Enhancement of biological fermented sludge dewaterability by inoculation of filamentous fungi *Mucor circinelloides* XY-Z and *Penicillium oxalicum* LY-1

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**ABSTRACT**

The two filamentous fungi of *Mucor circinelloides* XY-Z and *Penicillium oxalicum* LY-1 isolated from citric acid wastewater sludge collectively enhanced sludge dewaterability by 85.83% to achieve the lowest value of normalized sludge specific resistance (SRF) to 6.8 $\times$ 10$^{11}$ m$^{-1}$L$^{-1}$g$^{-1}$TSS. The results showed that 75.77% of slime extracellular polymeric substances (EPS), 42.99% of protein in slime EPS and 60.27% of polysaccharide in LB-EPS were degraded during activated sludge treatment by the two mixed fungi of *Mucor circinelloides* XY-Z and *Penicillium oxalicum* LY-1, contributing to the conversion of 64.61% of bound water wrapped in EPS into free water, thereby improving activated sludge dewaterability.

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**Introduction**

Wastewater treatment will have many by-products which are very complex, containing not only high concentrations of organic matter but also N, P, K, and other plant nutrients.\cite{1} The excess sludge is one of the main by-products, which has high water content and poor dewaterability, bringing great difficulties to the transportation and disposal of sludge.\cite{2,3} The cost of sludge treatment and disposal accounts for 50% of the total cost of sewage treatment.\cite{4} Therefore, conditioning prior to mechanical dewatering to improve sludge dewaterability and reduce sludge volume is key to reducing sludge transportation and disposal costs.\cite{5-7} At present, chemical flocculants are commonly used during sludge treatment to achieve slurry separation.\cite{8,9} Although flocculants can improve sludge dewaterability,\cite{10,11} there are some problems associated with their use, such as their toxic and corrosive properties, their potential to exert adverse effects on the environment and their high cost.\cite{12,13} Therefore, it has become urgent to seek effective, sustainable, economic, and environmentally-friendly conditioning methods to improve sludge dewaterability. Accordingly, many researchers have evaluated biological conditioning methods in recent years.\cite{14} Liu et al.\cite{15} found that the dewatered sludge after bioleaching has the faster evaporation rate compared with conventional dewatered sludge, which is beneficial to the sludge drying in the later period.

Biological fermented sludge with a high content of organic matter has poorer dewaterability than municipal sludge. However, the moisture content is still higher than 80% after conditioning with cationic polyacrylamide (CPAM) and mechanical dewatering. It has been reported that bacteria and filamentous fungi can significantly improve the dewaterability of municipal sewage sludge.\cite{16,17} Moreover, microorganisms have greater waste degradation capacity if they are separated or collected from the same or similar waste sources as the sludge.\cite{18} Studies have also shown that, when compared with bacteria, fungi have the advantages of being able to grow on medium at low pH, low temperature and low nitrogen content.\cite{19} Filamentous fungi can also entrap solid particles from sludge and compress the sludge with its filamentous mycelia, thus modifying the porosity structure of biosolids and enhancing the bioseparation, dewaterability, and filterability of the fungal treated sludge.\cite{20-22} It has been reported that some different fungal strains can be combined, forming mycelia...
mixtures, possibly with synergic effects.\textsuperscript{19} According to a study conducted by Molla et al.\textsuperscript{23} and Fleury et al.\textsuperscript{24}, the symbiotic association of mixed fungal culture improved fungal colonization of the substrate with increased yield and cellulase production, which could enhance productivity and lead to better adaptability. Many studies have investigated the performance of sludge dewaterability with mixed fungal culture of \textit{Asperillus niger} and \textit{Penicillium corylophilum}. A series of studies by Alam\textsuperscript{25} and Fakhrul-Razi\textsuperscript{26} found that SRF (1.08 × 10\textsuperscript{12} m/kg) was reduced by 98.7\% compared with untreated sludge (84 × 10\textsuperscript{12} m/kg) after 6 days of treatment of municipal sludge with mixed fungal culture of \textit{Asperillus niger} and \textit{Penicillium corylophilum}. However, researchers investigating this aspect were focused on the treatment of municipal sludge by mixed fungal culture of \textit{Asperillus niger} and \textit{Penicillium corylophilum}, and it is not clear whether other types of mixed fungal culture can improve the dewaterability of biological fermented sludge with high organic content.

The moisture in the sludge can be divided into free water and bound water. Free water is not bound by sludge flocs and can be removed from the sludge by concentration or mechanical dewatering. Bound water has a strong bond with sludge flocs and is therefore difficult to remove through mechanical methods.\textsuperscript{27} Research has focused on enhancing the removal of bound water, because removal of bound water limits the dewatering rate.\textsuperscript{28} There is a close relationship between bound water and the extracellular polymer (EPS) in sludge. Most of the water in sludge is bound together in EPS and difficult to remove.\textsuperscript{29,30} In addition, the increase in EPS usually leads to poorer sludge dewaterability, possibly because of the steric force generated by EPS inhibiting the contact between cells.\textsuperscript{12,31} Hence, a lower EPS content is more beneficial for improvement of sludge dewaterability. In addition, Yu et al.\textsuperscript{32} divided EPS into slime EPS, loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). Studies have shown that the ratio of protein to polysaccharide (PN/PS) in EPS has an effect on sludge dewaterability.\textsuperscript{33} The protein content plays an especially important role, with high protein content being beneficial to sludge dewatering.\textsuperscript{34} Previous studies have found that Slime EPS in sludge were degraded by filamentous fungus \textit{Mucor} sp. GY-1, which contributed to the improvement of municipal sludge dewaterability.\textsuperscript{17} However, some questions still remained in the mechanisms of dewaterability improvement by filamentous fungi. For example, the change of water distribution in sludge during the treatment is unclear. What’s more, many previous studies focused on municipal sludge, with little research on biological fermented sludge.

Citic acid production often adopts biological fermentation processes, resulting in the production of excess sludge with high levels of microorganisms and organic matter. In this study, citric acid wastewater sludge was selected as a representative of biological fermented sludge and two filamentous fungi that could improve the dewaterability of sludge were isolated. The SRF, CST, SV\textsubscript{30}, EPS, and bound water were investigated during sludge treatment using the mixed filamentous fungi to determine whether the mixed filamentous fungi could help improve the dewaterability of biological fermented sludge and the mechanisms of action. The results present in this study will improve understanding of the role of mixed filamentous fungi in biological fermented sludge and facilitate development of biological fermented sludge treatment technology using mixed filamentous fungi.

\section*{Materials and methods}

\subsection*{Biological fermented sludge sample}

Biological fermented sludge used in this study was collected from the secondary sedimentation tank of the sewage treatment plant of a citric acid production enterprise in Shandon Province, China and preserved in a plastic container in a cold room at 4°C for immediate use. Thereafter, the sludge pH (7.85 ± 0.08), solid content (1.27 ± 0.02\%), and organic matter content (62.5 ± 1.36\%) of the sludge were determined using the Standard Methods.\textsuperscript{35}

\subsection*{Isolation and screening of filamentous fungi}

Filamentous fungi capable of enhancing sludge dewaterability were screened out from the activated sludge by serial dilution techniques using Sabouraud agar (SDA), which resulted in isolation of seven filamentous fungal strains. Cultures were then grown on SDA at the center of the petri dishes and their growth rates observed. Among them, the average growth rates of two filamentous fungi (N1 and N2) in the first 3 days were (13.3 ± 1.1) mm/day and (10.7 ± 0.9) mm/day, respectively. Furthermore, fungal mycelium in Martin Broth modified medium of N1 and N2 had more developed mycelium than the other five strains. Their influence on the dewaterability of activated sludge was then preliminarily determined in 500 mL conical flasks each containing 270 mL of municipal sewage sludge...
and 30 mL of actively growing culture of isolated N1 and N2. The mixtures were cultivated on a gyratory shaker at 28 °C and 180 rpm, and 50 mL aliquots of the sludge samples were collected at 24 h intervals and used for determination of the sludge capillary suction time (CST) using a TR04-304M analyzer. The two strains decreased the sludge CST by more than 60% in only 3 days, indicating they had good ability to enhance sludge dewaterability, and better than other strains (20–30%). Therefore, N1 and N2 were selected out because of their high growth rate, flourishing mycelia and better ability to enhance sludge dewaterability. Isolates N1 and N2 were identified through molecular phylogenetic analysis based on the 18S rRNA gene. Sequencing of the purified PCR product was conducted by Beijing S Bo Sing C Biological Technology Co. Ltd., China. The resulting sequence was compared with available sequences in the GenBank database using the BLAST system. Multiple sequence alignment was performed using the Clustal X 2.1 software, after which a phylogenetic tree was drawn using the neighbor joining method.

**Preparation of filamentous fungi inoculum**

The selected N1 and N2 were cultured on SDA for 3 days at 30 °C. The spores were then washed from the agar using sterilized distilled water, after which the cell suspensions obtained were filtered through autoclaved columns packed with glass wool. Next, flasks containing sterilized Martin broth modified medium were inoculated with 1% (v/v) spore suspension of two strains at a density of $1 \times 10^5$ spores/mL. The mixture was then incubated in a gyratory shaker at 150 rpm and 25 °C for 48 h, after which the fungal culture was filtered through #1 Whatman paper and washed several times with sterilized water to remove the culture medium. The isolated fungal mycelium was re-suspended in sterilized water to its original volume, after which these fungal mycelia with a dry weight of 5.29 mg/mL for N1 and 4.46 mg/mL for N2 were used as fungal inocula in the following experiments.

**Experimental design and procedure**

A total of 30 mL of N1 inoculum or 30 mL of N2 inoculum were added into 500 mL Erlenmeyer flasks each containing 270 mL of activated sludge, while 15 mL of N1 plus 15 mL of N2 were added into 500 mL Erlenmeyer flasks each containing 270 mL of activated sludge. The control flasks contained 300 mL activated sludge that were not inoculated with filamentous fungi. All flasks were incubated at 25 °C for 5 days at 180 rpm in an orbital shaker. During the treatment period, three flasks as parallel samples were removed at 24 h intervals from each treatment to measure the sludge SRF, sludge CST, SV$_{30}$, sludge EPS content, and bound water. All measurements were done in triplicate unless otherwise noted, and data presented were the mean values of the triplicate samples with standard deviations.

**Analytical methodology**

In this study, the sludge dewaterability was characterized in terms of CST and normalized sludge SRF. The CST was measured using a CST analyzer (TR04-304M, England). The normalized sludge SRF value was calculated by dividing the sludge SRF by the TSS concentration in the corresponding sludge sample and expressed in m-L/kg-g-TSS to minimize the dilution effect of the fungal inoculum on the sludge SRF. The SV$_{30}$ of the sludge was determined using the Standard Methods. The bound water was measured by a dilatometric test, which defines the bound water in sludge as nonfreezing water at a certain minus temperature. The sludge EPS was determined according to the method described by Zhang et al. and Wong et al. Briefly, sludge samples (50 mL) were harvested by centrifugation at 550 g for 15 min, after which the supernatant collected was used to determine the slime-EPS. Additionally, the pellet retrieved after centrifugation was washed twice with 0.1 mol/L NaCl solution, then resuspended to its original volume (50 mL) using 0.1 mol/L NaCl solution. Next, the suspension was transferred and centrifuged at 5000 g for 15 min, after which the supernatant and sediments were collected separately. This supernatant was used as the LB-EPS fraction. The residual sludge pellets were resuspended to their original volume (50 mL) using 0.1 mol/L NaCl solution, after which the solution was transferred to an extraction beaker to extract the TB-EPS. An ultrasound (20 kHz, 120 W) was then performed on the suspension three times for 2 min each, after which the TB-EPS were harvested by centrifugation at 20,000 g at 4 °C for 20 min. The supernatant was used as the TB-EPS fraction. The particulates present in the soluble EPS, LB-EPS, and TB-EPS solutions were removed using polytetrafluoroethylene membranes with a pore size of 0.45 µm prior to chemical composition analysis. All extracted EPS solutions were analyzed for total organic carbon (TOC) using a TOC analyzer (Vario TOC, Germany).
Protein content in the EPS solution was measured using the Lowry method and albumin was used as a standard solution. The polysaccharide content was measured by the anthrone method.

Statistical analysis

The mean and standard deviation for the three replicate experiments for each treatment are reported. The data were analyzed using one-way analyses of variance. Least-significant difference tests were used to determine the significance of differences between means. Pearson correlation analysis between the CST, SV30, bound water, and sludge EPS of the treated sludge by mixed culture of M and P were performed using SPSS 22.0.

Results and discussion

Isolation and identification of filamentous fungi

Two dominant filamentous fungal strains that can enhance sludge dewaterability were isolated from citric acid waste sludge. The colonies and their mycelial morphologies are shown in Supplementary material Figure 1 (see Appendix). Neighbor-joining trees depicting the phylogenetic relationship of the isolated strains with related species based on the 18S rRNA gene sequence are shown in Supplementary material Figures 2 and 3 (see Appendix). The results showed that the strains with the highest homology with N1 and N2 were Mucor circinelloides and Penicillium oxalicum, respectively. Therefore, they were then named Mucor circinelloides XY-Z and Penicillium oxalicum

Evaluation of the effect of inoculation of M and P on activated sludge dewaterability

Different researchers often select different indexes to evaluate the dewaterability of sludge. The specific resistance to filtration (SRF) and the capillary suction time (CST) tests are both commonly used to estimate sludge dewaterability. The SRF is a good simulation of the actual production of the filtration process, with a larger SRF value indicating harder filtration and worse sludge dewaterability. Figure 2 shows the change in normalized sludge SRF during the treatment of activated sludge with single and mixed fungal cultures. The normalized SRF of activated sludge decreased in the first 3 days, then increased slightly in the control system without inoculation. This phenomenon may be caused by the aerobic digestion of the original microorganisms in the sludge. The treatment systems inoculated with a single culture of M, single culture of P and a mixed culture of M and P exhibited similar changes in normalized sludge SRF as the control system, which achieved the best dewaterability after 2 or 3 days of incubation, then deteriorated slightly. However, in the treatment system inoculated with mixed culture of M and P, the normalized SRF of activated sludge decreased to the lowest value of 0.68 \times 10^{-12} \text{ m}^2\text{kg}^{-1}\text{g-TSS} on the second day.
with a reduction rate of 85.83%. The normalized SRF was decreased to $2.05 \times 10^{12}$ m$^{-1}$L/g-TSS and $1.22 \times 10^{12}$ m$^{-1}$L/kg-TSS on the second day in the treatment system inoculated with a single culture of $M$ and $P$, respectively, with a reduction rate of 76.39% and 74.25%. Significant differences were found of the SRF among the sludge with different culture ($p = 0.028$, $p < 0.05$). In addition, the SRF showed highly significant differences among sludge with different culture and control system ($p = 0.008$, $p < 0.01$). This indicated that filamentous fungi reduced sludge SRF, and the results showed that the effects of mixed culture of $M$ and $P$ on SRF reduction were better than those of single culture of $M$ or $P$. Alam et al.\cite{25} found that the SRF of municipal sludge was reduced to the lowest value after 6 days and the reduction rate was as high as 98.35%. Thus, the improvement of dewaterability of activated sludge by mixed culture of $M$ and $P$ with a shorter treatment time was not as good as improvement of the dewaterability of municipal sludge by $A. niger$ and $P. corylophilum$. This phenomenon may be because the activated sludge is more difficult to deal with, and there may be more suitable mixed culture; however, this remains to be further studied.

Because of the complex measurement process and great operative errors of SRF, CST has been recognized by researchers for its advantages of simple measurement and accurate results.\cite{6,43} The United States has standardized the methods for determination of CST.\cite{35} Generally speaking, a lower CST value is better for sludge filterability and dewaterability, and CST values between 15 s and 20 s are acceptable for sludge dewatering when using a filter press.\cite{44} The changes in sludge CST during treatment of activated sludge by different fungi are shown in Figure 3. The overall trend in CST is similar to that of SRF. The CST value of culture sludge was obviously lower than that without culture sludge, but both increased gradually after achieving the minimum value. No significant differences were found of the CST among the sludge with different culture ($p = 0.506$, $p > 0.05$), but highly significant differences were found among sludge with different culture and control system ($p = 0.008$, $p < 0.01$). The results showed that the CST achieved the minimum value of 9.6 s after 3 days during the treatment of activated sludge using the filamentous fungus $M$, while during the treatment of activated sludge using the filamentous fungus $P$, the CST achieved the minimum value of 10.9 s after 2 days. However, the CST achieved the minimum value of 9.7 s with a reduction rate of 69.59%, which rose to 12.9 s in 5 days. The lowest value of CST in the control system was still as high as 17.2 s throughout the treatment process. Previous studies showed that the CST of municipal sludge decreased from 31.5 s to 13.4 s after 3 days of treatment with $Mucor sp.$ GY-1.\cite{17} The enhancement of dewaterability of municipal sludge by filamentous fungi was worse than that of activated sludge, and the effect of mixed culture of $M$ and $P$ on CST reduction was better than that of single culture.

### Evaluation of the effect of inoculation of $M$ and $P$ on activated sludge settleability

Alam and Fakhru’l-Razi\cite{25} reported that pretreatment of sludge using a mixed fungal culture of $Aspergillus niger$ and $Penicillium corylophilum$ significantly increased the sludge settling rate. The change in SV$_{30}$ during the
treatment of activated sludge using the fungal mixed culture of M and P is shown in Figure 4. No significant differences were found of the SV30 among the sludge with different culture ($p = 0.323, p > 0.05$), but significant differences were found among sludge with different culture and control system ($p = 0.011, p < 0.05$). The results showed that the SV30 value of culture sludge was significantly lower than that without fungal culture sludge, indicating that filamentous fungi improved the settleability of the sludge. The SV30 of the control system was above 88% throughout the experimental period, and the sludge treated with the single strain was reduced to 70–78% on the fifth day. However, the SV30 of the sludge treated with the mixed strains could be reduced to 54.73% on the fifth day. Therefore, the settleability of sludge was improved by addition of filamentous fungi, which was consistent with the results of previous studies. Subramanian et al. [45] found that *Penicillium expansum* BS30 was useful in sludge-mass reduction and sludge settling. The fungal biomass enhanced floc formation with contaminants entrapped in it. Moreover, the fungal mycelium, which interacted with sludge solids and other natural microbes forming strong and large flocs, resulted in enhanced sludge settling. [19]

![Figure 4. Change in SV30 during the treatment of activated sludge using *Mucor circinelloides* XY-Z, *Penicillium oxalicum* LY-1, mixed *Mucor circinelloides* XY-Z, and *Penicillium oxalicum* LY-1.](image)

**Degradation of dissolved organic matters by mixed M and P**

Extracellular polymeric substances (EPS) are the most important constituents of activated sludge, accounting for 60–80% of the total biomass. [33] Changes in the sludge slime-EPS content during the treatment of activated sludge using the fungal mixed culture of M and P are shown in Figure 5. The slime EPS of both the control system and the treatment system showed a decreasing trend and there was a significant difference ($p = 0.000, p < 0.01$) between the control sludge and mixed M and P inoculated sludge. It might be that slime EPS was degraded by mixed culture of M and P. The slime EPS in the control system decreased to a minimum of 28.0 mg/g VSS on the third day with a reduction rate of 41.54%, then slightly increased. The slime EPS in the treatment system decreased to 22.67 mg/g VSS on the first day with a reduction rate of 75.77%, while it was maintained at 19–20 mg/g VSS after the second day. Therefore, the mixed culture of M and P degraded slime EPS in activated sludge.

EPS are the products of hydrolysis and dissolution of macromolecules, which are primarily composed of polysaccharides and proteins. [46] The changes in protein and polysaccharide content in EPS and their ratio in the process of mixed culture treatment activated sludge are shown in Figure 6. As illustrated in Figure 6a, the protein content in the total sludge EPS fluctuated between 44.39–55.42 mg/g VSS in the control system and 38.28–60.26 mg/g VSS in the treatment system. The protein in the slime EPS decreased significantly from 33.37 mg/g VSS to 20.24 mg/g VSS on the second day, with a reduction rate of 39.35% in the control system, while during treatment of activated sludge using the mixed culture of M and P, the protein in the slime EPS decreased from 32.03 mg/g VSS to 18.26 mg/g VSS on the second day, with a reduction rate of 42.99%. This phenomenon indicates that the mixed culture of M and P degraded the protein in slime EPS. The content of protein in LB-EPS was very small, showing an initial decrease followed by an increase that fluctuated from 2.66–6.50 mg/g VSS for the control system and 3.58–7.18 mg/g VSS for the treatment system. The protein in the TB-EPS slowly

![Figure 5. Change in sludge slime EPS content during the treatment of activated sludge with fungal mixed culture of *Mucor circinelloides* XY-Z and *Penicillium oxalicum* LY-1.](image)
increased in the control system, while an unstable increase occurred in the treatment system. For the protein in the slime EPS, there was a significant difference ($p = 0.038$, $p < 0.05$) between the control system and mixed fungi inoculated sludge. The changes in polysaccharide content in EPS in the process of mixed culture treatment of activated sludge are shown in Figure 6b. The polysaccharide content in slime-EPS was almost unchanged in the control system, but decreased from 4.24 mg/g VSS to 2.08 mg/g VSS with a reduction rate of 50.94%, then increased to 4.44 mg/g VSS on the fifth day in the treatment system. The content of polysaccharide in LB-EPS decreased slowly in both the control system and treatment system, while it decreased by 60.27% in the treatment system. Polysaccharide content in the TB-EPS of the treatment system decreased from 3.60 mg/g VSS to 2.56 mg/g VSS with a reduction rate of 28.89% on day 2, then increased to 3.58 mg/g VSS on day 5, while polysaccharide content in TB-EPS increased first, then decreased in the control system. In addition, studies have shown that the hydrophobicity of the sludge surface is positively correlated with PN/PS in EPS, which affects sludge dewaterability.\[47,48\]

As shown in Figure 6c, the PN/PS of the LB-EPS remained stable in the first 4 days during activated sludge treatment by the mixed culture of $M$ and $P$, then increased sharply on the fifth day. The PN/PS of slime EPS and TB-EPS increased, then decreased, and then increased again after 4 and 5 days. This phenomenon may be caused by the destruction of cell walls and the release of intracellular proteins, carbohydrates and other organic materials after cell death.\[49\]

Several studies have shown that there is a significant positive correlation between SRF and CST.\[42\] In the present study, Pearson correlation analysis between the CST, SV$_{30}$, and sludge EPS was performed and the results are shown in Table 1. The CST was positively correlated with the slime EPS content ($R > 0.915$, $p < 0.01$) and the protein content in slime EPS ($R > 0.938$, $p < 0.01$), as well as the polysaccharide content in LB-EPS ($R > 0.593$, $p < 0.05$). The SV$_{30}$ was positively correlated with the slime EPS content ($R > 0.691$, $p < 0.05$) and protein content in slime EPS ($R > 0.669$, $p < 0.05$), as well as the polysaccharide content in LB-EPS ($R > 0.613$, $p < 0.05$). It should be noted that there are some different views about the contributions from polysaccharide and protein in sludge EPS. Although previous studies found that the content of slime EPS or polysaccharide content in slime EPS significantly influenced the dewaterability of sludge,\[17\] the present study revealed that the content of slime EPS, protein content in slime EPS or polysaccharide content in LB-EPS significantly influenced the dewaterability and settleability of activated sludge. The reason was that the biological fermented sludge with more protein content and the polysaccharides were a major component in LB-EPS, and they can be more easily degraded by mixed culture of $M$ and $P$.  

![Figure 6](image-url)
As discussed above, the mixed culture can degrade three types of EPS. Among them, the degradation of slime EPS and polysaccharides in TB-EPS was particularly high. The proteins and polysaccharides of slime EPS were degraded by 46.52% and 50.59% by the mixed culture of $M$ and $P$, respectively, while the polysaccharides in TB-EPS were degraded by 27.58%. In addition, the PN/PS of the EPS increased; thus, the hydrophobicity of the sludge surface was increased. These results indicated that their degradation in the treatment system resulted from the growth of strains of $M$ and $P$, and that overall trends were consistent with earlier studies.\[17\] Therefore, the degradation of polysaccharides and proteins in slime EPS and polysaccharides in TB-EPS by the mixed culture of $M$ and $P$ contributed to the improvement of sludge dewaterability during the mixed fungal treatment of activated sludge using the mixed culture of $M$ and $P$.

### The change in bound water during the treatment of activated sludge using the fungal mixed culture of $M$ and $P$

The change of bound water during the treatment of activated sludge using the fungal mixed culture of $M$ and $P$ is shown in Figure 7. The content of bound water in the activated sludge decreased sharply on the first day, then further decreased from 28.31 g/g DS to 10.02 g/g DS on the second day, after which it increased slightly during treatment with the mixed culture of $M$ and $P$. Conversely, the bound water of activated sludge in the control system fluctuated slowly, decreasing to a minimum value as high as 19.35 g/g DS on day 4. There is a huge gap of the bound water between the control system and mixed fungi inoculated sludge ($p = 0.019$, $p < 0.05$), indicating the growth of mixed strains of $M$ and $P$ greatly reduced the content of bound water in the activated sludge. In addition, studies have shown that most of the bound water in the sludge is absorbed by EPS.\[50\] Pearson’s correlation analysis between the content of bound water and sludge EPS was performed and the results are shown in Table 2. The content of bound water was significantly positively correlated with the slime EPS ($R > 0.827$, $p < 0.01$) and the content of protein in slime EPS ($R > 0.856$, $p < 0.01$), as well as with polysaccharide in LB-EPS ($R > 0.582$, $p < 0.05$). Therefore, it was possible that the mixed strains of $M$ and $P$ degraded the EPS in activated sludge and converted part of the bound water wrapped in EPS into free water, which is easy to separate from solid particles, thus improving the dewaterability of the activated sludge (Table 2).\[51\]

### Conclusion

The filamentous fungi $M$ and $P$ isolated from citric wastewater sludge were used for the fungal treatment of biological fermented sludge. The results showed that, when compared with single inoculation, mixed inoculations can more effectively reduce the CST, SRF, and $SV_{30}$ of activated sludge, as well as improve its dewaterability.

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**Table 1.** Coefficient of Pearson correlation between CST, $SV_{30}$, and slime EPS, LB-EPS, or TB-EPS during the treatment of citric sludge using mixed strains of *Mucor circinelloides* M37 and *Penicillium oxalicum* LY-1.

| Sludge composition | EPS content | Protein content | Polysaccharide content | EPS content | Protein content | Polysaccharide content |
|--------------------|--------------|-----------------|------------------------|--------------|-----------------|------------------------|
| Slime-EPS          | 0.915**      | 0.938**         | 0.253                  | 0.691*       | 0.669*          | –0.45                  |
| LB-EPS             | –            | –0.01           | 0.593*                 | –            | –0.366          | 0.613*                 |
| TB-EPS             | –            | –0.099          | 0.303                  | –            | –0.168          | 0.122                  |

*Two-tailed significance:* $**p < 0.01$, $*p < 0.05$, $n = 12$.

**Table 2.** Coefficient of Pearson correlation between bound water in sludge and slime EPS, LB-EPS, or TB-EPS during the treatment of citric sludge using the fungal mixed culture of *Mucor circinelloides* M37 and *Penicillium oxalicum* LY-1.

| Sludge composition | EPS content | Protein content | Polysaccharide content |
|--------------------|--------------|-----------------|------------------------|
| Slime-EPS          | 0.827**      | 0.856**         | 0.260                  |
| LB-EPS             | –            | –0.070          | 0.582*                 |
| TB-EPS             | –            | –0.041          | 0.376                  |

*Two-tailed significance:* $**p < 0.01$, $*p < 0.05$, $n = 12$.

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**Figure 7.** Change in bound water during the treatment of activated sludge using the fungal mixed culture of *Mucor circinelloides* XY-Z and *Penicillium oxalicum* LY-1.
and settleability. On one hand, the proteins and polysaccharides of EPS in the activated sludge were degraded to different degrees by the mixed fungi of M and P, resulting in an increase of PN/PS, which increased the hydrophobicity of the sludge surface. On the other hand, the mixed strains of M and P degraded the protein in slime EPS and polysaccharides in LB-EPS of the activated sludge, thereby releasing and converting part of the bound water wrapped in EPS into free water, which is easy to separate from solid particles, thus improving the dewaterability of the biological fermented sludge.

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Disclosure statement

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