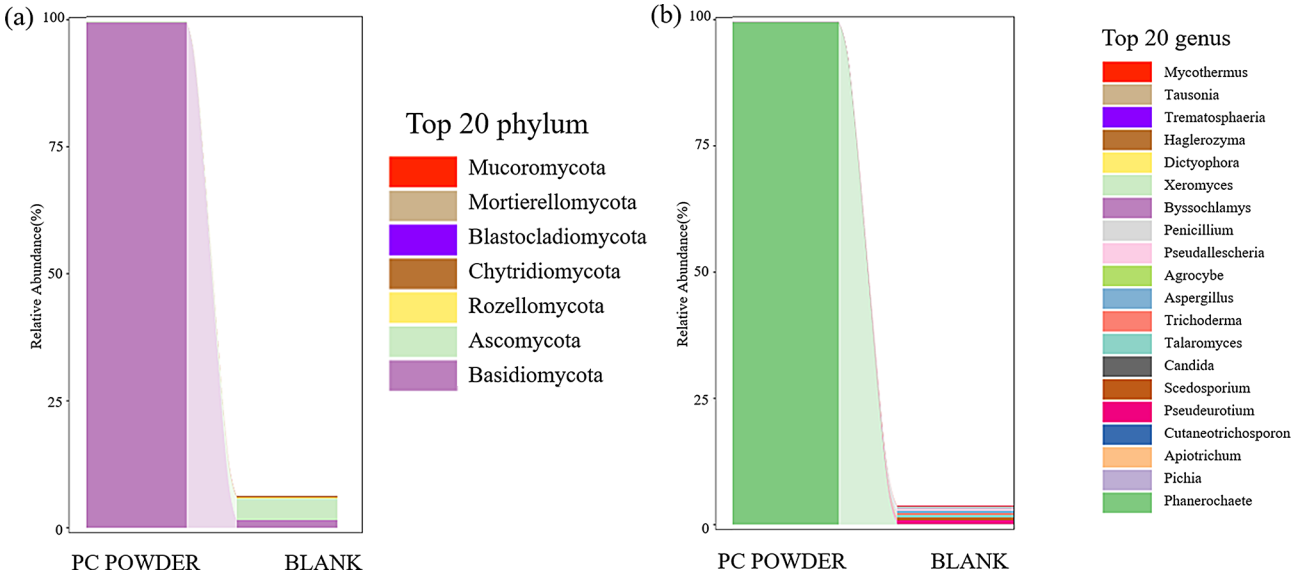

Fig. 9 Relative abundance of fungi at the phylum level and genus level in sludge samples

Table 2 Index of alpha diversity of bacteria and fungi in different sludge samples

	Sludge type	Ace exponent	Chao exponent	Shannon exponent	Simpson exponent	Pd exponent	Coverage rate(%)
Bacteria	Blank sludge	513.9095	511.4118	4.77097	0.01954	91.9245	99.9856
	Fungal Sludge	530	530	4.68938	0.02512	93.0406	100
Fungi	Blank sludge	374.9819	374.8571	3.39439	0.10238	70.2075	99.9961
	Fungal sludge	419	419	3.52206	0.08605	72.7491	100

Effect of WRF treatment on bacterial communities in actual anaerobically digested sludge

Figure 8 shows the changes of bacterial populations in the actual anaerobically digested sludge before and after treatment with *Phanerochaete chrysosporium* at the phylum level (a) and genus level (b). As shown in Fig. 8(a), the top 20 clades in terms of abundance at the bacterial clade level contained basically all the bacteria in the sludge samples. The phylum *Proteobacteria* was the most abundant bacterial phylum, followed by the phylum *Chloroflexi* and the phylum *Actinobacteria*, which accounted for about 80% of the total bacterial abundance. After treatment, the abundance of *Proteobacteria* phylum increased, but the abundance of *Chloroflexi* phylum and *Actinobacteria* phylum decreased. Figure 8(b) shows the variation in abundance at the genus level, with the top 20 genera accounting for about 50% of the total abundance, and the rest being the less abundant and unclassified bacterial genera. Among them, the abundance of *RBG-13-54-9*,*Dyella*, *Rhodanobacter* genus and *Gaiella* genus increased significantly. *Ellin6067*, *Candidatus_Competibacter*, *SJA-15* and *Denitratisoma* genus were significantly reduced in abundance. *Denitratisoma* genus can oxidize nitrite to nitrate, providing nitrate material for denitrifying bacteria [29].

Analysis of microbial richness and diversity of different sludges by WRF treatment

Alpha diversity indices of bacteria and fungi in blank and fungal sludge were shown in Table 2. Among them, Ace and Chao indices were used to reflect the richness information of microbial communities; Shannon and Simpson indices were used to represent diversity information of microbial communities.

The addition of *Phanerochaete chrysosporium* minimally impacted bacterial abundance in the sludge, while having a moderately greater effect on fungal abundance. The introduction of *Phanerochaete chrysosporium* increased the Ace and Chao indices of sludge fungi from 374 in the control sludge to 419, suggesting a greater enhancement in fungal abundance with the addition of *Phanerochaete chrysosporium*. In terms of sludge diversity, the addition of *Phanerochaete chrysosporium* had less effect on sludge Shannon and Simpson indices. The addition of *Phanerochaete chrysosporium* modestly decreased bacterial diversity, as indicated by the Shannon's index, while enhancing fungal diversity, suggesting

that the introduction of WRF fosters fungal competitiveness at the expense of bacterial populations. The Pd index measures the phylogenetic diversity within the sample, while the Coverage index indicates the proportion of species within the sample that have been successfully captured. Both sludge groups achieved over 99.9% coverage of bacterial and fungal species, suggesting that the sequencing results accurately represented the true microbial diversity.

Conclusions and prospect

In response to the issues present after anaerobic digestion of sludge, this study leverages the broad and efficient degrading capabilities of WRF and their enzyme systems to apply WRF in the treatment of anaerobically digested sludge, thereby verifying the feasibility of using WRF for this purpose. This experiment provides a new approach for the subsequent resource utilization of sludge and serves as a reference for the treatment of other types of sludge with white-rot fungi. The research investigates the impact of white-rot fungi treatment on the organic matter and physicochemical properties of sterilized anaerobic digestion sludge and preliminarily explores the effects on non-sterilized actual anaerobic digestion sludge, laying the foundation for the practical engineering application of white-rot fungi in this field. The following are the main conclusions drawn from the study.

Different forms of *Phanerochaete chrysosporium* and *Trametes versicolor* both demonstrated certain abilities to remove organic components from sterilized anaerobic digestion sludge after 10 days of treatment. *Trametes versicolor* powder showed the best effect on sludge COD removal, with the soluble COD removal rate of up to 71% and Total COD removal rate of 13%. In contrast, the *Phanerochaete chrysosporium* powder was less effective in sludge COD removal compared to the *Trametes versicolor* powder, with the soluble COD removal rate of 52% and Total COD removal rate of 11%. Both mycelium and immobilized forms had lower COD removal effects than the powder form. The removal effects on UV_{254} by different forms of *Phanerochaete chrysosporium* and *Trametes versicolor* were similar, with *Phanerochaete chrysosporium* powder showing the best removal effect, approximately 45%. Treatments by different fungal species and their forms all resulted in a decrease in the content of polysaccharides and proteins in sludge EPS. Infrared spectroscopy indicates that the impact of different species and forms of fungi treatment on the functional groups of sludge components varies. Among them, *Trametes versicolor* powder has the most significant effect on the functional groups of sludge, significantly reducing the peak intensity of C-O bonds, which represents the degradation of carbohydrate substances, and enhancing the intensity of amide peaks, which represents the production of protein substances. In contrast, the effects of *Phanerochaete chrysosporium* powder, *Trametes versicolor* pellet, and immobilized forms on sludge functional groups are not obvious. Research on the treatment of actual non-sterilized anaerobic digestion sludge with *Phanerochaete chrysosporium* powder and *Trametes versicolor* powder for 10 days found that *Phanerochaete chrysosporium* powder has removal rates of 33% for the soluble COD and 66% for UV_{254}, while *Trametes versicolor* is less effective in sludge treatment compared to *Phanerochaete chrysosporium*, with removal rates of 21% for the soluble COD and 53% for UV_{254}. Microbial community analysis results show that WRF treatment greatly inhibits the growth and reproduction of other fungi in sludge and has a significant impact on bacterial populations. In terms of the diversity of bacteria and fungi in sludge, WRF can also promote the regulation of microbial communities.

The feasibility of using WRF for the treatment of anaerobically digested sludge has been validated in the current study, but the treatment period is relatively long, and the effect on actual anaerobically digested sludge is slightly poor. Further in-depth research is needed in this field to improve the efficiency and effectiveness of the treatment process. In future studies, we can further explore their application mechanisms and optimize the treatment conditions. By combining multiple strains of white-rot fungi or synergizing white-rot fungi with bacteria for the treatment of anaerobically digested sludge [9], we aim to develop a more efficient, economical, and environmentally friendly treatment method for anaerobically digested sludge.

Abbreviations

COD	Chemical oxygen demand
SCOD	Soluble chemical oxygen demand
PC	Phanerochaete chrysosporium
TV	Trametes versicolor
PC I	Phanerochaete chrysosporium immobilization
TV I	Trametes versicolor immobilization
EPS	Extracellular polymeric substances
UV_{254}	Ultraviolet radiation
WRF	White-rot fungi
Mnp	Manganese peroxidase
Lip	Lignin peroxidase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02611-x.

Supplementary Material 1

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Author contributions
Conception or design: 1st and Corresponding Author (Xuefeng Zhu), 6th Author and Corresponding Author (Hongbo Liu) and 9th Author and Corresponding Author (Xuedong Zhang); Acquisition, analysis, or interpretation: 1st Author (Xuefeng Zhu), 2nd Author (Shicai Cheng); Draft manuscript preparation: 1st Author (Xuefeng Zhu), 2nd Author (Shicai Cheng); Critically revise the manuscript: 3rd Author (Zexian Fang), 4th Author (Guangyin Zhen), 5th Author (Xueqin Lu), 6th Author and Corresponding Author (Hongbo Liu), 7th Author (Jing Qi), 8th Author (Zhen Zhou), 9th Author and Corresponding Author(Xuedong Zhang), 10th Author(Zhichao Wu). All authors reviewed the results and approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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