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Addition of chitosan improves the efficiency of total phosphorus removal from wastewater using the D-A^{2}O reactor and metagenomic analysis

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ABSTRACT

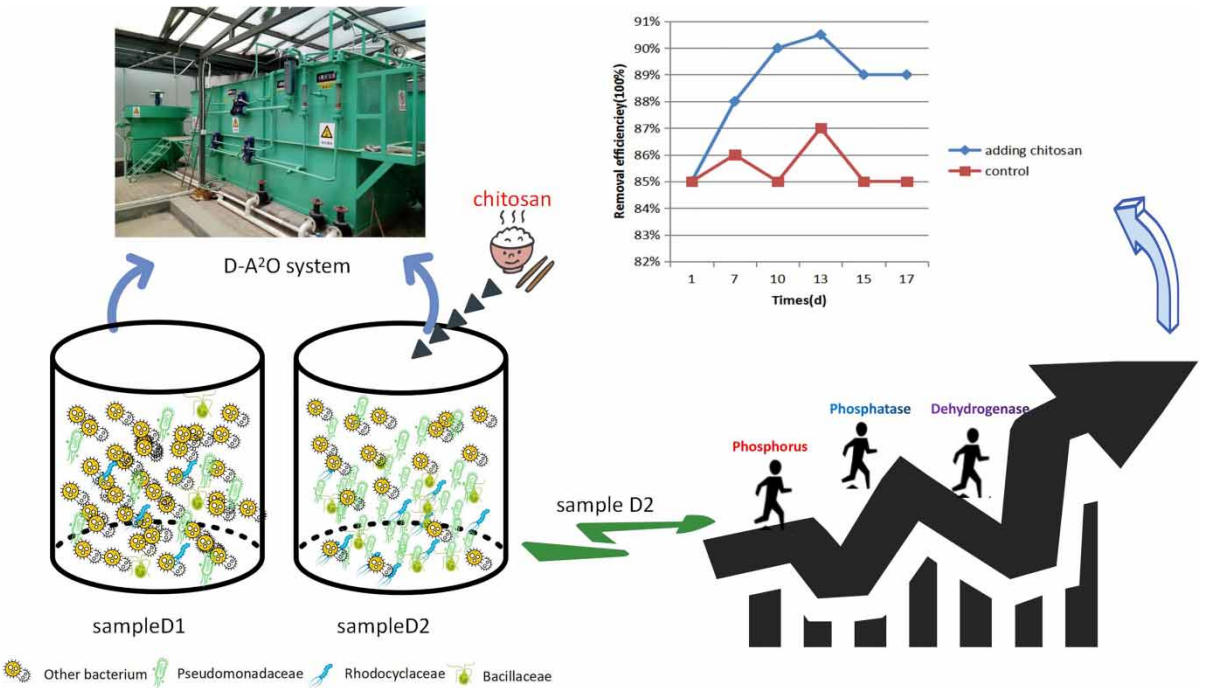
Microbial phosphate removal from wastewater sewage is a promising and feasible technique that increases the ability of a sewage treatment system to remove phosphate from wastewater. Maintaining a healthy population of phosphate-solubilizing bacteria is the key premise of biological sewage treatment. Chitosan is used to remove dissolved phosphorus from the water column during wastewater treatment. The present study found that chitosan has another function in phosphorus removal, affecting the diversity and community composition of phosphate-solubilizing bacteria. We obtained 16S rRNA genetic data by using a shotgun metagenomic sequencing method. Data indicated that phosphate-solubilizing Pseudomonadaceae was the dominant bacteria population, after adding chitosan to the dynamic water treatment process. In chitosan-enhanced treatments, populations were 35.11% larger than the control group. Chitosan addition also caused some increases in the population sizes of Rhodocyclaceae, Bacillaceae, and Enterobacteriaceae, but the addition of chitosan had little effect on Hyphomicrobiaceae and Sphingomonadaceae in the activated sludge. Moreover, the Chao1 estimator, the abundance-based coverage estimator (ACE), and Shannon index all indicated a very high diversity of bacteria when chitosan was added. Finally, we determined that chitosan increased the activity of the enzymes phytase, dehydrogenase, and phosphatase, which enhance the degradation rate of phosphorus in the activated sludge of a D-A^{2}O system. We suggest that chitosan plays an important role in dissolving organophosphorus during sewage treatment.

Key words: chitosan, enzyme activity, phosphate-solubilizing bacteria, species diversity

HIGHLIGHTS

- We report that we found another function of chitosan, that is, chitosan could affect the bacterial diversity and community composition in wastewater treatment.
- We solved the problem that to increase the total phosphorus removal efficiency of biological nutrient removal process for wastewater treatment in low temperatures.

GRAPHICAL ABSTRACT



INTRODUCTION

The world is progressing at a rapid pace, and this progression is exerting a lot of pressure on natural resources. The resources are limited, but demand has been increased to an all-time high. The nations are facing many issues including climate change and extinction of natural flora and fauna (Neetu *et al.*, 2019). Water is important for life, and climate change is expected to produce significant effects on global water resources and freshwater ecosystem sand. There are growing concerns around the impact of climate change on the water resources. Wastewater treatment effluent is a major global environmental problem, which is increasingly affecting our precious freshwater resources.

Over the past several decades, biological nutrient removal (BNR) processes have been widely used to treat wastewater containing nitrogen and phosphorus, as well as chemical oxygen demand (COD) to prevent eutrophication due to their economic advantages compared with chemical treatment methods (Yong *et al.*, 2006). For effective removal of both N and P, it is important that the bioreactor system can degrade N and P concentrations in the wastewater. In conventional wastewater treatment, N and P are removed from wastewater in two separate processes. Usually, N is converted to N_2 gas through coupled nitrification–denitrification, whereas P is precipitated with metal salts. The biological removal of phosphorus, called enhanced biological phosphorus removal (EBPR), is a two-step process involving polyphosphate-accumulating organisms (PAOs). The A^2O process is a sequential wastewater treatment process that uses anaerobic, anoxic, and oxic chambers for nitrogen and phosphorus removal. The A^2O is globally one of the most widely used biological sewage treatment processes, but it also has some challenges, such as the P and N removal capacity and the balance between beneficial and detrimental organisms in the treatment plants (Tian *et al.*, 2015). A^2O systems also face other challenges, such as

being able to maintain a sufficient supply of carbon sources for both the PAOs and nitrogen-removing microorganisms.

The D-A^{2}O process was developed to treat municipal wastewater, by removing nitrogen (N), phosphorous (P), and organic carbon from sewage before the effluent discharges to a receiving water (Ye *et al.*, 2018). The novel D-A^{2}O system creates enhanced anoxic environments and simultaneously removes organic matter and nutrients and improves effluent quality. The system's unique alternating operation mode (A/B series) and divisional influent tanks were useful in microbial regulation and timely carbon source supplementation (Ye *et al.*, 2016).

The D-A^{2}O system, which incorporated aspects of the traditional A^{2}O system with a modified-SBR system, results in a hybrid wastewater management system that creates enhanced anaerobic/anoxic environments that can simultaneously remove organic matter and nutrients and improve effluent quality. However, the phosphorus removal rate of the system is not stable, and the average removal efficiency ranges up to 90.03% in the 25 °C summer, and down to 80% in winter. It is important to further study how to increase the total phosphorus removal efficiency in winter, when it is cold. This article provides a good enlightenment that application of an effective microorganism product as a cyanobacterial control enhances water quality (Zati *et al.*, 2020). The phosphate-solubilizing bacteria is the best choice for enhancing consequent oxic uptakes of phosphorous.

Chitosan is a natural biopolymer modified from chitin, which is the main structural component of squid pens, cell walls of some fungi and shrimp and crab shells (Boonlertnirun *et al.*, 2008). Many reports have shown chitosan can adsorb water extractable phosphorus (WEP) (Sivakami *et al.*, 2013; Simpson, 2014). Chitosan, as an effective adsorptive agent, could remove excess phosphate from water and purify the water (Zou, 2012). Some phosphate-solubilizing bacteria have a gene promoting chitosan production (*csn*), for example, *Bacillus subtilis* and *Pseudomonas* sp. This research tries to add chitosan to the D-A^{2}O reactor to promote the biological cell synthesis of phosphate-accumulating organisms in activated sludge, thus increasing phosphorus removal in wastewater and improving water quality.

MATERIALS AND METHODS

Sample collection and processing

Chitosan was added to an oxic tank (50 L) of one D-A^{2}O reactor, sample D2, in the proportion of 1:100. The activated sludge concentration was 3,000 mg/L. The other D-A^{2}O reactor, sample D1, without chitosan, was used as the control. The two reactors were inoculated with activated sludge from the Yuxi Sewage treatment plant (Yunnan, China). The wastewater used in our experiments came from the campus of Yuxi Normal University (24_2003900N, 102_3303300E). The characteristics of the sewage were as follows: COD was 288–405 mg/L, NH_3-N was 28.26–47.62 mg/L (average value of 31.22 mg/L), TN was 37.26–58.45 mg/L (average value of 52.23 mg/L), TP was 2.86–7.43 mg/L (average value of 4.75 mg/L), pH value was 7.45–8.32 (average value of 7.68). In the process, the temperature was 15 $\pm$ 1 °C, the return ratio of sludge (r) was 80%, the return ratio of mixture (R) was 200%, the hydraulic retention time (HRT) was 8 h, and the double anoxic alternative operating time (DAOT) was 1 h.

Analytical methods and data analysis

TP was analyzed according to the details in APHA (2003). The experiment was performed in triplicate each week. The statistical analysis of the results was carried out using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

DNA extraction

DNA was extracted from 0.25 g aliquots of activated sludge samples in quadruplicate using the Mobio Biometry Project DNA extraction protocol and a Power Soil DNA isolation kit (MoBio, Carlsbad, CA, USA, catalog#12888-100). Quadruplicate samples were combined after extraction.

16S rRNA gene sequencing and analysis

The 16S rRNA gene from the DNA extracts was amplified in triplicate with archaeal and bacterial primers of 515F and 806R, which target the V4 region of *E. coli* according to the details in Caporaso *et al.* (2010). Samples were analyzed using the Prinseq version 0.20.4 and QIIME version 1.8.0-devpipeline. Raw sequences were demultiplexed, then quality-filtered with the default parameters in QIIME (Mason *et al.*, 2014; Stolze *et al.*, 2015). Sequences were then clustered into operational taxonomic units (OTUs, 97% similarity) using Mothur version 1.30.1. Alpha diversity analysis was employed to measure sample species diversity, to calculate ACE, the Chao1 estimator, Shannon, and Simpson diversity indices. Beta diversity analysis was used to compare samples. Phylogenetic analysis was conducted using MEGA 5.7.1.

Analysis of enzyme activity

We determined activity of the enzymes that enhance the degradation rate of phosphorus in D-A^{2}O systems with the following method.

Phytase activity was assayed by measuring the amount of inorganic phosphate (Pi) released by hydrolysis using sodium phytate. 100 mL of phytase preparation were added to a 1.0 mL assay mixture containing 100 M sodium acetate buffer (pH 4.5) and 1 mM sodium phytate, and incubating the mixture at 37 °C for 1 h. The reaction was terminated by the addition of 0.5 mL 10% trichloroacetic acid (CCl_3COOH). One unit of phytase activity was defined as the amount of enzyme that released 1 μmol Pi min^{-1}.

The dehydrogenase activity in activated sludge was determined as described by Klein *et al.* (1971). Briefly, 1 g of air-dried sludge sample was saturated with 0.2 mL of 3% triphenyltetrazolium chloride (TTC) solution and incubated for 24 h at 28 ± 0.5 °C, then 10 mL of methanol was added and the mixture was shaken vigorously. The clear pink supernatant was withdrawn after 6 h and the absorbance at 485 nm was measured in a spectrophotometer. The amount of triphenylformazan (TPF) formed in each sample was calculated from the standard curve drawn in the range of 10–90 μg TPF mL^{-1}. The dehydrogenase activity is expressed as the amount (μg) of TPF formed per gram of sludge per hour.

Centrifuged activated sludge was used to colormetrically estimate phosphatase activity following the procedure of Tabatabai & Bremner (1969).

RESULTS

The efficiency of TP removal after adding chitosan

Excess phosphorus in water is responsible for several possible types of problems. Phosphorus removal can significantly reduce eutrophication. Minimizing phosphorous in wastewater treatment plant discharges and treating chemical waste containing phosphorous is a necessary priority. Numerous research papers discussing phosphorus in wastewater and the use of phosphate-solubilizing bacteria as treatment have been published so far. Increasing the population density of phosphate-solubilizing bacteria is an efficient method for the treatment of many types of wastewater. To determine chitosan's role in TP removal, this experiment was conducted six times in two separate reactors. As shown in Figure 1, sample D2 with chitosan has a higher efficiency for TP removal than sample D1, without chitosan. The average efficiency for TP removal in the two reactors varies from 86 to 89%. Therefore, we suggest that the addition of chitosan is helpful to enhance the efficiency of TP removal.

Differences in bacteria community composition and abundance

Alpha diversity mainly measures species number in one uniform habitat. Statistical results of every sample's alpha diversity value are shown in Table 1. The results show that all the samples have an alpha diversity value higher than 94%, which means our experimental data is reliable because it effectively reflects a diversity of samples. The

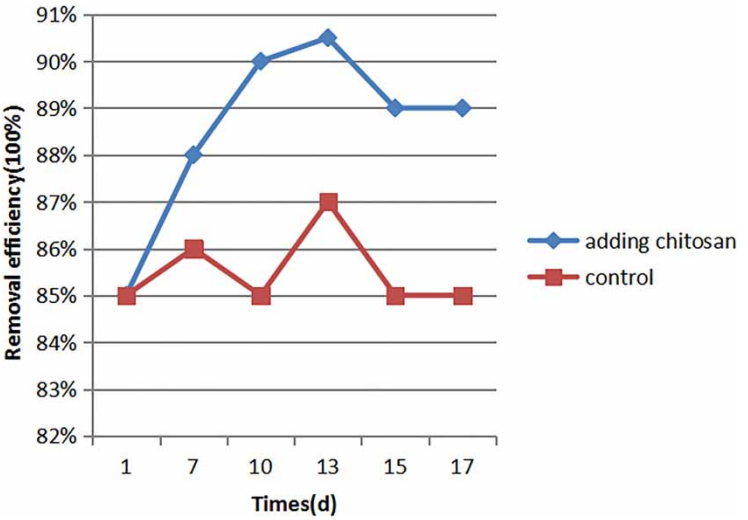


Fig. 1. | Removal efficiencies of TP at different time intervals.

Table 1. | Community richness and diversity indices for samples D1 and D2.

Sample	No. of sequences	OTUs	Shannon_in	ACE_index	Chao_index	Simpson	Coverage
D1	80,298	8588	6.46	48518.42	28484.79	0.05	0.99
D2	129,814	11246	5.58	98894.98	48338.98	5.6×10^{-03}	1

number of diverse sequences in the samples with chitosan added (D2) was higher than the sequence diversity in samples without chitosan (D1). A total of 80,298 and 129,814 sequences of 16S rRNA from bacteria were recovered from D1 and D2 samples, respectively. The community analysis and libraries of these samples contained 11246 and 8588 OTUs.

In this study, the major phyla had a relative abundance $\geq$1%. The phylum comprising the D1 samples are shown in Figure 2. The sample was mainly composed of unclassified bacteria (28.94%), Rhodosporillaceae (9.6%), Anaerolineaceae (3.29%), Comamonadaceae (2.88%), and Rhodocyclaceae (2.51%). Sample D2 was primarily composed of Pseudomonadaceae (35.16%), unclassified bacteria (18.6%), Rhodocyclaceae (4.19%), Anaerolineaceae (3.12%), Rhodospirillaceae (2.49%), and Bacillaceae (2.24%). Therefore, the major bacterial groups were slightly different between the two samples. The difference among samples is more evident at the class level. Pseudomondaceae, Rhodocyclaceae, Anaerolineaceae, and Rhodospirillaceae were dominant in both samples, but their abundances varied greatly. Pseudomonadaceae were dominant in the D2 sample (Figure 3), being 35.11% higher in abundance than the control. Furthermore, the population density of Rhodocyclaceae increased from 2.51% in the D1 sample (Figure 2) to 4.19% in the D2 sample (Figure 3) after adding chitosan. The population of Bacillaceae slightly increased from 1.8 to 2.24% in D1 and D2 samples, respectively. With chitosan added, the community number of the typical degrading bacteria such as Anaerolineaceae (3.12%) in the sewage treatment system had unaffected population structures. Therefore, the addition of chitosan significantly increased the population density of Pseudomonadaceae and Rhodocyclaceae in the activated sludge. This result explained the higher efficiency of TP removal after adding chitosan.

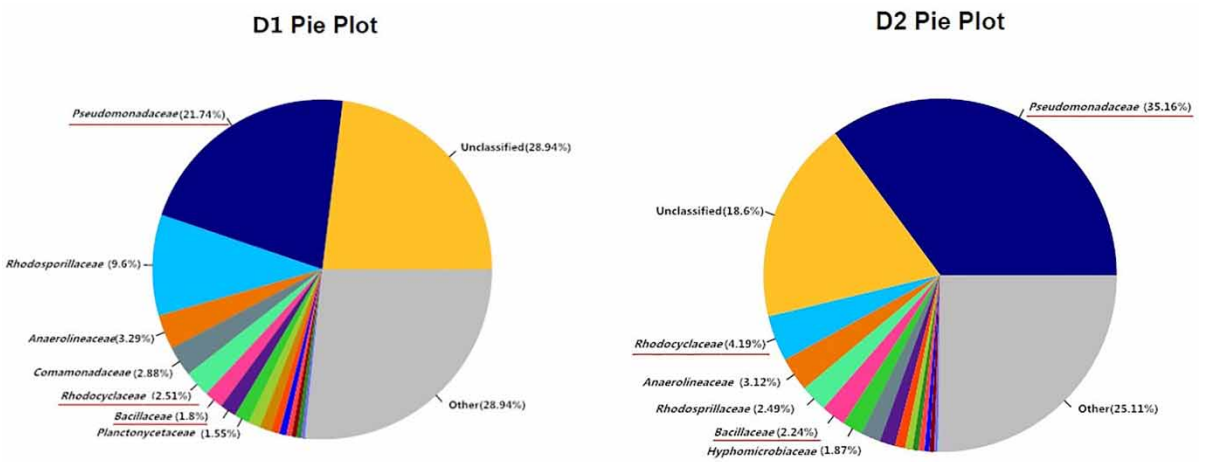


Fig. 2. | Genus level D1 and D2 group sample abundance pie.

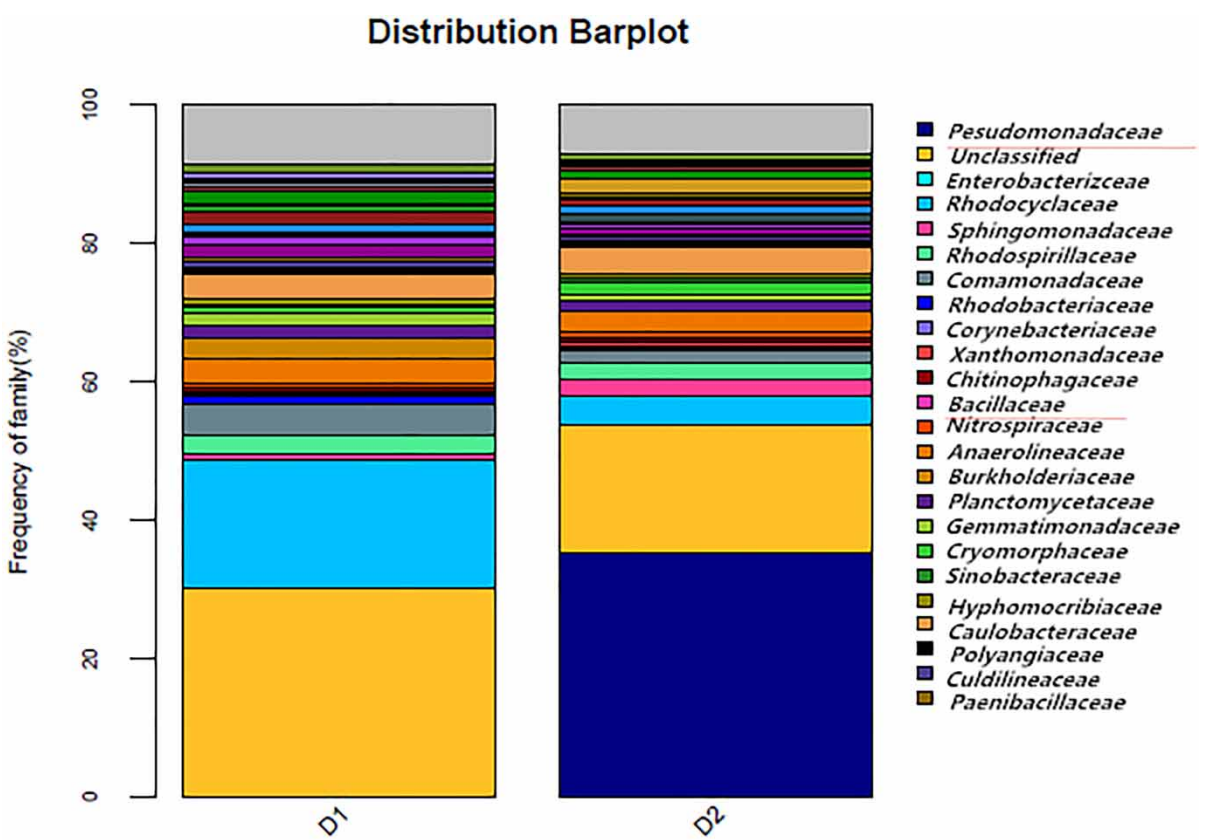


Fig. 3. | Sample groups D1 and D2 community structure distribution charts.

A large number of studies have shown Pseudomonadaceae to be a phosphate-solubilizing bacteria family, which can degrade organophosphorus into inorganic phosphorus. The common phosphate-solubilizing bacteria include *Bacillus*, *Pseudomonas*, *Rhodocyclales*, *Erwinia*, *Agrobacterium*, *Serratia*, *Flavobacterium*, and *Enterobacter*. In this study, the addition of chitosan had a clear effect, inducing and promoting the growth of phosphate-solubilizing bacteria of the Pseudomonadaceae, Rhodocyclaceae, Bacillaceae, and Enterobacteriaceae families in the activated sludge. The P bioaccumulation by the members relating to *Pseudomonas* of γ-proteobacteria had been reported early by Sresb *et al.* (1985) and confirmed by many researchers, e.g. Lin *et al.* (2003). Many studies showed that the Pseudomonadaceae could produce gluconic acid to break down mineral phosphate in the soil, and gluconic acid is produced by catalyzing the conversion of glucose with glucose dehydrogenase in the periplasmic space of its cells. Some papers have shown that Pseudomonadaceae can produce the exocellular chitosan enzyme (Li & Nesbet, 1966; Huang *et al.*, 2009; Wang *et al.*, 2014). The addition of chitosan could provide some bacteria with enough ingestible chemicals, and accelerate the growth and reproduction of the community. However, Pseudomonadaceae can also produce some active enzymes, including the phytases and phosphatase. Therefore, the degradation rate of insoluble phosphate in the reactor is raised. This may be the reason for the increased degradation rate of organophosphorus in the D-A^{2}O reactor containing chitosan. On the other hand, Rhodocyclus belongs to 'Betaproteobacteria' that were originally isolated as chitinase-producing bacteria. In a study by Hesselsoe *et al.* (2009), *Rhodocyclales* were identified in a nitrifying–denitrifying, phosphorus removing activated sludge process with unique substrate-utilization profiles.

In addition, the percentages of Hyphomicrobiaceae and Sphingomonadaceae increased in the D2 sample. More importantly, bacteria can promote the degradation of some harmful substances, and Sphingomonadaceae can degrade aromatic compounds. Therefore, the bacteria effectively degrade organic matter in sewage. Hyphomicrobiaceae can promote the denitrification of sewage. The increased frequency of Pseudomonadaceae is also evident in the community distribution bar plot (Figure 4).

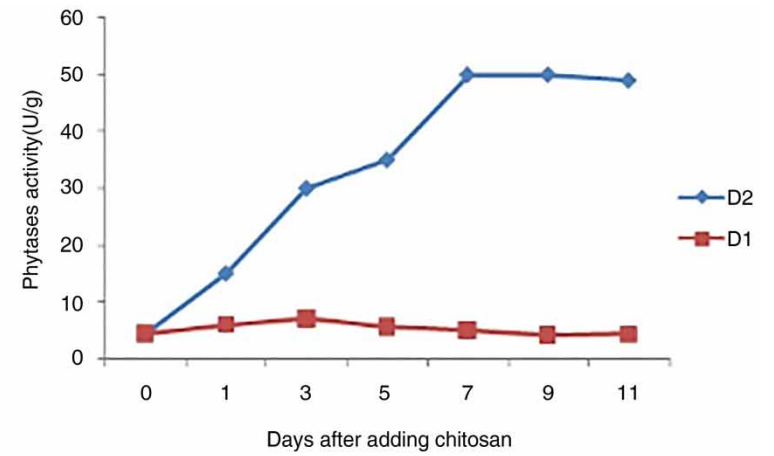
Analysis of enzyme activity

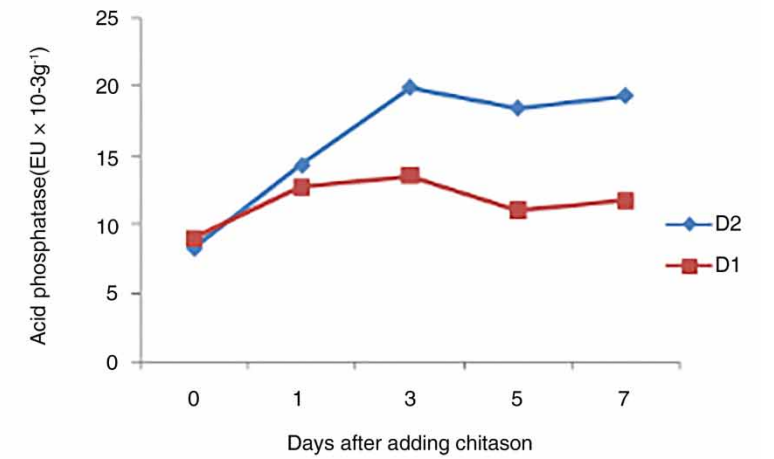
In order to verify the induced effect chitosan has on phosphate-solubilizing bacteria, the phosphate-related enzyme activities of the D1 and D2 samples in the D-A^{2}O reactors were determined.

Phosphorus in the soil exists mainly in the form of organic phosphorus such as phytate anions, nucleic acid, and phospholipids. Among them, phytate anions account for 50–80% of the organic phosphorus in the soil. Phytase enzyme activity was comparable between samples (Figure 4). Specifically, the phytase activity of the D2 sample increased significantly from 4.3 to 15 U/g after day 1, to a maximum value of 50 U/g after adding chitosan for 7 days. The addition of chitosan is an important influencing factor, as it regulates phytase to release P.

Phosphatase plays an important role in studying the phosphate-solubilizing mechanism of organic phosphates. Microbial degradation of organic phosphates is achieved by secreting phosphatase. Microorganisms were found to be highly efficient at the hydrolysis of organic P compounds via the phosphatase enzyme. In general, sample D2 had higher acid phosphatase activity than sample D1. Acid phosphatase activity increased markedly after day 1 (8.3–14.34 U/g), then reached its maximum value after day 3 (20 U/g) (Figure 4). The results of the present study show that the addition of chitosan can promote the release of the enzyme. Many reports have shown that phosphatase and phosphate-solubilizing bacteria play major roles in lowering phosphate concentrations (Zhang *et al.*, 2014). Several bacteria, especially phosphate-solubilizing bacteria such as Pseudomonadaceae, can produce phosphatase and phytase enzymes (Li *et al.*, 2017). Therefore, the increasing phosphatase and phytase content observed in the present study may be related to the increase of the Pseudomonadaceae community.

Dehydrogenase activity is an important indicator of the biological activity of sludge. Variations in dehydrogenase activity were recorded in different sample groups (Figure 4). At day 1, the two groups had similar values, and





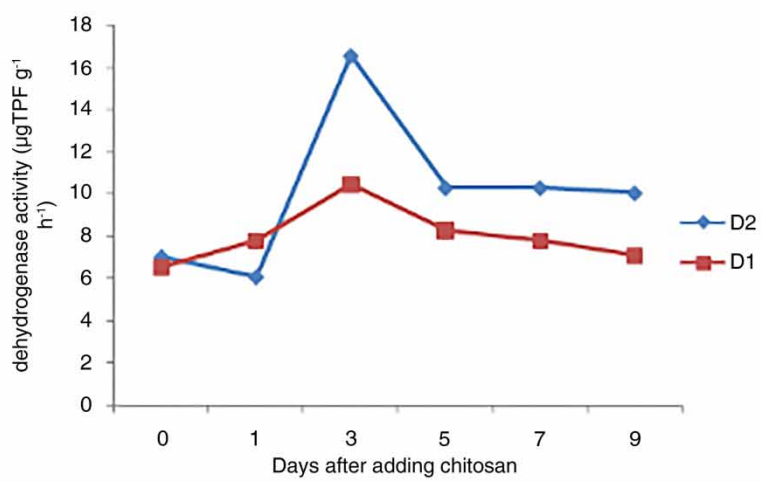


Fig. 4. | Differences in three enzymes activity between the two samples.

D2 and D1 were at 6.984 and 6.501 μg TPF g^{-1} h^{-1}, respectively, in sewage sludge. The dehydrogenase activity of sample D2 was 1.5 times higher than sample D1, after adding chitosan for 3 days. The highest value, 16.547 μg TPF g^{-1} h^{-1}, appeared after day 3. Dehydrogenase activity is considered to reflect the total range of oxidative activity of sludge microflora and to be a good indicator of microbial activity (Benefield *et al.*, 1977). The enzyme activities in the soil are generally closely related to the organic matter content (Beyer *et al.*, 1993). The application of chitosan also lowered levels of other nutrients in sewage sludge, which corresponds to higher enzyme activity.

The above results show that the activity of three phosphate-solubilizing enzymes in the D2 samples has increased with the addition of chitosan. The increased activity of phytase, phosphatase, and dehydrogenase promoted the degradation of organophosphorus in the D-A^2O wastewater treatment reactor, and phytase had the highest increased activity.

The results show some fluorescent *Pseudomonas* species can produce highly active phytase. So the significantly increased phytase activity may be related to the increased density of fluorescent *Pseudomonas* species. Dehydrogenase activity reflects microbial cell synthesis – the greater the dehydrogenase activity, the stronger the microbial cell synthesis. In this research, according to metagenomics sequencing, the population density of Pseudomonadaceae, Rhodocyclaceae, Bacillaceae, and Enterobacteriaceae increases, which exactly verifies this result. Pseudomonadaceae and Rhodocyclaceae are well-known as phosphorus-accumulating bacteria with aerobic and phosphorus-absorbing features. While under hypoxic conditions, they also have a strong phosphorus-accumulating function, especially Pseudomonadaceae. In the case of adding chitosan, chitosan will act as an organic carbon source for phosphorus-accumulating microorganisms such as Pseudomonadaceae, Rhodocyclaceae, Bacillaceae, and Enterobacteriaceae, providing these bacteria with better growth conditions, strengthening their ability to grow and reproduce, as well as enhancing the dehydrogenase activity. Among others, for the increasing microbial population Pseudomonadaceae and Bacillaceae, existing research has supported their secretion of dehydrogenase in growth and multiplication.

DISCUSSION

Chitosan is produced by the deacetylation of chitin and becomes aqueous in solution when deacetylation reaches about 50%. Chitosan is a biodegradable polymeric compound, which is abundant in nature, and is nontoxic, biodegradable, highly reactive and can form chelates.

Several studies on the use of chitosan for lake management have been published, because it is efficient at adsorbing P (Funes *et al.*, 2014; Mucci *et al.*, 2017). Lin Cheng reported that chitosan and lanthanum hydroxide composite aerogel beads could be prepared for phosphorus adsorption via a co-precipitation and supercritical carbon dioxide drying technique (Lin *et al.*, 2018). During the acidification of chitosan, $-NH_2$ and H^+ combine to form $-NH_3^+$, which can bond to $H_2PO_4^-$. In this study, we first confirmed that chitosan can promote phosphate-solubilizing bacterial growth.

In this study, metagenomic sequencing showed that the addition of chitosan increased the population densities of some phosphate-solubilizing bacteria in the activated sludge, such as Pseudomonadaceae, Rhodocyclaceae, Bacillaceae, and Enterobacteriaceae. There are many species of phosphate-solubilizing microorganisms including bacteria, fungus, and actinomycetes. Among them, bacteria have been studied the most, and research focuses mainly on *Pseudomonas*, *Serratia*, *Erwinia*, *Agrobacterium*, *Flavobacterium*, and *Escherichia*. It was reported that some bacteria have the gene for chitinase, such as Pseudomonadaceae, Rhodocyclaceae, Bacillaceae, and Enterobacteriaceae. We suggest that chitosan provides a source of carbon, resulting in increased population densities of these bacteria, and also increased enzyme activity, which dissolves organophosphorus.

The degradation of organic matter primarily relies on microbial communities and extracellular enzymes produced by them (Sinsabaugh *et al.*, 1993). The levels of enzymes in environmental systems vary in amount

primarily due to the variety of living organisms and differing intensities of the biological processes. Phytase proved to be efficient in hydrolyzing phytate and delivering inorganic P to growing plants (Trouillefou *et al.*, 2015). Phosphatases are a broad group of enzymes that are capable of catalyzing hydrolysis of esters and anhydride of phosphoric acid (Schmidt & Laskowski, 1961). Some studies showed that soluble phosphorus and phosphorus utilization efficiency are concerned with the content and activity of phosphatase; however, dehydrogenase activity is more commonly used as an indicator of biological activity in the soil. In summary, the present study's experiments on three enzymes showed phytase, phosphatase, and dehydrogenase activities increased after adding chitosan, and also explained that chitosan plays a certain role in the degradation of insoluble phosphate.

In the D-A^2O biological nitrogen and phosphorus removal process, under the alternate environment of anaerobic, anoxic, and aerobic, heterotrophic bacteria, nitrifying bacteria, denitrifying bacteria and phosphorus-accumulating bacteria coexisting in the system can take the organics in the sewage as a carbon source, and realize the simultaneous removal of COD, N, and P. In this study, only the effect of chitosan addition on phosphorus removal efficiency was explored. The results showed that chitosan contributes to increasing the population density of phosphorus-accumulating bacteria in activated sludge, and increases the activity of dehydrogenase, phytase and other enzymes, thereby achieving the purpose of improving dephosphorization efficiency. With the addition of chitosan, from the data on reactor water quality monitoring, the effluent of its water quality reached the standard of first-level A. However, this paper did not specifically consider the effect of chitosan addition on the denitrification function of the sewage treatment reactor, which requires further study.

FUNDING

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CONTRIBUTIONS

Y.C. conceived the idea. Z.J. designed and carried out the experiments. L.M. helped review the manuscript. L.Q. and Z.Z. performed data analysis and prepared the figures.

CONFLICTS OF INTEREST

All the authors declared that they have no conflicts of interest.

COMPLIANCE WITH ETHICS REQUIREMENTS

This article does not contain any studies with human or animal subjects.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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