Conditioning Effect of Lysozyme Combined with Freeze-Thaw on the Municipal Sludge Dewatering Performance

Feng Lin, Jigeng Li, Xiaolin Zhu, Peiran Yu, and Mengru Liu *

The effects of lysozyme (LZM) and freeze-thaw conditioning, alone or in combination, on sludge dewatering performance were comparatively investigated. After the optimization of the dewatering conditions using response surface methodology (RSM), the co-conditioning exhibited obvious superiority to the separate conditioning in improving the dewaterability of municipal sludge, with the capillary suction time (CST) and the water content (W) of the dewatered sludge reduced to 12 ± 0.5 s and 52.0% ± 0.4% from 61.8 s and 73.0%, respectively. The co-conditioning appeared not only to destroy the structure of extracellular polymeric substance (EPS) and microbial cell wall by virtue of enzymatic conditioning, but it formed larger particles and compact sludge floc structure with the help of freeze-thaw conditioning. Additionally, the bound water content of sludge decreased by 47.5% after co-conditioning, consistent with the enhancement in sludge dewaterability. All the results showed that LZM in combination with freeze-thaw conditioning had a great potential in sludge reduction, providing more opportunity of resource utilization for the dewatered sludge.

Keywords: Lysozyme; Freeze-thaw; Response surface methodology; Sludge dewaterability; Resource utilization

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INTRODUCTION

The widespread application of municipal sludge processing in wastewater treatment has increased the quantity of sludge with the passage of years. As a valuable biomass resource, the main options for utilization of municipal sludge are combustion to generate electricity, as a conditioner and fertilizer for soil, and as material for making building materials; such utilization not only can solve the problem of large sludge output, but also produce huge environmental and economic benefits (Tay and Show 1997). However, the reduction, detoxification, and resource recovery have been regarded as the optimal treatment sequence for dealing with the waste (Chen *et al.* 2015). As nearly all the resource utilization of sludge usually requires the water content (*W*) to be below 60%, sludge dewatering as an effective means of sludge reduction is the key to tackling the challenge of sludge treatment and disposal (Wei *et al.* 2018).

Together the extracellular polymeric substance (EPS) and water within sludge account for about 80% of the total municipal sludge mass (Liu *et al.* 2016), which is thought to be the most crucial factor in sludge dewatering. EPS usually occurs in the form of highly hydrated capsules surrounding cell walls. The capsules contain large amounts of hydrophilic functional groups, which could change the surface characteristics of sludge particles, thus increasing their viscosity and hydrophilicity (Liang *et al.* 2016). As a

consequence, it is necessary to destroy the EPS structure for better sludge dewaterability. Conventional coagulant and flocculation agents usually improve the sludge filtering performance, but they hardly influence the destruction of the EPS structure, which may account for the almost unchanged dewatering extent after chemical conditioning (Yan *et al.* 2013). As a natural biodegradable enzyme, LZM could destroy the microbial cell wall and EPS structure, leading to the improvement of sludge dewaterability (He *et al.* 2014). LZM was shown to increase the cake solids content from 7% to 28.7%, while it could not change the capillary suction time (CST) of sludge, which indicates the filtration performance (Liang *et al.* 2016). However, Bonilla *et al.* (2015, 2018) found that LZM could reduce CST of the paper mill sludge and reported that inactive LZM provides the same dewatering effect as active LZM. In comparison, freeze-thaw conditioning environmentally reduced the water content of dewatered sludge, accompanied by better filtration performances. For instance, the *W* value of municipal sludge was dewatered to 66.5% after freezing and thawing at -12 °C (Haung *et al.* 2008), which is still behind the requirement of resource utilization. Since each approach has its own pros and cons, the combination of LZM and freeze-thaw conditioning was conducted in this work to obtain both superior filtration and dewatering performance of municipal sludge.

Considering the respective advantage of LZM and freeze-thaw in sludge dewatering, this study aimed to investigate the effect of the above-described procedures alone or in combination conditioning on sludge dewaterability. What's more, the mechanism of co-conditioning was preliminarily evaluated by observing the changes in sludge physicochemical properties.

EXPERIMENTAL

Materials

Municipal sludge samples were collected from the secondary sedimentation tank of Lijiao wastewater treatment plant in Guangzhou, China. They were stored at 4 °C. The TSS and VSS contents were 21.61 g/L and 8.38 g/L, respectively. The main pollution and nutrient elements of sludge are listed in Table 1. The dosages of lysozyme are listed in Table S1.

Table 1. Main Pollution and Nutrient Elements of Sludge

Sludge sample	Zn	Cu	Pb	Ni	Cd	As	$N+P_2O_5+K_2O$ (g/kg DS)	Calorific value (kJ/kg DS)
Raw sludge	480	280	27	110	0.69	20	55.11	10170

RSM Optimization Experiment

On the basis of single factor experiment, the Box-Behnken design (BBD) was employed to optimize the sludge dewatering conditions as shown in Table 2.

Table 2. Range and Levels of Natural and Corresponding Coded Variables for RSM

Variable	Symbols	Range and levels		
		-1	0	1
LZM dosage (×10^6U/g DS)	X_1	3.2	4.8	6.4
Freezing temperature (°C)	X_2	-30	-25	-20
Freezing time (h)	X_3	4	5	6

Analytical methods

Lysozyme activity assay

The activity of LZM was measured using a detection kit (Sigma-Aldrich Co. LLC., US) (Chipman and Sharon 1969). The operational process is in the Test S1.

Determination of the sludge dewaterability

A lab-scale pressure filtration system based on static pressure, featuring a hydraulic drive piston which could move up and down in an enclosed space, was used for sludge dewatering (Fig. S1). The *W* of sludge was examined by a Halogen Moisture Analyzer (HX204, Mettler Toledo, UK). CST was measured with a CST instrument (Model 304M, Triton Electronics Ltd, Dunmow, UK). The bound water content of sludge was determined by differential scanning calorimetry (DSC) (Liu *et al.* 2016).

Characterizations of protein, polysaccharide and DNA in EPS

The protein concentration in the soluble EPS (S-EPS), loosely bound EPS (LB-EPS), and tightly bound EPS (TB-EPS) was measured by the Coomassie brilliant blue method with bovine serum albumin (BSA) as a standard (Bradford 1976). The polysaccharide concentration of the EPS was determined using the anthrone method with a glucose standard (Raunkjær *et al.* 1994). And a blank test with only LZM was used to exclude the effect of LZM on EPS concentration. The DNA content in the EPS was analyzed using the modified diphenylamine method with calf thymus DNA sodium as the standard.

Analysis of sludge floc structure

A particle size analyzer (MS3000, Malvern, UK) was employed to measure the median diameter (Dv [50]) of the sludge particle size. The floc images were acquired by a Stereo Microscope (SZX12, Olympus, Japan) and the projection area A, perimeter P and long axis La were measured using the Image-Pro Plus software. Finally, the one-dimensional fractal dimension and two-dimensional fractal dimension were obtained according to the lg P-lg La and lg A-lg P (Zhao *et al.* 2013).

RESULTS AND DISCUSSION

Effects of LZM and Freeze-thaw on the Sludge Dewaterability

The enzyme activity of LZM is 1.6×10^4 U/mg. As displayed in Fig. S2a, the *W* of dewatered sludge decreased to 58.5% from 73.0% at a dosage of 4.8×10^6 U/g DS LZM, and subsequently it remained almost stable when continuing to increase the LZM dosage. Additionally, the change of CST had the same tendency as *W* of dewatered sludge, and CST decreased from 61.8 s to 37.3 s, which means CST went down to 14.9 s/g DS from

24.7 s/g DS, still more than 10.1 s/g DS, suggesting a poor sludge filtering performance (Yan *et al.* 2013).

Both *W* and CST of the sludge simultaneously reached the minimum at the freezing temperature of -25 °C and freezing time of 5 h in Fig. S2 b and c, with CST down to 15 s. Despite the obvious enhancement of sludge filtration, the resulting minimum *W* of 62.8% was not satisfactory. In view of the requirement of sludge resource utilization and their respective advantage, freeze-thaw in combination with LZM conditioning was carried out in the further study.

Combined Conditioning

According to the single factor experiment, LZM dosage (X_1), freezing time (X_2), and freezing temperature (X_3) all evidently affected the sludge dewatering performance. Considering the interaction between above parameters in joint conditioning, response surface methodology (RSM) was used to analyze the problems affected by multiple variables and optimize the response values (Qi *et al.* 2013). Therefore, X_1, X_2, and X_3 were taken as three factors, and *W* (Y_1), as well as CST (Y_2), was considered to be the response value to optimize the sludge dewatering conditions in the combined conditioning process. The experimental data obtained by BBD were analyzed by RSM using second-order polynomial equations as Eqs. 1 and 2:

$$Y_1=53.26 - 2.36X_1 + 1.61X_2 - 2X_3 + 0.71X_1X_2 - 0.16X_1X_3 + 0.12X_2X_3 + 3.53X_1^2 + 2.82X_2^2 + 0.71X_3^2 \quad (1)$$

$$Y_2=13.75 - 0.37X_1 + 3.11X_2 - 1.91X_3 - 0.025X_1X_2 - 0.34X_1X_3 - 0.71X_2X_3 + 1.88X_1^2 + 2.80X_2^2 + 1.03X_3^2 \quad (2)$$

From Fig. 1, the actual and predicted experimental values were distributed along the linear function, indicating that these values were in an obvious correlation. What's more, the model established with *W* of the dewatered sludge as the response value was significant ($P< 0.05$) with a coefficient of determination of $R^2= 0.99$ according to the results of the variance analysis in Table S2. The independent factors X_1, X_2, and X_3 all had prominent effects on the water content, and X_1X_2 in the quadratic and interaction terms also showed remarkable effects ($P<0.05$). Meanwhile, the model of CST was significant ($P<0.05$) with a coefficient of determination of $R^2= 0.97$ in Table S3. The independent factors X_2 and X_3 had prominent effects on CST, and the quadratic effects (X_1^2, X_2^2, and X_3^2) also exhibited important influence. Therefore, the above factors had notable interaction and synergistic effect to improve the dewatering performance of sludge.

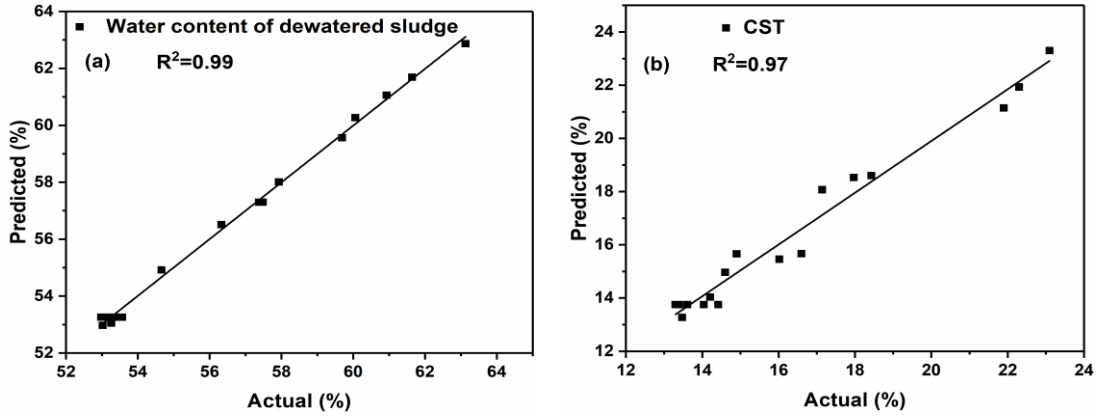
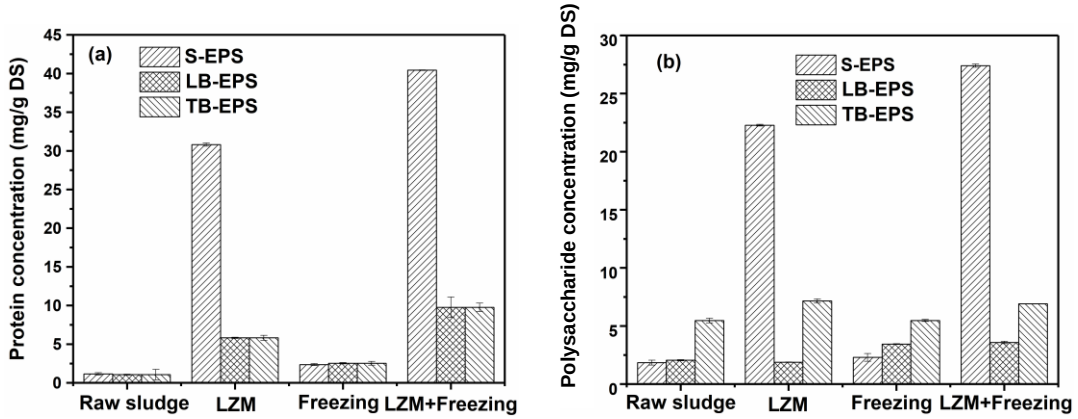


Fig. 1. Comparison of predicted and actual results: (a) Water content of the dewatered sludge and (b) CST

The optimal conditions were derived by software analysis as follows: the LZM dosage of 5.9×10^6 U/g DS, the freezing temperature of -26.56 °C, and the freezing time of 5.78 h, with *W* of 51.68% and CST of 12.83 s. Parallel tests were carried out under the optimal conditions, which obtained a CST of 12 ± 0.5 s and dewatered sludge with *W* of 52.01% ± 0.4%, respectively. These values were not only consistent with the model predictions. This was superior to the previous study (Wang *et al.* 2001), which reported that the *W* of the dewatered sludge could be reduced to 62.8% after cationic surface active agent in combination with freeze-thaw conditioning.

Effects of EPS Properties on Sludge Dewatering Performance

The chemical composition and spatial distribution of EPS had dominant effects on the bio-flocculation, settling, and dewatering performance of the activated sludge (Wei *et al.* 2018). In Fig. 2a and b, compared with the LZM and freeze-thaw conditioning alone, the concentration of protein and polysaccharide in S-EPS increased sharply from 1.15 mg/g DS to 40.43 mg/g DS and 1.84 mg/g DS to 27.4 mg/g DS after combined conditioning. Moreover, as shown in Fig. 2c and d, DNA content increased by 402%, and bound water content of sludge decreased by 47.5% after co-conditioning, which was observably lower than that by separate conditioning. It could be inferred that the breakdown of cell walls and EPS structure not only released the organic substances, but also conduced to the removal of bound water, and thus improved the dewatering performance of sludge (Chen *et al.* 2015).



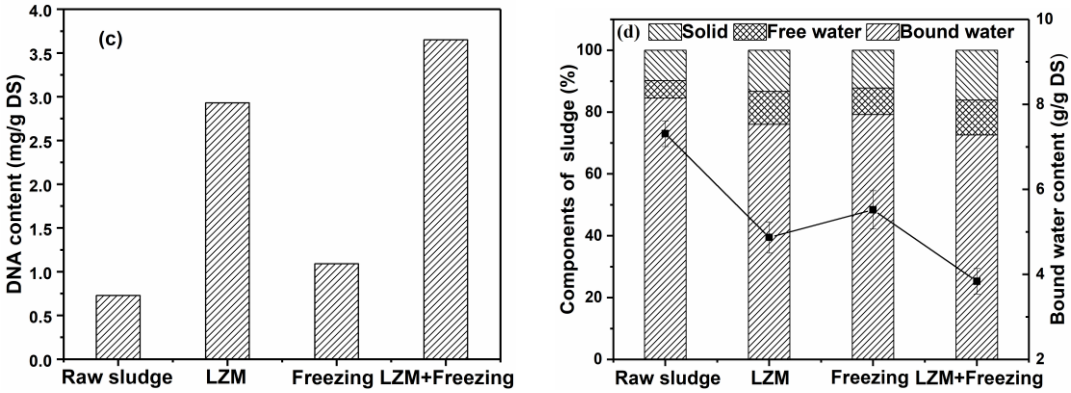


Fig. 2. Effects of various conditioning methods on EPS components, bound water and DNA content: (a) Protein; (b) Polysaccharide; (c) DNA and (d) Bound water

Effects of Sludge Particle Size Distribution on Sludge Dewatering Performance

As illustrated in Fig. 3a, the median diameter (D_v [50]) of the sludge particle increased a little after enzymatic conditioning compared with the raw sludge, in contrast, it rose to 175.6 μm from 49.1 μm after freeze-thaw conditioning alone, and accordingly, it grew to 170 μm after co-conditioning.

In general, the one-dimensional fractal dimension (D_1) indicates whether the flocs surface is smooth, and the two-dimensional fractal dimension (D_2) indicates whether the interior flocs structure is compact (Zhao *et al.* 2013). As shown in Fig. 3b, D_1 decreased to 1.08 and D_2 increased to 1.49, which was beneficial for the sludge flocs to become more smoother with a more compact structure. Additionally, the raw sludge and LZM-conditioned sludge exhibited small and loose floc structure with an indistinct boundary (Fig. S3 a, b). In contrast, the sludge floc structure became compacter after freeze-thaw conditioning alone or in combination with LZM (Fig. S3 c, d). This phenomenon could be attributed to the ice crystals hitting the sludge particles during the freezing process; the hydrophilic ice particles were expelled from the sludge particles (Haung *et al.* 2008). The number of particles contained in the flocs was increased, and the small granular sludge particles were aggregated into large flocs or large particles; therefore, the sludge particle size increased markedly and the floc structure became more compact.

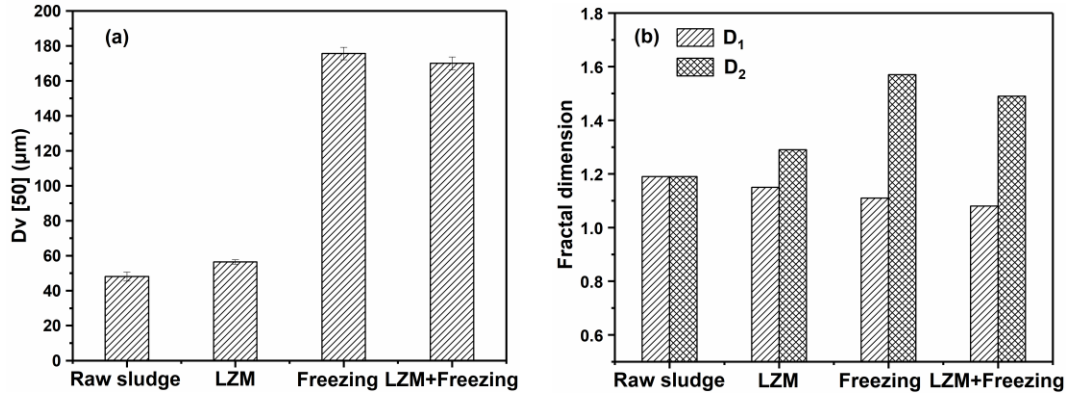


Fig. 3. Sludge particle size distribution (a) Dv [50]; (b) Fractal dimension

CONCLUSIONS

1. The dewatering performance of the activated sludge was improved mainly by the additive effect of the lysozyme in combination with freeze-thaw conditioning. After RSM optimization, CST and *W* dropped to 12 ± 0.5 s and 52.01% ± 0.4%. Such improvements in performance have the potential to broaden the ways in which sludge resources can be utilized.
2. After co-conditioning, the enhancement of sludge dewaterability was mainly due to the destruction of microbial cell walls as well as EPS structure and charge neutralization by LZM conditioning, resulting in the release of the intracellular polymers and bound water. Subsequently, the filtering performance of enzymatic-conditioned sludge was improved by freeze-thaw conditioning.
3. Combined conditioning produced sludge flocs with large size and compact structure, owing to the continuous formation of ice crystals during the freezing process. Finally, the above results were confirmed by the comparison of the sludge microstructure before and after the conditioning.

ACKNOWLEDGMENTS

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APPENDIX

Lysozyme Activity Assay

The cell suspension used in the study has an absorbance at 450 nm (A450) between 0.6 to 0.7 versus the reaction buffer blank. Immediately before use, a solution was prepared containing 200 to 400 units/mL of LZM in a cold reaction buffer (catalog number L9295). First, 800 µL of the micrococcus cell suspension was pipetted into a cuvette, one as a blank and one as a control, and one for each sample. Then, the cuvettes were equilibrated to 25 °C, and A450 was monitored until constant. Afterwards, 30 µL of reaction buffer was added to the blank cuvette, 30 µL of the LZM solution was added to the control cuvette, and 30 µL of the test sample was added to the remaining cuvettes. The mixtures were immediately mixed by inversion and the decrease in A450 was recorded for 5 min. Finally, the maximum linear rate (ΔA450/min) was obtained for each of the test samples and the blank, respectively.

The enzyme activity (units/mL) was calculated as follows:

$$r = \frac{(\Delta A_{450}/\min test - \Delta A_{450}/\min blank)(d_f)}{0.001 \times 0.03} \quad \text{(S1)}$$

where d_f indicated the dilution factor, 0.001 was the per unit definition (one unit will produce an ΔA_{450} of 0.001 per minute at pH 6.24 at 25 °C), and 0.03 was the enzyme solution volume (in milliliters).

EPS Extraction

Approximately 45 mL of the sludge suspension was placed in a 50 mL centrifuge tube and centrifuged at 3000 rpm for 15 min. Then, the supernatant was collected as S-EPS. The remaining deposit in the tube was then resuspended in a buffer solution, which was mixed with 2 mmol Na_3PO_4, 4 mmol NaH_2PO_4, 9 mmol NaCl, and 1 mmol KCl. The resulting mixture was centrifuged at 7400 rpm for 15 min to separate the solid and liquid phase. The collected organic matter was called the LB-EPS. Afterward, the residual sludge pellet in the centrifuge tube was suspended again in a buffer solution, sonicated for 5 min, then heated at 80°C for 30 min, and subsequently centrifuged to collect TB-EPS at 12000 rpm for 10 min. Finally, all the fractions of the EPS were filtered through acetate cellulose membranes (0.22 µm) and subsequently analyzed.

Table S1. Dosage of Lysozyme

Dosage / Conditioner							
Lysozyme (×10⁶ U/g DS)	0	1.6	2.4	3.2	4.8	6.4	8
Lysozyme (g/g DS)	0	0.1	0.15	0.2	0.3	0.4	0.5

Table S2. RSM Regression and Variance Analysis of Water Content of Dewatered Sludge

Source	Sum of squares	df	Mean Square	F Value	p-Value	R^2
Model	194.76	9	21.64	262.35	<0.0001	0.99
X_1	44.56	1	44.56	540.19	<0.0001	
X_2	20.74	1	20.74	251.41	<0.0001	
X_3	32.08	1	32.08	388.93	<0.0001	
X_1X_2	2.00	1	2.00	24.27	0.0017	
X_1X_3	0.11	1	0.11	1.28	0.2951	
X_2X_3	0.06	1	0.06	0.73	0.4218	
X_1^2	52.39	1	52.39	635.10	<0.0001	
X_2^2	33.42	1	33.42	405.16	<0.0001	
X_3^2	2.11	1	2.11	25.53	0.0015	
Residual	0.58	7	0.082			
Lack of Fit	0.35	3	0.12	2.06	0.2486	
Pure Error	0.23	4	0.057			
Cor Total	195.33	16				

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

Table S3. RSM Regression and Variance Analysis of CST

Source	Sum of Squares	df	Mean Square	F Value	p-Value	R^2
Model	167.18	9	18.58	27.00	0.0001	0.97
X_1	1.10	1	1.10	1.60	0.2461	
X_2	77.31	1	77.31	112.36	<0.0001	
X_3	29.11	1	29.11	42.30	0.0003	
X_1X_2	2.500E-003	1	2.500E-003	3.633E-003	0.9536	
X_1X_3	0.46	1	0.46	0.66	0.4426	
X_2X_3	2.00	1	2.00	2.91	0.1318	
X_1^2	14.81	1	14.81	21.52	0.0024	
X_2^2	32.90	1	32.90	47.82	0.0002	
X_3^2	4.45	1	4.45	6.47	0.0385	
Residual	4.82	7	0.69			
Lack of Fit	3.94	3	1.31	5.99	0.0583	
Pure Error	0.88	4	0.22			
Cor Total	172.00	16				

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

Determination of the Sludge Dewaterability

Firstly, 300 mL sludge sample was placed into centrifuge tubes, followed by 5 min centrifugation at 3000 rpm to discard the supernatant. Subsequently, the residual precipitate in the centrifugal tube was covered with the filtering cloth and transferred into the container before operating the pressure filtration system. The squeezing pressure was adjusted to approximately 5 to 6 MPa, and the filter-pressing time was controlled for 5 min. Finally, the water content of the dewatered sludge was examined by a Halogen Moisture Analyzer (HX204, Mettler Toledo, UK).

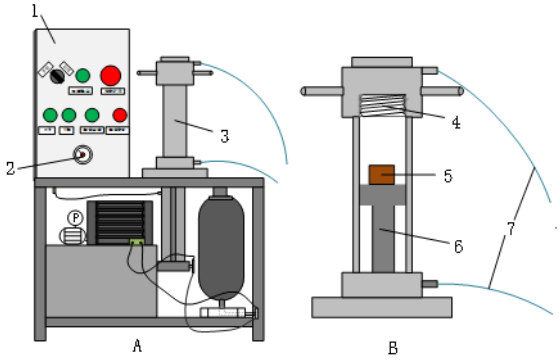

Fig. S1. The lab-scale dewatering system

(1) the control panel; (2) the pressure controller; (3) the sample room; (4) the filter cloth; (5) the sludge sample; (6) the piston; (7) the drainage exit

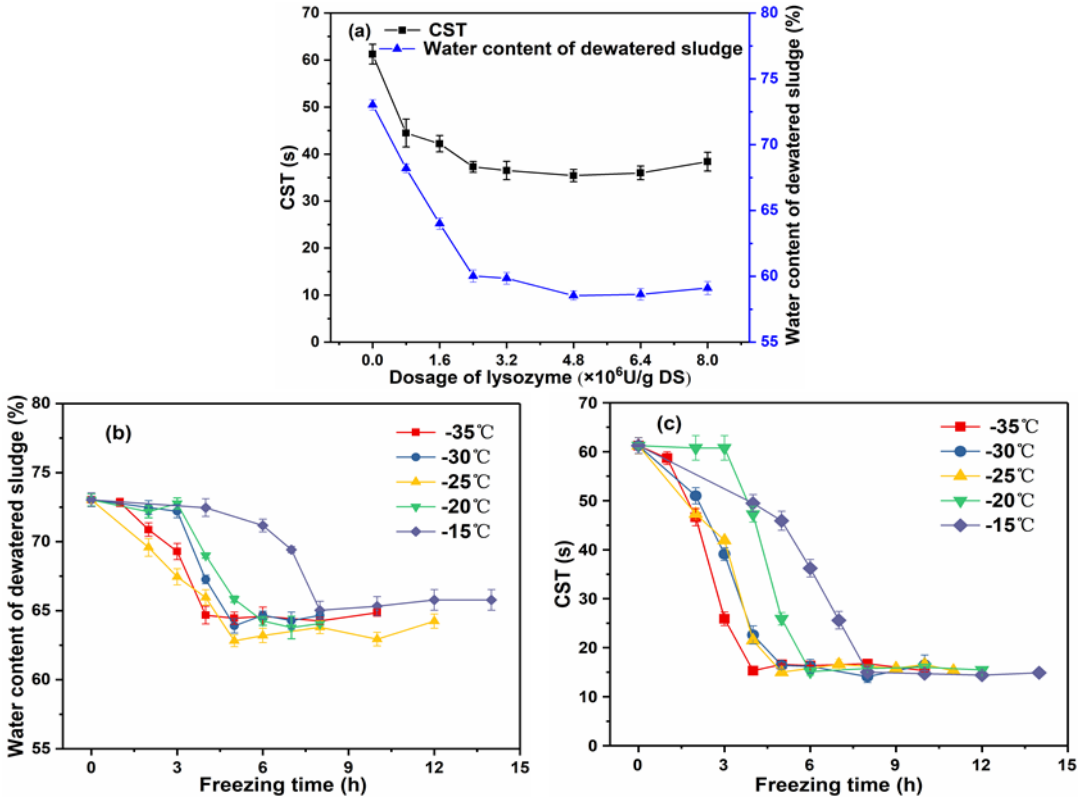

Fig. S2. Effects of separate conditioning on sludge dewatering performance
(a) LZM; (b) and (c) Freeze-thaw

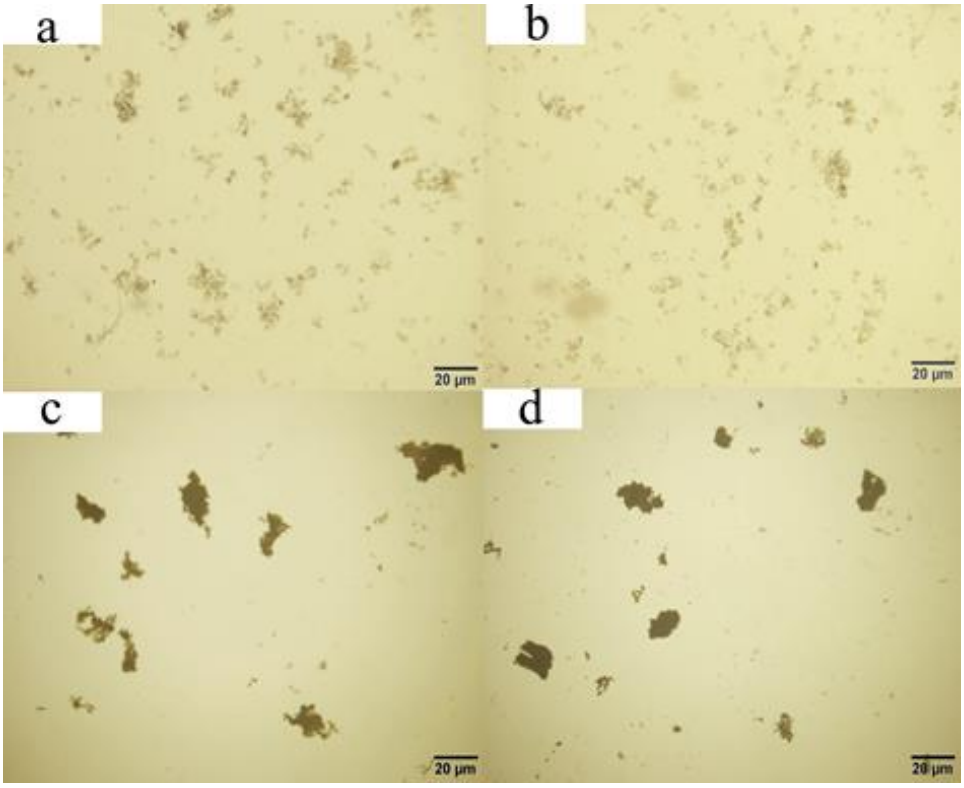

Fig. S3. Micrographs of sludge treated under various conditions (a) Raw sludge; (b) LZM; (c) Freezing; (d) LZM + freezing