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Isolation, production and optimization of endogenous alkaline protease from in-situ sludge and its evaluation as sludge hydrolysis enhancer

Jie Hou, Wenbin Liu, Wanrong Hu, Jing Chen, Jinqiong Wang, Panyu Li and Yonghong Li

ABSTRACT

Bioconversion (e.g. anaerobic fermentation and compost) is the common recycling method of waste activated sludge (WAS) and its hydrolysis, as the rate-limiting step of fermentation, could be accelerated by protease. However, the commercial protease was unstable in a sludge environment, which increased the cost. An endogenous alkaline protease stable in sludge environment was screened in this study and its suitability for treating the sludge was analyzed. The optimal production medium was determined by Response Surface Methodology as starch 20 g/L, KH_2PO_4 4 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, sodium carboxy-methyl-cellulose 4 g/L, casein 4 g/L and initial pH 11.3, which elevated the yield of protease by up to 15 times (713.46 U/mL) compared with the basal medium. The obtained protease was active and stable at 35 °C–50 °C and pH 7.0–11.0. Furthermore, it was highly tolerant to sludge environment and maintained high efficiency of sludge hydrolysis for a long time. Thus, the obtained protease significantly hydrolyzed WAS and improved its bioavailability. Overall, this work provided a new insight for enzymatic treatment of WAS by isolating the endogenous and stable protease in a sludge environment, which would promote the resource utilization of WAS by further bioconversion.

Key words | endogenous alkaline protease, process optimization, sludge hydrolysis, stability assessment, waste activated sludge

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HIGHLIGHTS

- A novel protease-producing bacterium was isolated from the sludge.
- The screened protease was highly tolerant to the sludge environment.
- Treatment of the sludge by the screened protease elevated its bioavailability.
- The screened protease was a promising biocatalyst for sludge treatment.

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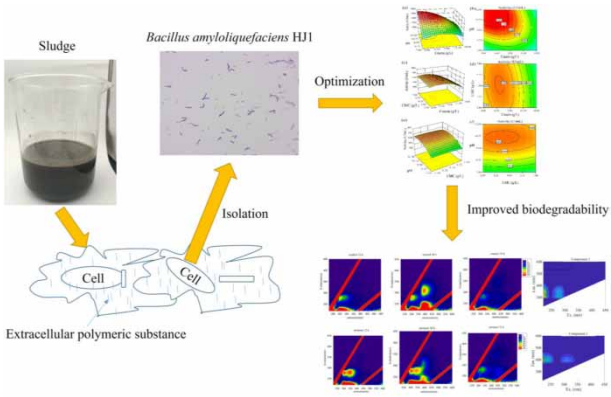
GRAPHICAL ABSTRACT



INTRODUCTION

As industrialization and urbanization developed rapidly, the amount of wastewater substantially increased significantly (Yang *et al.* 2010). The activated sludge process was widely used for wastewater treatment, which generated a vast amount of waste activated sludge (WAS). This was enriched with harmful substances (e.g. organic matters, heavy metals and pathogens), which could seriously threat the ecological environment if not handled properly (Rai *et al.* 2004). In addition to the characteristics of pollution, WAS contains abundant biomass (e.g. protein and polysaccharide), which showed the potential for resource and energy recovery.

Bioconversion or microbial utilization processes are typical sludge recycling methods (e.g. anaerobic fermentation and compost). However, these processes are restrained by the hydrolysis of WAS, which is known to be the rate-limiting step. Generally, complex biomacromolecules (e.g. protein and polysaccharide) and microbial cell walls hinder the release of organics (Lee *et al.* 2009). Therefore, a pretreatment for disintegrating sludge is needed. Currently, many physical and chemical pretreatment methods have been reported, including ultrasound (Erden & Filibeli 2010), microwaves (Lee *et al.* 2015), oxidative treatment (Hu *et al.* 2018), alkaline treatment (Li *et al.* 2008) and acid treatment (Devlin *et al.* 2011). Among them, alkaline treatment is a preferable method for its simple device, high efficiency and easy operation (Li *et al.* 2013). However, it suffers from some limitations, such as high cost, secondary pollution and extreme operational conditions. To diminish the limitations described above, there is a prerequisite to couple alkaline treatment with other methods (e.g. the use of surfactants or enzymes) (Chen *et al.* 2013; Pang *et al.* 2020b).

Protein is the predominant organic component in WAS (Yu *et al.* 2008) and the degradation of protein plays a crucial role in fermentation of WAS, which raised the hypothesis that protease could effectively enhance the biodegradability of WAS. Hence, alkali–protease coupling treatment has drawn much attention for its minimal byproducts and mild operation conditions (Hasegawa *et al.* 2000; Guo *et al.* 2016). Gessesse *et al.* (2003) found that enzymes were abundant in in-situ sludge. Furthermore, Yang *et al.* (2010) investigated the treatment of WAS using a commercial protease and concluded that the hydrolysis could be significantly enhanced by protease. However, the easy inactivation of the above commercial protease during enzymatic treatment made it economically unfeasible (Odnell *et al.* 2016). Commercially available enzymes are not derived from bacteria naturally existing in the WAS, thus it made sense that these enzymes were markedly vulnerable to lose activity in the sludge environment (Speda *et al.* 2017). Consequently, the alternative way to alleviate these limitations has been to isolate endogenous protease producers from in-situ sludge, in order to obtain protease with high activity and stability.

Given the facts above, the present research aimed to provide a robust endogenous protease for sludge pretreatment by isolating indigenous protease-producing bacteria from the sludge. Moreover, the optimal medium for protease production was determined by Response Surface Methodology (RSM). To comprehensively analyze the process of enzymatic treatment of WAS, it was necessary to determine

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the dynamics of protease activity over time. To the best of our knowledge, there have been few studies considering the dynamics of enzyme activity during its application in WAS. Therefore, we investigated the stability of the obtained protease in the sludge environment and the efficiency of sludge solubilization, which would manifest the application potential of the screened protease in WAS treatment.

MATERIALS AND METHODS

Sludge characteristics

The WAS samples were collected from a local Wastewater Treatment Plant in Chengdu which adopted conventional activated sludge processes to treat 100,000 m^3/day of municipal wastewater. Prior to use, the samples were stored at 4 °C. The characteristics of WAS were: pH = 6.79 ± 0.50, total solids (TS) = 12,720 ± 109 mg/L, volatile solids (VS) = 7,130 ± 114 mg/L, TCOD = 11,403.00 ± 450.00 mg/L, SCOD = 50.13 ± 4.80 mg/L.

According to the previous publications, enzymes are inherent in the sludge (Yu *et al.* 2008). To eliminate the interference of these endogenous enzymes, the sludge samples for enzymatic treatment were heated at 121 °C for 30 min, then centrifuged at 10,000 × g for 10 min. The sediment was re-suspended to the original volume in deionized water, which was then adjusted to pH 10.5 according to properties of the protease.

Isolation and identification of alkaline protease-secreting bacteria

Briefly, 1 mL of the sludge was serially diluted and streaked on a plate containing selective medium (pH 10.0) consisting of 10 g/L casein and 20 g/L agar, followed by culture at 30°C for 48 h. The colonies with a clearance zone were identified as protease-producing ones, which were sub-cultured until a uniform colony was obtained. The isolate was identified by morphology and biochemistry analyses on the basis of Bergey's manual (Holt *et al.* 1994). Analysis of 16S rRNA gene sequences was further conducted to identify bacterial species (Karan *et al.* 2011; Sonali *et al.* 2020).

Preparation of inoculum and protease production

The isolate *Bacillus amyloliquefaciens* HJ1 was maintained and preserved in selective medium at 4 °C before the inoculation. Protease production was carried out in two steps. First, seed culture (i.e. inoculum) was prepared by inoculating a loop of the isolate into 50 mL of YEPD medium (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L), and then incubated at 40 °C and 180 rpm for 12 h. Second, the seed culture was inoculated (10%, V/V) into 50 mL of basal medium (peptone 10 g/L, glucose 5 g/L, K_2HPO_4 2.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.35 g/L). After incubation at 40 °C and 180 rpm for 48 h, the sample was centrifuged (10,000 × g, 10 min, 4 °C) and the supernatant was recovered as crude protease for further study.

Optimization of the medium for protease production

Selection of suitable nutritional factors by single-factor experiment

Initially, the effects of various carbon sources (lactose, starch and sucrose) and nitrogen sources (yeast extract, casein and beef extract) were evaluated by replacing peptone and glucose in the basal medium. In the investigation of surfactant, sodium dodecyl sulfate (SDS), Tween 80 and sodium carboxymethylcellulose (CMC) were separately supplemented at the concentration of 2.5 g/L into the basal medium based on the previous studies (Liu *et al.* 2004; Yao *et al.* 2009; Amore *et al.* 2015; Baweja *et al.* 2016). Next, the effect of pH on protease production was investigated by adjusting the basal medium to different initial pH (7.0–12.0) using 2 M NaOH or HCl.

Selection of the significant factors by Plackett–Burman design (PBD)

Following the single-factor experiment, PBD was adopted to rapidly select the significant factors regulating protease production (Liu *et al.* 2010). The experiment was conducted according to the design shown (Table 2). Analysis of variance (ANOVA) was applied to assess the significance of these variables.

Optimization of significant factor using central composite design (CCD)

CCD was performed to investigate the simultaneous effects and to optimize the concentration of the significant variables. A five-level-three-factor experiment was performed according to the design shown (Table 4). The experimental design of PBD and CCD, as well as data analysis were carried out using the software Design-Expert Trial (Stat-Ease Inc., USA). To study the relationship between bacterial

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growth and protease production, the dynamics of cell growth and protease production of the isolate were studied under the optimized conditions. Briefly, batch fermentation was conducted in 1 L flasks containing 250 mL of the optimized medium. The isolate was incubated at 30 °C and 180 rpm. The sample was extracted every 4 h to measure cell density (OD_{600}) and protease activity.

Determination of key properties of the screened protease

The effect of temperature on the screened protease was determined at different temperatures (25–60 °C). The protease activity at 50 °C was considered as 100%. To evaluate the thermal stability of the screened protease, it was incubated at 25–60 °C for 30 min and the residue activity was assayed. Similarly, the optimal pH of the screened protease was determined in different pH buffer (200 mM phosphate buffer (pH 6.0–8.0) and 50 mM borax-NaOH buffer (pH 9.0–11.0)). The protease activity at pH 9.3 was considered as 100%. With regard to pH stability, the screened protease was diluted with different pH buffer solutions and incubated at 40 °C at pH 9.3. Next, the solution was adjusted to pH 10.5 and the residual activity was assayed.

Enzymatic treatment of WAS by the screened protease

The obtained protease was partially purified by ammonium sulfate to reduce the interference of the high COD in the enzyme solution. According to our preliminary test, ammonium sulfate at 70% saturation could salt out almost all protease. After ammonium sulfate precipitation, the sample was centrifuged (10,000 × g, 4 °C, 10 min). The precipitate was dissolved in 50 mM borate buffer (pH 10.5) and dialyzed at 4 °C for 24 h against the same buffer. Then, protease was concentrated using PEG20000 and the concentrated protease was used for enzymatic treatment of WAS. For enzymatic treatment, 5 mL of partially purified protease was added to 45 mL of WAS in a 250 mL flask, with 5 mL of deionized water added as control group. Both groups were incubated in a reciprocal shaker (40 °C and 120 rpm) and sampled over time. Thereafter, the sludge was immediately centrifuged (5,000 × g, 4 °C, 10 min) and the dissolved organic matter (DOM) in the supernatant was determined.

Analytic methods

TS, VS and chemical oxygen demand (COD) were analyzed according to standard methods (APHA *et al.* 1998). SCOD is defined as the COD of the filtrate through a 0.45 μm membrane. The biomass was determined by measuring OD_{600} and dry cell weight (DCW). Optical density at 600 nm (OD_{600}) was defined as the light absorbance of the fermentation broth at 600 nm, which was positively correlated with biomass (Francois *et al.* 2005). DCW was measured according to Bhunia *et al.* (2012).

The content of soluble polysaccharide was determined by phenol–sulfuric acid method using glucose as the standard, and the content of soluble protein was determined by the Lowry method using bovine serum albumin as the standard (Ibrahim *et al.* 2015). The COD conversion factors were 1.5 g-COD/g protein and 1.06 g-COD/g polysaccharide (Zhou *et al.* 2013). Protease activity was determined according to the modified method of Haddar *et al.* (2009). One unit (U) of protease activity represented the quantity of protease required to release 1 μg of tyrosine. Excitation-emission matrix (EEM) was determined using a Shimadzu RF-6000 spectrofluorometer (Kyoto, Japan). The emission spectra of 220–600 nm (2 nm increment) were scanned at the excitation wavelength of 220–450 nm (5 nm increments) (Pang *et al.* 2020b). The Parallel factor analysis (PARAFAC) algorithm was used to identify the component of the fluorophore according to Stedmon & Bro (2008).

RESULTS AND DISCUSSION

Isolation and identification of the alkaline protease producer originated from WAS

Among various proteases, alkaline protease was the promising choice for sludge pretreatment due to its high hydrolytic activity and stability, and is widely used in the detergent industry (Yu *et al.* 2019). In this study, the bacteria were isolated from WAS based on whether they could grow and hydrolyze casein in alkaline environment (pH 10). Four morphologically different protease-producing bacteria were isolated. One strain, named as HJ1, exhibited the biggest size of clearance zone on agar plates and high protease activity (48.00 U/mL) in the broth. Therefore, it was chosen for further studies.

The morphological and biochemical characteristics of HJ1 are listed in Table 1, which showed a closely related characteristic to *Bacillus amyloliquefaciens* (Balows 1975).

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Table 1 | Morphological and biochemical characteristics of the isolate HJ1

Test	Result	Test	Result
Shape	Rod	D-tagatose	−
Gram stain	+	N-acetyl glucose	−
Spore formation	+	Fructose	+
Starch hydrolysis	+	Mannitol	+
Casein hydrolysis	+	Galactose	−
Amylase	+	Gentiobiose	+
Catalase activity	+	Mycose	−
Arginine	+	Arabinose	−
Sodium Citrate	+	Sucrose	+
2-Nitrophenyl β-D-galactopyranoside	+	Cellobiose	+
Ribose	+	Phaseomannite	+
Sorbitol	+	Soya peptone	−

+, positive results; −, negative results.

Especially, HJ1 grew well and secreted a large amount of protease at pH 8–11 (Fig. S1d), which was a typical characteristic of alkaliphile. Generally, the condition of alkaline treatment of the sludge was pH 10 (Pang *et al.* 2020b). Hence, it could be expected that the obtained protease would function well in alkaline treatment of WAS. Furthermore, the result of molecular evolutionary relationship revealed that HJ1 showed the highest similarity with *Bacillus amyloliquefaciens* (Figure 1). Likewise, it was reported that the genus *Bacillus* was predominant producer of alkaline protease (Deng *et al.* 2010; Guleria *et al.* 2016). The 16S rRNA gene sequence was deposited in the GenBank database (Accession number: MT678828) and this strain was named as *Bacillus amyloliquefacien* HJ1.

Optimization of the medium for protease production

Selecting the suitable nutritional and environmental factors in favor of protease production

For large-scale enzymatic treatment of WAS, a large amount of protease was a requisite. However, protease production was regulated by various conditions (e.g. the carbon and nitrogen sources, C/N ratio, pH and temperature) (Ibrahim *et al.* 2015). Hence, the composition and concentration of medium should be optimized to increase the yield of protease. As shown in Figure S1 (supplementary information), the optimal medium components were starch (278.12 U/mL), casein (106.08 U/mL), CMC (356.21 U/mL) and initial pH 11.0 (552.16 U/mL), respectively. When simple substrates like glucose or peptone were used, protease

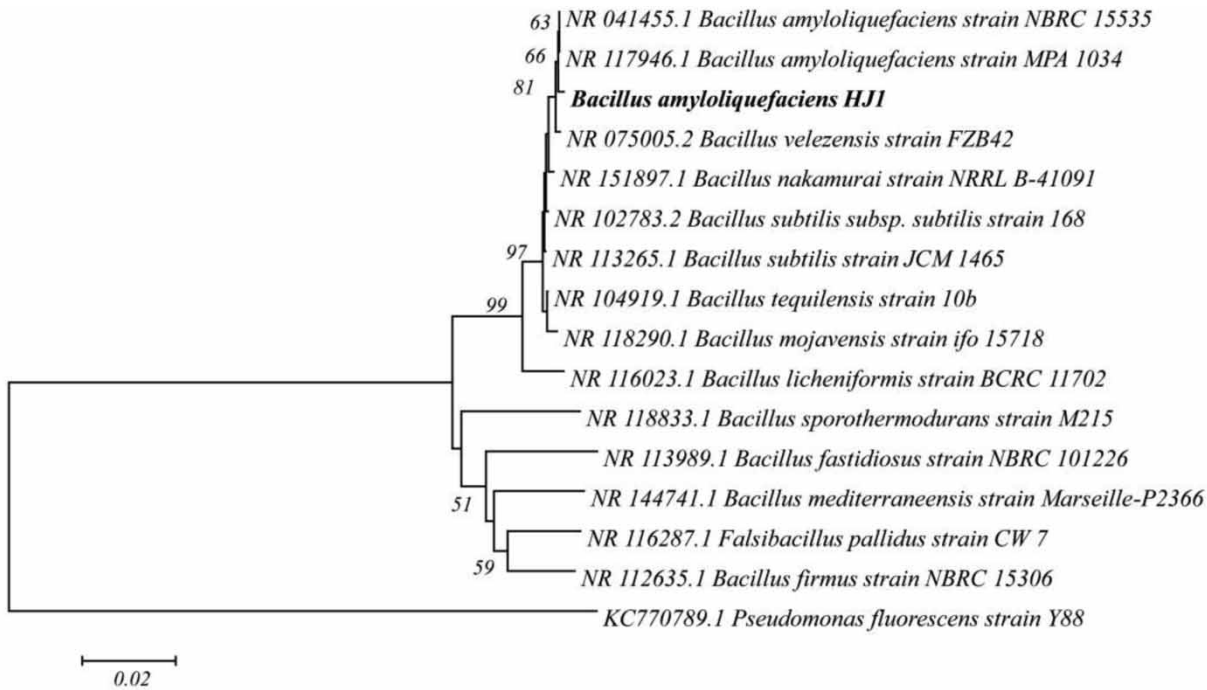

Figure 1 | Phylogenetic tree showing the relationship between the isolate HJ1 and the related *Bacillus* strains based on 16S rRNA gene sequence. The number on the node is based on the percentage of the bootstrap value for 1000 repetitions.

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production dramatically declined. This could be explained by the catabolic repression of simple carbon and nitrogen sources (Ibrahim *et al.* 2015). In contrast, the casein and starch could not be assimilated directly by bacteria, which stimulated alkaline protease production (Stammen *et al.* 2007; Geisseler & Horwath 2008). Surfactants were reported to change cellular permeability, so that the alkaline protease could be released out of the cell in a timely manner (Reese & Maguire 1969). In our study, different surfactants had different effects (i.e. promoting or inhibiting) on enzyme production, which was also found in the previous studies (Liu *et al.* 2004; Yao *et al.* 2009; Amore *et al.* 2015; Baweja *et al.* 2016). In addition, protease production was promoted in alkaline conditions (pH 8–11), which inferred that *Bacillus amyloliquefacien* HJ1 was an alkaliphilic microorganism. Likewise, alkaliphilic *Bacillus* sp. NPST-AK15 produced massive amounts of alkaline protease within the pH range from 8 to 12 (Ibrahim *et al.* 2015). Thus, four variables (starch, casein, CMC and initial pH 11.0) were selected for further study.

Establishing a model for selecting of the significant variables affecting protease production

Based on the above results, various nutrients (e.g. carbon and nitrogen sources) were selected for protease production, which affected protease production at different levels. Hence, PBD was employed to identify the variables significantly affecting protease production (Karan *et al.* 2011). Six variables (starch, casein, CMC, $MgSO_4{\cdot}7H_2O$, KH_2PO_4 and initial pH) were studied at two levels (−1 and +1) (Table 2). A first-order equation model was constructed to analyze the significance of these variables based on the experimental data:

$$Y = 297.68800 - 19.33227X_1 - 3.73653X_2 + 52.28267X_3 + 13.32978X_4 + 29.46489X_5 - 49.31200X_6 \quad (1)$$

where Y is the yield of protease, X_1 is casein, X_2 is starch, X_3 is pH, X_4 is CMC, X_5 is KH_2PO_4 and X_6 is $MgSO_4{\cdot}7H_2O$. Based on the correlation coefficient in Equation (1), it clearly demonstrated that there were three variables (casein, starch and $MgSO_4{\cdot}7H_2O$) negatively correlated with the yield of protease, while three others (pH, CMC and KH_2PO_4) correlated positively. As shown in Table 3, the *P*-value of casein, pH and CMC were less than 0.1, implying that they significantly affected protease production (Karan *et al.* 2011). Thus, they were selected for further optimization using CCD.

Establishing a model for accurately predicting protease production

It was reported that interactions between factors significantly affected protease production (Reddy *et al.* 2008). However, the constructed first-order equation model by PBD only contained linear terms and neglected the interactions between variables. Thus, CCD was further used to analyze interactions among variables (casein, pH and CMC) and to determine the optimal condition. The experimental results along with their corresponding predicted values were presented in Table 4. Based on the experimental

Table 2 | Plackett–Burman design matrix for evaluating the variables on protease production

Run	Casein (g/L)	Starch (g/L)	pH	CMC (g/L)	KH_2PO_4 (g/L)	$MgSO_4{\cdot}7H_2O$ (g/L)	Protease activity (U/ml)
1	12.5	20	11.0	4	4	0.5	707.74 ± 11.43
2	10	25	11.0	4	1	0.5	676.19 ± 59.54
3	10	25	11.0	1	4	1	649.54 ± 14.34
4	12.5	20	8.0	1	4	0.5	523.87 ± 15.74
5	10	20	11.0	1	4	1	705.02 ± 25.91
6	10	20	8.0	1	1	0.5	439.28 ± 39.18
7	12.5	20	11.0	4	1	1	637.02 ± 17.01
8	10	25	8.0	4	4	0.5	631.86 ± 52.9
9	12.5	25	8.0	4	4	1	424.05 ± 37.79
10	12.5	25	8.0	1	1	1	467.30 ± 27.37
11	12.5	25	11.0	1	1	0.5	471.92 ± 53.29
12	10	20	8.0	4	1	1	420.24 ± 15.5

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Table 3 | Analysis of variance (ANOVA) for Plackett–Burman design

	Effect	Sum of squares	*F*-value	*P*-value
Casein	48.33066667	7007.560021	8.8361601	0.0589
Starch	18.68266667	1047.126101	1.3203703	0.3338
pH	156.848	73803.88531	93.062771	0.0024
CMC	39.98933333	4797.440341	6.0493169	0.0909
KH_2PO_4	88.39466667	23440.85129	29.557666	0.0122
$MgSO_4{\cdot}7H_2O$	−24.656	1823.755008	2.299658	0.2267

Table 4 | Design and the corresponding results of central composite design

	Casein (g/L)	CMC (g/L)	pH	Observed (U/ml)	Predicted (U/ml)
1	6.00	2.00	7.00	470.56 ± 111.51	498.05
2	6.00	6.00	11.00	716.99 ± 40.18	754.66
3	10.00	4.00	12.36	703.94 ± 51.26	737.88
4	10.00	4.00	5.64	402.30 ± 151.90	406.89
5	10.00	0.64	9.00	675.92 ± 55.54	636.50
6	10.00	7.36	9.00	636.75 ± 47.59	629.25
7	6.00	2.00	11.00	703.94 ± 51.26	652.42
8	14.00	6.00	7.00	443.09 ± 32.85	442.31
9	10.00	4.00	9.00	686.26 ± 66.75	655.00
10	16.73	4.00	9.00	396.58 ± 65.75	390.77
11	14.00	2.00	7.00	472.19 ± 55.23	467.70
12	3.27	4.00	9.00	676.19 ± 66.92	635.08
13	10.00	4.00	9.00	620.43 ± 36.62	655.00
14	6.00	6.00	7.00	513.54 ± 115.17	510.47
15	10.00	4.00	9.00	713.18 ± 66.06	655.00
16	14.00	2.00	11.00	479.26 ± 47.37	515.50
17	14.00	6.00	11.00	488.78 ± 45.95	494.47
18	10.00	4.00	9.00	690.34 ± 95.86	655.00
19	10.00	4.00	9.00	591.87 ± 89.86	655.00
20	10.00	4.00	9.00	619.89 ± 45.82	655.00

data, a second-order polynomial equation was constructed to illustrate the relationship between protease activity and the independent variables:

$$Y = -1307.81522 + 103.36620X_1 + 23.93438X_2 + 294.84937X_3 - 1.18150X_1X_2 - 6.00100X_1X_3 + 0.27200X_2X_3 - 3.13949X_1^2 - 1.95558X_2^2 - 11.07938X_3^2 \quad (2)$$

where Y is the predicted protease yield, X_1 is casein, X_2 is CMC and X_3 is initial pH.

Furthermore, ANOVA was performed to evaluate the statistical significance of the independent variables and the fitted quadratic equation (Table 5). Generally, a larger *F*-value and smaller *P*-value ($P < 0.1$) suggested a more significant independent variable (Haaland 1989). Hence, it could be found that X_1 -casein, X_3-initial pH, X_1^2, X_3^2, X_1X_3 were significant. According to the evolution-economy principle (Allison *et al.* 2011), bacteria would elevate protease production when simple nutrients were scarce and complex nutrients were abundant. In this study, bacteria cannot directly utilize complex nitrogen source (i.e. casein), which stimulated the synthesis of protease. In addition, the isolated alkaliphile relied heavily on an alkaline environment for cell growth and enzyme production. Furthermore, it was reported that the structure of casein became more refractory at lower pH, but looser at higher pH (Liu & Guo 2008). It

Table 5 | Analysis of variance (ANOVA) for the variables and the regression model

Source	SS	DF	MS	*F*-value	*P*-value
Model	222,931.01	9.00	24770.11	10.89	0.0004
A-casein	72,049.47	1.00	72049.47	31.69	0.0002
B-CMC	63.40	1.00	63.40	0.03	0.8707
C-pH	72,771.40	1.00	72771.40	32.01	0.0002
AB	714.72	1.00	714.72	0.31	0.5874
AC	18,438.14	1.00	18438.14	8.11	0.0173
BC	9.47	1.00	9.47	0.00	0.9498
A^2	36,363.01	1.00	36363.01	15.99	0.0025
B^2	881.81	1.00	881.81	0.39	0.5474
C^2	28,304.40	1.00	28304.40	12.45	0.0055
Residual	22,735.51	10.00	2273.55		
Lack of Fit	10,722.37	5.00	2144.47	0.89	0.5481
Pure Error	12,013.14	5.00	2402.63		
Cor Total	245,666.52	19.00			

$R^2 = 0.9075$; Adj $R^2 = 0.8242$; CV = 8.15%; Adeq precision = 10.793; SS, sum of squares; DF, degree of freedom; MS, mean square.

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was clear that the pH-dependence of casein affected its availability. Consequently, the interactive effect between pH and casein was significant. Three-dimensional response surface and contour graph were further plotted to gain a visual interpretation of the interactive effect of variables on protease production (Figure 2). As expected, there were significant interactions between casein and pH and a clear peak inside the design boundary conditions, which indicated that the obtained optimal conditions were credible. The optimal level of three variables were as follow: initial pH 11.3, CMC 4 g/L, and casein 4 g/L, respectively. It also proved that RSM was an efficient method for protease optimization, which achieved a 15-fold increase in the optimized medium (713.00 U/mL) compared with the basal medium (48.00 U/mL). It was reported by Karan *et al.* (2011) that protease production from *Geomicrobium* sp. EMB2 was improved from 37 U/ml to 721 U/ml by means of RSM.

Growth kinetics and protease production of *Bacillus amyloliquefacien* HJ1

The dynamics of cell growth and alkaline protease production varied greatly with culture conditions (Geisseler & Horwath 2008). Therefore kinetic analysis was performed in the optimized production medium (starch 20 g/L, KH_2PO_4 4 g/L, $MgSO_4{\cdot}7H_2O$ 1 g/L, CMC 4 g/L, casein 4 g/L and initial pH 11.3). As shown in Figure 3, *Bacillus amyloliquefacien* HJ1 grew rapidly but scarcely produced protease in phase I. In phase II (12–36 h), the yield of protease increased substantially to 713.46 U/mL. According to above results, it could be concluded that protease production was partially associated with microbial cell growth. Similar dynamics of protease production have been also reported in previous studies (Rao *et al.* 2009; Jain *et al.* 2012).

The determination of key characterization of the protease for sludge pretreatment

Previous studies have reported that alkali-protease coupling treatment under mesophilic conditions effectively promoted the deflocculation of WAS (Haddar *et al.* 2009; Jie *et al.* 2014). Hence, it is a prerequisite for the obtained protease to be stable under alkaline and mesophilic conditions before being applied in sludge treatment. As shown in Figure 4(a), the protease showed marked activity at pH 7.0–11.0 and reached the highest at pH 9.0, which demonstrated that it was a typical alkaline protease (Yu *et al.* 2019). In addition, the protease retained almost all protease activity at the broad range of pH 6–11 (Figure 4(b)) after being incubated at 40 °C for 30 min. As shown in Figure 4(c) and 4(d), the protease was highly active and stable at broad ranges of temperature from 30 to 60 °C with an optimal protease activity at temperature 50 °C, which was in line with a previous report (Yu *et al.* 2019). The above results confirmed that the isolated protease was highly stable in alkaline and mesophilic conditions, which ensured high efficiency of sludge treatment.

The dynamics of protease activity and solubilization of WAS during enzymatic treatment

Until recently, many attempts have been made to improve WAS solubilization using protease (Yang *et al.* 2010; Pang *et al.* 2020b). However, it was reported that the activity lifetime of commercialized protease in sludge was short (<12 h), which limited the application of enzymatic treatment (Odnell *et al.* 2016). Indeed, the stability of enzyme in sludge environment was crucial to the practical application. Hence, enzymatic treatment of WAS by the screened protease was conducted to verify its stability and efficiency of sludge solubilization. COD was the sign of WAS solubilization. As shown in Figure 5(a), there were two distinct phases. First, both CODs in enzymatic treatment and control group increased rapidly within the first 12 h. For enzymatic treatment, the net increase in COD was 2,431.06 mg/L, while for control group this was only 1,378.44 mg/L. The reason for the enhanced efficiency of sludge hydrolysis might be that hydrolase breaks down the cell wall of the microorganisms and hydrolyzes biomacromolecules into simpler ones (Song *et al.* 2010; Yang *et al.* 2015). Similarly, the endogenous cellulase improved the hydrolysis rate of cellulose and thus elevated the biogas production (Speda *et al.* 2017). Second, COD trended to decline up to the end of treatment (12–72 h). This phenomenon could be explained by the active cryptic growth of spore-producing microorganisms like *Bacillus*, whose spores could withstand high temperatures without being killed (Jonghe *et al.* 2010). Spore-producing microorganisms started to utilize organic nutrients for growth once the spore was activated after 6 h incubation. When the rate of organics utilization was greater than the rate of release, COD started to decline. Meanwhile, the reduction of COD in the enzyme treatment group was significantly higher than in the control group, indicating that more organics were consumed by the microorganisms. Thus, it could be concluded that treatment by the screened protease resulted in higher bioavailability of WAS.

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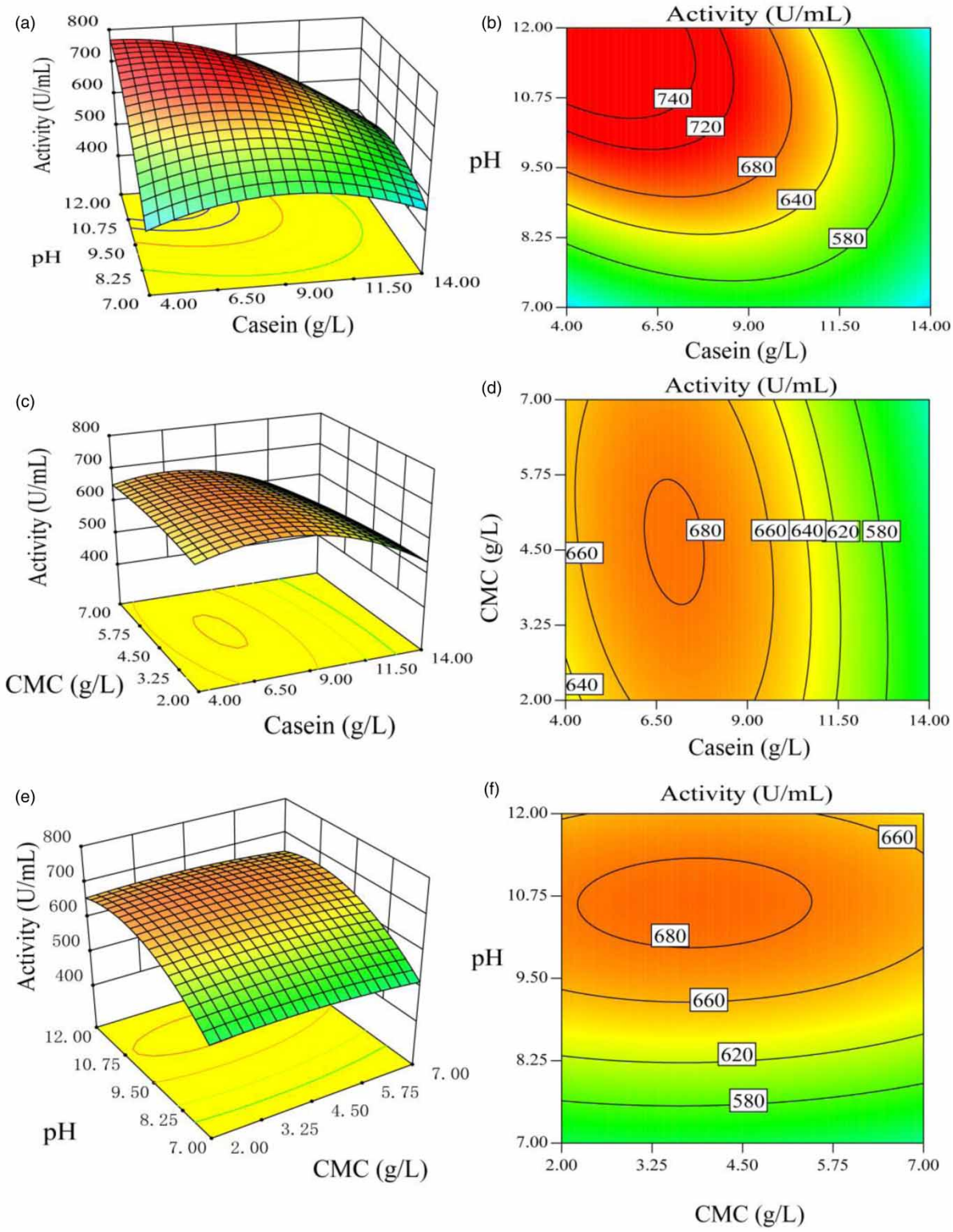

Figure 2 | 3D-response surface and contour graph of protease production showing the interactive effect between (a and b) casein and pH, hold value: CMC 4 g/L; (c and d) CMC and casein, hold value: pH 9.0; (e and f) CMC and pH, hold value: casein 10 g/L. $K_2H_2PO_4$ was fixed at a high level (4 g/L) while $MgSO_4 \cdot 7H_2O$ and starch were maintained at a low level (0.1 g/L and 20 g/L). *Bacillus amyloliquefacien* HJ1 was incubated at 30 °C and 180 rpm for 48 h.

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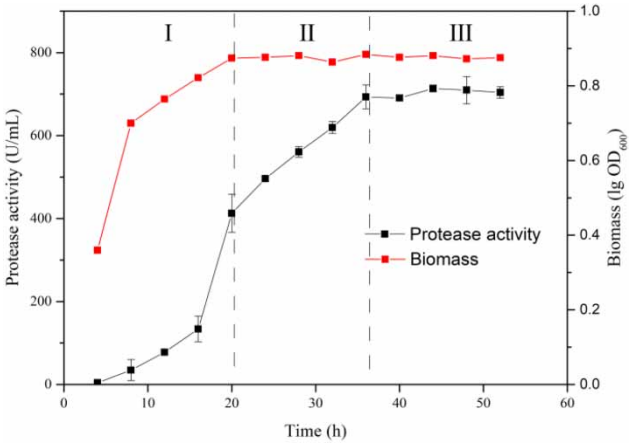

Figure 3 | Dynamics of protease production and growth of *Bacillus amyloliquefacien* HJ1 in the optimal medium (starch 20 g/L, KH_2PO_4 4 g/L, $MgSO_4 \bullet 7H_2O$ 1 g/L, CMC 4 g/L, casein 4 g/L and initial pH 11.3). I: exponential growth phase; II: early stage of stationary phase; III: late-stationary phase.

In addition, the variation in DOM was further characterized by the variation in protein and polysaccharide. The protein showed a similar pattern with COD (Figure 5(c)). In the control group, the protein decreased from 893.30 mg/L at 6 h to 590.05 mg/L at 72 h. However, the protein in the enzymatic treatment group decreased rapidly from 3,278.02 mg/L at 6 h to 1,180.10 mg/L at 72 h, which indicated that the addition of protease significantly promoted the utilization of protein. It is worth noting that the total reduction of protein from 6 to 72 h (303.25 mg/L for the control group, 2,097.92 mg/L for enzymatic treatment) was almost equal to that of COD (751.88 mg/L for the control group, 2,681.69 mg/L for enzymatic treatment), which indicated that protein was the main nutriment utilized by the microorganisms in WAS (Yu *et al.* 2008). The dynamic pattern of polysaccharide was completely different to those of

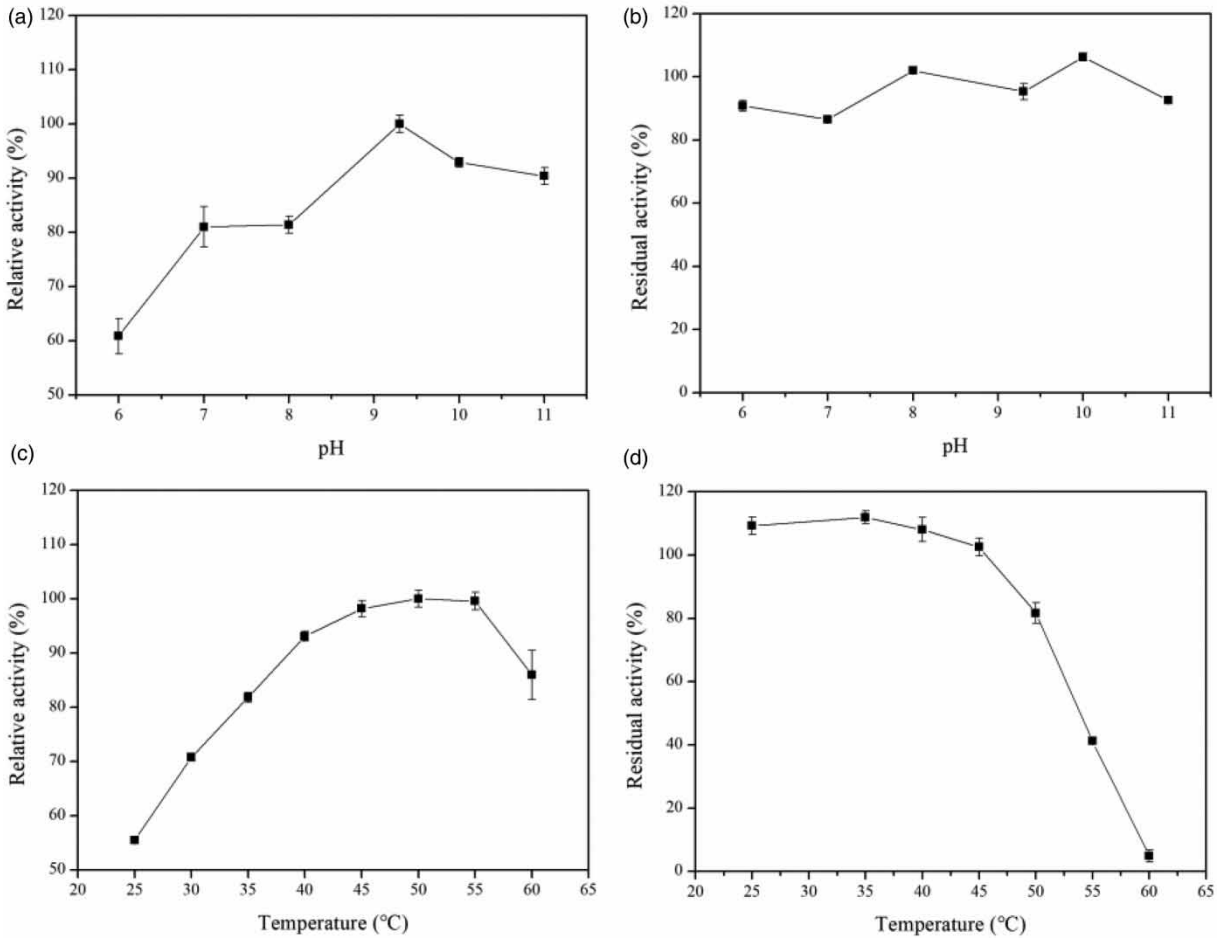

Figure 4 | Effect of pH on protease (a) activity and (b) stability. The pH stability was determined by incubating the protease in different pH buffer solutions at 40 °C for 30 min and the residual activity was assayed. Effect of temperature on protease (c) activity and (d) stability. Thermal stability was determined by pre-incubating the crude protease at 25–60 °C for 30 min and the residual activity was assayed. Relative activity was calculated as the ratio of protease activity under different conditions (pH or temperature) to the optimal condition (i.e. pH 9.3 or 50 °C). Residual activities were calculated as the ratio of protease activity after incubation to the protease activity without incubation.

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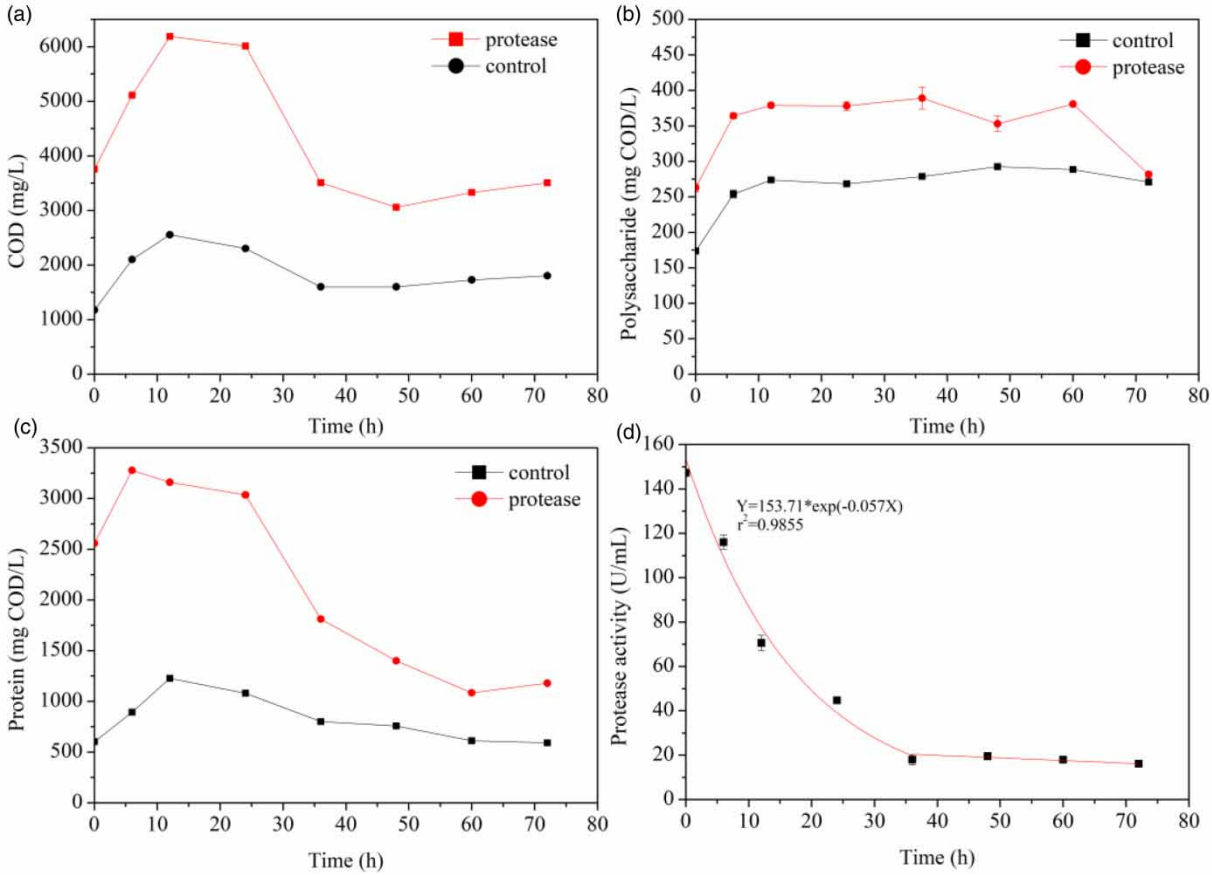

Figure 5 | Dynamics of (a) COD, (b) polysaccharide, (c) protein and (d) protease activity in liquid phase of WAS during sludge treatment.

protein and COD (Figure 5(b)). Polysaccharide increased rapidly from 0–12 h, beyond which it remained almost constant. In general, the amount of released polysaccharide was less than that of protein. This was in accordance with the fact that protein is the main organic in WAS (Yu *et al.* 2008). Similarly, Kavitha *et al.* (2017) also observed a higher level of release of protein than polysaccharide. Thus, it could be speculated that the elevated bioavailability was mainly caused by the release of protein.

Furthermore, the protease activity in sludge supernatant was also measured to determine the stability of the screened protease in the sludge (Figure 5(d)). In agreement with the trends of COD and protein, the dynamics of protease activity could be distinctly divided into two stages (stage I and stage II). In stage I (0–36 h), the protease activity decreased exponentially with time, while in stage II (36–72 h) the protease activity remained relatively constant. The screened protease had a 47.92% activity at 12 h, 30.33% at 24 h and 10.94% at 72 h. In a previous report, Odnell *et al.* (2016) treated the sludge with commercial protease (subtilisin and trypsin). They found that the enzymes had almost lost all activity at about 6 h and were completely inactivated after 12 h. The improvement of the stability of the screened protease might be explained by the endogeneity (Speda *et al.* 2017). The endogenous protease was defined as protease naturally existing in the sludge, which was evolutionarily adapted to the sludge environment. Based on the result above, it could be concluded that the screened protease was more stable than commercial enzymes in the sludge environment. This enabled a lower dosage to achieve the similar effect as commercial protease, resulting in high cost–effectiveness for sludge treatment. In addition, DOM solubilization of enzymatic treatment and bio-utilization by the microorganisms were reported to be two simultaneous and competitive processes (Xin *et al.* 2016). Thus, protease activity could also be used to explain the change of DOM in sludge. For example, the protease activity in stage I was relatively high, so DOM solubilization exceeded the bio-utilization capacity by the microorganisms, which resulted in the rapidly increased COD. Therefore, it could be concluded that the protease screened from the sludge was highly tolerant to the sludge environment, which will be helpful for enzymatic treatment of WAS.

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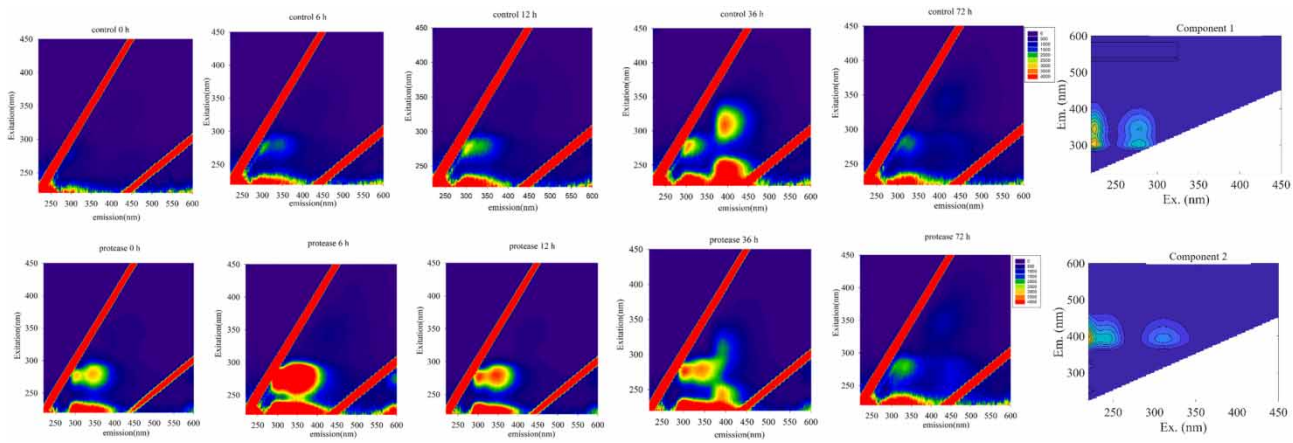

Figure 6 | EEM spectra of liquid phase of WAS during enzymatic treatment. All samples were diluted by 200 times.

It was reported that the biodegradability of protein depended on its type and chemical property (Pang *et al.* 2020a). Thus, EEM was applied to characterize the dynamics of fluorophores (e.g. protein and humid acid). PARAFAC confirmed two main components (Figure 6). Component I (aromatic protein-like substances) located at the Ex/Em of 225/300–350 nm and 275/300–350 nm, which was the preferred substrate for the microorganisms (Luo *et al.* 2013). Component II (lignin-like substance) located at the Ex/Em of 240/400 nm and 300/400 nm, which could be biodegraded into acid-precipitable polymerized lignin (APPL) (Zeng *et al.* 2017). With the release of DOM, fluorescence intensities (FI) of components I and II gradually increased within 6 h beyond which FI decreased significantly. In addition, FI of aromatic protein-like substances in enzymatic treatment was significantly higher than control group, which indicated that the screened protease effectively improved the hydrolysis of WAS and promoted the utilization of aromatic protein. A similar phenomenon was also observed by Luo *et al.* (2013) who found that the treatment of WAS by commercial protease and α-amylase released more protein-like substances, which were the fermentation substrates of volatile fatty acids (VFA). Thereby, enzymatic treatment elevated the bioavailability of WAS. Taken together, the screened protease offered a robust source for alkaline protease-based pretreatment of WAS considering its stability and high efficiency of sludge hydrolysis.

CONCLUSION

This study provided a new insight into the isolation of new enzyme and enzymatic treatment of WAS. The protease isolated from native community in the sludge was active and stable in the sludge environment. Furthermore, the yield of protease was elevated using RSM. Treatment of WAS by the screened protease was shown to significantly promote the hydrolysis of WAS and elevate its bioavailability. The screened protease was endowed with superior properties: (1) good catalytic performance and stability over broad pH and temperature ranges, (2) high stability in the sludge environment and (3) an alkaline nature with high production level, laying the foundations for its application as a sludge hydrolysis enhancer.

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DATA AVAILABILITY STATEMENT

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

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