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Original scientific paper

Enhancing the hydrolysis of excess sludge using thermophilic *Bacillus* sp. Hnu under different oxygen supply conditions

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Abstract: A thermophilic *Bacillus* strain was isolated from excess sludge in the present study. A 16S rDNA analysis indicated that this strain was a *Bacillus* sp. that had not been previously reported (named *Bacillus* sp. Hnu). The aim of the present study was to investigate the enhanced efficiency of excess sludge hydrolysis by the addition of thermophilic *Bacillus* sp. Hnu under different oxygen supply conditions. The results indicated that higher temperature and a greater oxygen supply were advantageous for the volatile suspended solid removal ratio, having the same effect to that of protease activity. The maximum volatile suspended solid removal ratio was achieved at 21.5, 42.5 and 54.4 % after 108 h digestion at pH 6.9 and 60 °C and increased by 17.2, 38 and 45.4 % under anaerobic, microaerobic, and aerobic conditions compared with the control test, respectively. The hydrolysis rate constants under anaerobic, microaerobic, and aerobic conditions were 3, 4.8, and 7 times (40 °C), 3.5, 9.8, and 11.8 times (50 °C) and 2.7, 7.2, and 10.3 times (60 °C), respectively. Hydrolysis performance indicated that the *Bacillus* sp. Hnu could accelerate the hydrolysis rate. The kinetic study showed that the hydrolysis of sludge with *Bacillus* sp. Hnu and the control test followed first-order kinetics except at 60 °C.

Keywords: thermophilic; excess sludge; microaeration; hydrolysis; first-order kinetics.

INTRODUCTION

The activated sludge process is the most widely used biological treatment for municipal and industrial wastewater worldwide.[1,2] This process uses microorganisms to transform dissolved and colloidal organic substances in the wastewater into biomass or carbon dioxide and water.[3] The production of the major byproduct, *i.e.*, excess sludge, is a serious disposal problem for treatment plants. The

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excess sludge contains considerable amounts of hazardous organic and inorganic materials, such as pathogens, parasite eggs, and a number of heavy metals. The mixture is frequently subjected to treatment prior to disposal to avoid posing a significant threat to ecological systems.[4]

The main treatment of the excess sludge presently employed in China depends on the landfill operation after coagulation filtration. However, the disposal of the excess sludge by this operation is not effective because it occupies a vast area of land. The costs associated with the treatment of excess sludge may cover up to 25 to 65 % of the total plant operational cost.[5,6]

The current technologies for sludge reduction can be roughly classified into the following three major categories: 1) a mechanical method such as mill or ultrasonification,[2,7,8] 2) oxidation using ozone[9–11] or chlorine[12] and 3) hydrolysis with or without enzymes.[13–15] Biological stabilization is considered as one of the most attractive methods for the optimal reduction of the organic fraction in excess sludge. The thermophilic bacteria treatment method is considered particularly advantageous because of its cost-effectiveness.[16,17] This type of treatments has also been reported in other studies,[3,18–22] whereas less information is available regarding the aerobic and anaerobic transition region, which is characterized by low levels of aeration.[23]

The objective of the present study was to investigate the enhanced efficiency of excess sludge hydrolysis by the addition of thermophilic *Bacillus* sp. Hnu under different oxygen supply conditions and analyze the kinetic parameters during this process.

EXPERIMENTAL

Source of excess sludge and culture media

The excess sludge used in the present study was obtained from the secondary sedimentation tank of a municipal wastewater treatment plant in Changsha, China. Concentrated sludge was obtained after the sludge was allowed to settle at 4 °C for 24 h. The supernatant was then removed. The sludge was filtered through a 1 mm×1 mm metal sieve and then stored at 4 °C until use. The main characteristics of the sludge after filtration are given in Table I.

TABLE I. Characteristics of the filtered excess sludge (mg L^{-1})

Parameter	Value
pH	6.9±0.1
Soluble chemical oxygen demand	150±10
Total chemical oxygen demand	14850±287
Concentration of total suspended solid	153840±148
Concentration of volatile suspended solid	71350±69
Concentration of ammonia nitrogen	18±1.5
Concentration of soluble phosphate	16±1

The following culture media were employed: Luria–Bertani (LB) solid medium containing: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride, 30 g agar powder and 1 L distilled water; starch agar containing: 5 g soluble starch, 1 g yeast extract, 2 g tryptone, 0.003 g calcium chloride, 0.1 g magnesium chloride, 0.36 g monopotassium dihydrogen phosphate, 1.3 g disodium hydrogen phosphate, 20 g agar powder, and 1 L distilled water; casein agar medium containing: 10 g casein, 3 g beef extract, 2 g disodium hydrogen phosphate, 5 g sodium chloride, 15 g agar, 12.5 mL bromothymol blue solution (0.4 %), and 1 L distilled water.

Isolation and identification of the thermophilic strain

Strain isolation. The thermophilic bacteria were isolated from the excess sludge. The fresh sludge was transferred into a 500 mL Erlenmeyer flask covered with a rubber stopper to prevent evaporation. The sludge was cultured at 60 °C in a water-bath with vibrator at a shaking rate of 100 rpm for one week. Then, 2/3 of the old sludge was discharged and replaced with an equal volume of fresh sludge. The same procedure was repeated for 6 months. The cultured sludge was diluted to an appropriate concentration, spread on an LB agar plate, and incubated at 60 °C for 48 h. After the growth of the bacterial colonies, the representative strains of all colony types that could be distinguished on the plates were isolated by sub-culturing onto the same LB agar plates at the same temperature until a single colony was eventually identified to ensure a pure culture. A typical isolated strain was inoculated onto LB agar and LB liquid medium, incubated at 60 °C for 48 h, and then preserved in a refrigerator at 4 °C.

Strain identification. The isolated bacteria were spread onto a standard nutrient agar plate and incubated at 60 °C for 48 h. The morphological characteristics, including: shape, colony, size, color and physiological and biochemical characteristics were determined (Table II).

TABLE II. Characteristics of thermophilic *Bacillus* sp. Hnu

Index	Characteristics	*Bacillus* sp. Hnu
Morphological characteristics	Shape	Bacilliform
	Colony	Smooth
	Size	Moderate
	Color	Semitransparent
Physiological and biochemical characteristics	Motility	+
	Sporiparous	+
	Gram stain	+
	Aerobic	+
	Catalase reaction	+
	Protease-producing	++
	Amylase-producing	+
	pH	5.5–8.5
	Temperature	40–65 °C
	Optimum temperature	60 °C

Casein agar medium and starch agar were used to test the target bacteria for the production of protease and amylase.

Moreover, 16S RNA gene sequence analysis was used to identify the species of the target strain. DNA was extracted from 2 mL of a pure strain solution (LB medium, 60 °C, 48 h), which was concentrated at 12000×*g* for 30 s and then extracted with a DNA extraction kit. The extracted genome was used as the template for 16S RNA amplification with PCR primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGA-

CTT-3'). The cycle program for the amplification was as follows: 5 min at 95 °C; 24 cycles each for 30 s at 95 °C, 30 s at 55 °C and 1.5 min at 72 °C, followed by a final 10 min extension at 72 °C. The PCR product was detected by agarose gel electrophoresis. The 16S rRNA gene from the isolated bacteria was purified, cloned, and sequenced by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China).

Bacillus *sp. Hnu-enhanced hydrolysis tests*

Experiments on the influence of *Bacillus* sp. Hnu in the enhanced hydrolysis of excess sludge were performed in four 1000 mL volumetric flasks. The temperature was controlled in each test using a water bath. Distilled water (50 mL) was added to 500 mL concentrated excess sludge in the control test. LB liquid medium (50 mL) was added to the 500 mL concentrated excess sludge in the other three reactors. The control test and anaerobic reactors were sealed with a rubber stopper with an inserted glass tube that reached the sludge, enabling sample withdrawal. Three glass tubes were placed in the rubber plugs of the microaerobic and aerobic reactors. The first tube was for sampling, the second was the air inlet, and the third tube was the gas outlet. The gas outlet was a covered condense pipe to prevent evaporation. Compressed air was allowed to pass through the microaerobic and aerobic reactors at ventilation rates of 20 and 70 mL min^{-1}, respectively. Under these aerating conditions, the dissolved oxygen (*DO*) ranged from 0.4 to 0.6 mg L^{-1} and 1.5 to 2.5 mg L^{-1}, respectively.

Using these experimental techniques, the effect of temperature on excess sludge digestion was investigated under control, anaerobic, microaerobic and aerobic conditions at 40, 50 and 60 °C with no pH adjustment (pH 6.9). The effect of pH on excess sludge digestion was investigated under control, anaerobic, microaerobic and aerobic conditions at pH values of 6.0, 7.0, 8.0 and 9.0, adjusted using 2 M NaOH or 2 M HCl. All flasks were mechanically stirred at 100 rpm.

The parallel experiments were performed simultaneously, and all experiments were repeated.

Analytical methods

The values of soluble chemical oxygen demand, total chemical oxygen demand, total suspended solid, volatile suspended solid, ammonia nitrogen and soluble phosphate were determined according to standard methods.[24] The pH was determined using a Multiline 330i pH meter standardized using buffer solutions of different pH values.

The protease activity was measured according to a universal protease activity assay (GB/T23527-2009): 1 mL of sample and 1 mL casein solution (10.00 mg mL^{-1}) were incubated for 10 min at 40 °C. The reaction was stopped by the addition of 2 mL 0.4 M trichloroacetic acid, and after standing for 10 min, the mixture was filtered though filter paper. 5 mL of sodium carbonate (0.4 M) and 1 mL of Folin reagent were added to 1 mL of the filtrate and the absorbance was detected at 680 nm. One unit of absorbance is expressed as one enzyme unit per milliliter (EU mL^{-1}) protease activity.

RESULTS AND DISCUSSION

Effect of temperature and oxygen supply on excess sludge digestion

Temperature influences the metabolic activities of microbial populations and characterizes the hydrolysis rates. The mesophilic temperature (40 °C) and thermophilic temperatures (50 and 60 °C) were chosen as target temperatures to determine the volatile suspended solid removal ratio as well as the presence of protease activity with and without the incubation of the *Bacillus* sp. Hnu under different oxygen supply conditions. The temperature remarkably influenced the volatile suspended solid removal ratio in the *Bacillus* sp. Hnu-inoculated sample (Figs. 1a and 1b). The highest volatile suspended solid removal ratio (54.4 %) was obtained at 60 °C after 108-h cultivation, whereas only 30 and 45.6 % were obtained under aerobic condition at 40 and 50 °C, respectively. The volatile suspended solid removal ratios for the microaerobic process were 22, 37.4 and 42.5 % at 40, 50 and 60 °C, respectively, whereas corresponding values for the anaerobic process were 14.6, 18.3 and 21.5 %. These effects were attributed to the autolysis of the mesophilic organisms, a major group in the excess sludge caused by the temperature shock. The protease exo-enzymatic activity produced by thermophilic processes can cause simultaneously lysis. The volatile suspended solid removal ratio, as seen in the control test (no *Bacillus* sp. Hnu inoculation and no oxygen supply), was 4.6 % at 60 °C after 6 h and only 0.8 % at 30 °C. In addition, the results demonstrated that 33.6 % of the volatile suspended solid were removed in the first 24 h at 60 °C under the thermophilic aerobic condition, which accounted for 66.7 % of the total removals. However, only 50.7 and 58.3 % of the total removals were obtained at 40 and 50 °C, similar to the anaerobic and microaerobic excess sludge processes.

Protease activity was important in the depolymerization of the excess sludge, lysis and hydrolysis of proteins. The variations in protease activity were evaluated during the volatile suspended solid degradation ratio test. The results of the protease activity at 50 and 60 °C are shown in Fig. 1c. The increase in the digestion temperature from 50 and 60 °C resulted in an increase of the protease activity in the supernatant. The protease activity increased with digestion time and the amount of oxygen supply (Fig. 1c). This activity reached 0.6 EU mL^{-1} at 60 °C under aeration after 24 h, and the highest activity reached 0.79 EU mL^{-1}, whereas the protease activities were only 0.354 and 0.396 EU mL^{-1} at 50 and 60 °C under anaerobic condition after 24 h. However, the amount of protease activity under the microaerobic condition was almost the same as that under the aerobic condition and increased to 0.58 EU mL^{-1} at 60 °C after 24 h. According to the equation $(K_\mathrm{d})_T = (K_\mathrm{d})_{20}q^{(T-20)}$, where K_d is reaction rate, T is temperature and q is reaction rate constant, an increase in the temperature of the reactor results in an increase in the reaction rate constant, which implies an increase in the digestion rate. A higher temperature leads to more bacteria lysis and release of endo-enzyme, resulting in an increased digestion rate. The aerobic digestion of the excess sludge could be considered as a continuation of the activated sludge process. The cell tissue is oxidized aerobically to carbon dioxide, water, and ammonia. The microaerobic oxidation, *i.e.*, a limited oxygen supply, could also produce gases, including carbon dioxide.[15] The supply of oxygen in the thermophilic excess sludge process could affect a decrease of the volatile suspended solid (Figs. 1a and 1b) compared with the anaerobic condition, as oxygen can increase

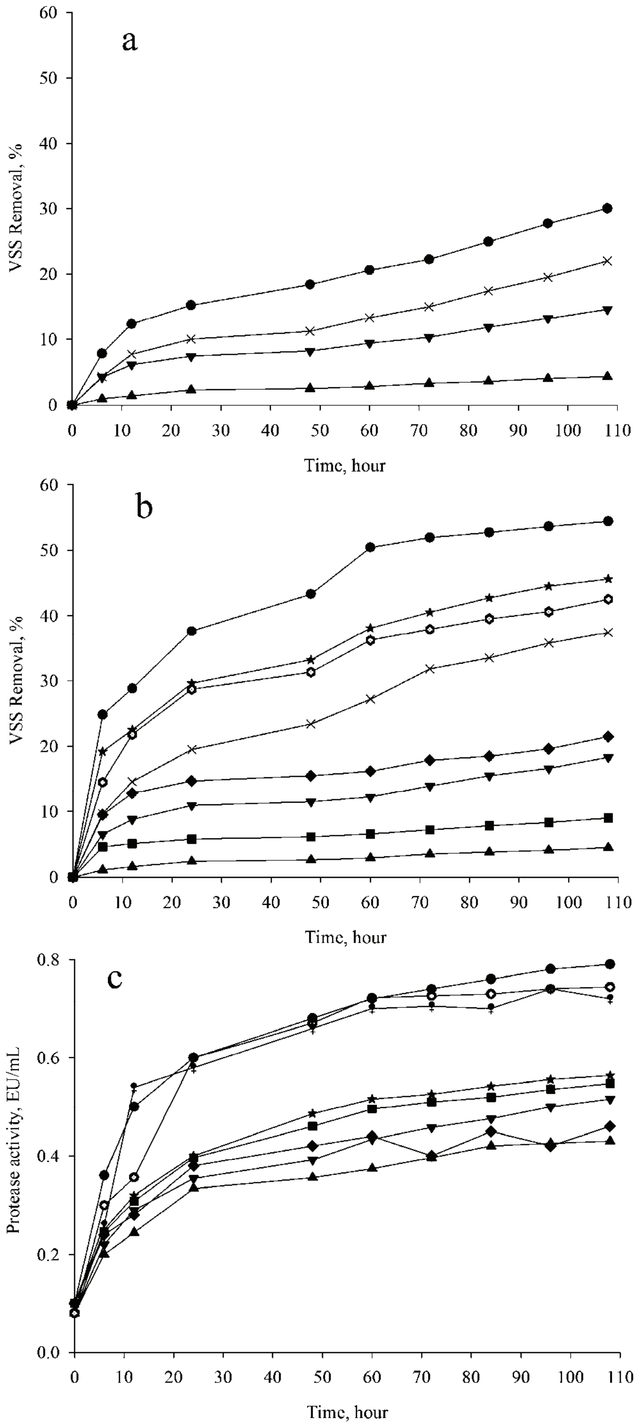

Fig. 1. Effect of temperature on anaerobic, microaerobic and aerobic digestion of waste activated sludge with and without inoculation of *Bacillus* sp. Hnu. a) Removal ratio of volatile suspended solid at 40 °C (–▲– – control, –▼– – anaerobic, –✕– – microaerobic, –●– – aerobic); b) removal ratio of volatile suspended solid at 50 and 60 °C (–▲– – 50 °C, control, –▼– – 50 °C, anaerobic, –✕– – 50 °C, microaerobic, –★– – 50 °C, aerobic, –■– – 60 °C, control, –◆– – 60 °C, anaerobic, –✪– – 60 °C, microaerobic, –●– – 60 °C, aerobic); c) variations of protease activity (–▲– – 50 °C, control, –▼– – 50 °C, anaerobic, –★– – 50 °C, microaerobic, –✪– – 50 °C, aerobic; –◆– – 60 °C, control, –■– – 60 °C, anaerobic, –✝– – 60 °C, microaerobic, –●– – 60 °C, aerobic).

increase the activity of the microorganism and accordingly increase the enzymatic activity. The above results demonstrate that protease activity has an important effect on the sludge digestion. The optimum temperature is 60 °C because *Bacillus* sp. Hnu cannot survive temperatures in excess of 65 °C.

Effect of pH on thermophilic excess sludge digestion

Bacillus sp. Hnu was inoculated into the excess sludge at pH values ranging from 6 to 9, which were adjusted with 0.1 M HCl or 0.1 M NaOH, and the effects of pH on the volatile suspended solid removal rate and protease activity were investigated. The volatile suspended solid removal rate and protease activity under different pH levels and oxygen supplies are shown in Figs. 2a and 2b, respectively. As can be seen from Fig. 2, within the range studied, the volatile suspended solid solubilization ratio was high at pH 7 and 8. The highest volatile suspended solid solubilization ratio (55 %) was obtained at pH 7 under aerobic

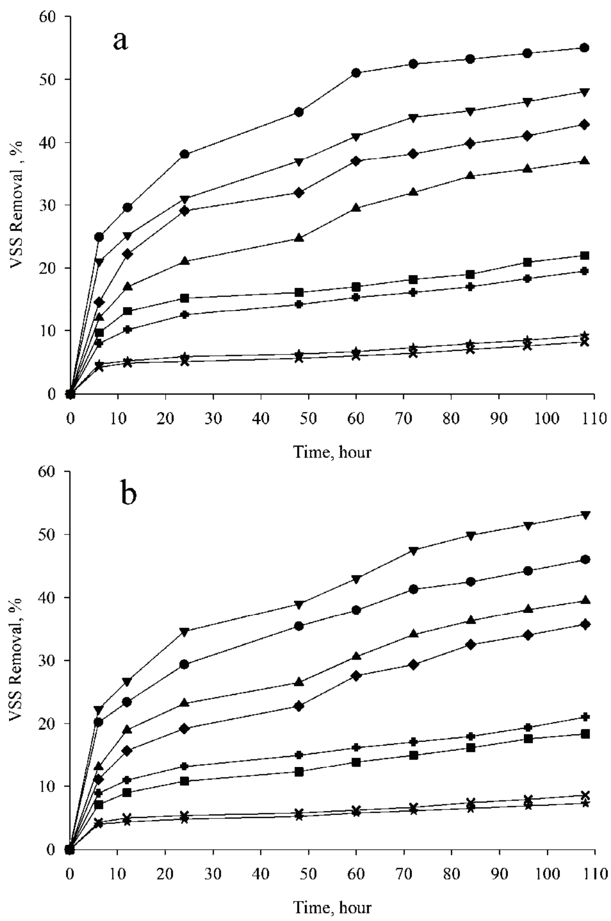

Fig. 2. Effect of pH on the volatile suspended solid removal under anaerobic, microaerobic and aerobic digestion of waste activated sludge with and without inoculation of *Bacillus* sp. Hnu at 60°C. a) pH 6 and 7 (–✱– – pH 6, control, –✚– – pH 6, anaerobic, –▲– – pH 6, microaerobic, –▼– – pH 6, aerobic, –★– – pH 7, control, –■– – pH 7, anaerobic, –◆– – pH 7, microaerobic, –●– – pH 7, aerobic); b) pH 8 and 9 (–✱– – pH 8, control, –✚– – pH 8, anaerobic, –▲– – pH 8, microaerobic, –▼– – pH 8, aerobic; –★– – pH 9, control, –■– – pH 9, anaerobic, –◆– – pH 9, microaerobic, –●– – pH 9, aerobic).

condition after 108 h. Moreover, the solubilization ratios of the volatile suspended solid were 42.9 and 22 % at pH 7 after 108 h under microaerobic and anaerobic conditions, respectively. These phenomena indicate that the oxygen supply is an important factor in volatile suspended solid degradation primarily because of the notable DO effect on the protease activity.

Bacterial life, *i.e.*, metabolism, growth and cellular division, is closely related to pH. The effect of pH on the transport of nutrients and organic components through the cytomembrane determines its toxicity action on bacteria. This condition also activates the hydrolytic enzyme alkaline phosphatase. The pH range suitable for the existence of most biological life is quite narrow and critical (typically 6 to 9).[25] The effects of pH on the protease activity were studied in the range of 6 to 9 under anaerobic, microaerobic, and aerobic digestions of the excess sludge with and without *Bacillus* sp. Hnu inoculation at 60 °C. The results are shown in Fig. 3. The protease activity increased with time under all sludge

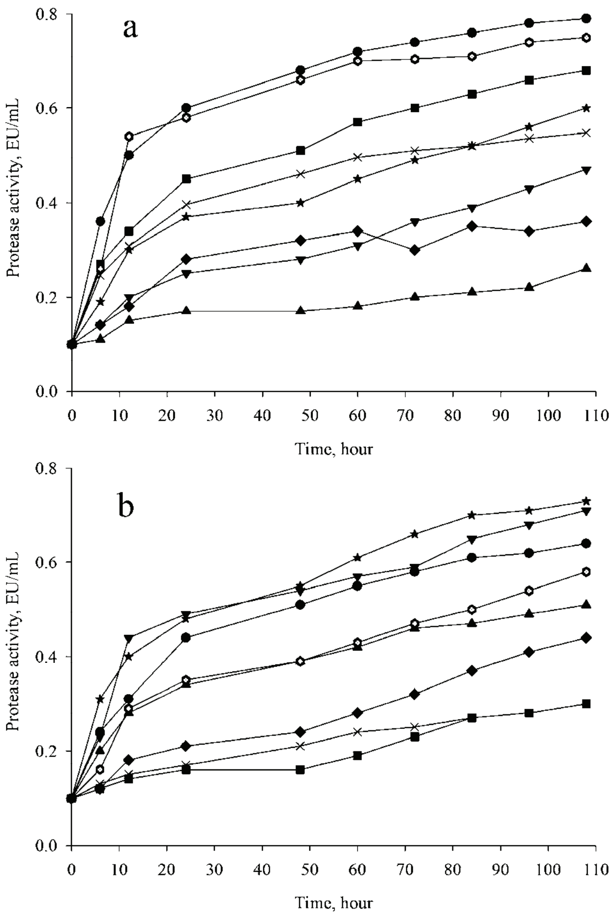

Fig. 3. Effect of pH on the protease activity under anaerobic, microaerobic and aerobic digestion of waste activated sludge with and without inoculated *Bacillus* sp. Hnu at 60 °C. a) pH 6 and 7 (▲ – pH 6, control, ▼ – pH 6, anaerobic, ★ – pH 6, microaerobic, ■ – pH 6, aerobic; ◆ – pH 7, control, ✕ – pH 7, anaerobic, ○ – pH 7, microaerobic, ● – pH 7, aerobic); b) pH 8 and 9 (✕ – pH 8, control, ▲ – pH 8, anaerobic, ▼ – pH 8, microaerobic, ★ – pH 8, aerobic; ■ – pH 9, control, ◆ – pH 9, anaerobic, ○ – pH 9, microaerobic, ● – pH 9, aerobic).

conditions and the highest activity of 0.79 EU mL^{-1} was reached at pH 7 under aerobic conditions. The activity of the sludge without inoculated *Bacillus* sp. Hnu at pH 7 was only 0.36 EU mL^{-1}. At the same pH, the protease activity increased with the increasing supply of oxygen. Thus, the inoculation of *Bacillus sp.* Hnu and the oxygen supply helped accelerate the hydrolysis rate.

Kinetic analysis of sludge hydrolysis

Different rates of hydrolysis, k_h, values were reported because the hydrolysis process was affected by various factors, such as pH, temperature, particle size and its distribution pattern, and sludge source.[26] Therefore, a comparison of the k_h values obtained in the present study with those in previous publications is quite challenging.

Feng *et al*.[27] analyzed excess sludge hydrolysis and short-chain fatty acids (SCFAs) production at pH 10, and observed that the hydrolysis of excess sludge particulate chemical oxygen demand (*COD*), as well as the accumulation of SCFAs followed first-order kinetics. Thus, the hydrolysis of volatile suspended solid in the present study could also be assumed to follow first-order kinetics. The first-order kinetic equation of the hydrolysis (volatile suspended solid reduction) can be described as:

$$\frac{\mathrm{d}M}{\mathrm{d}t} = -k_h M \qquad (1)$$

$$\ln M = -k_h t + b \qquad (2)$$

where dM/dt is the rate of change of the volatile suspended solid per unit time and b is the integration constant. By plotting ln M *versus* t, the slope and the intercept, corresponding to the values of $-k_h$ and b, respectively, could be obtained. The regression curves are illustrated in Fig. 4, and a summary of the values of the volatile suspended solid hydrolysis rate constants are given in Table III.

The goodness of fit values for the different types of treatment at the temperature 40 °C were generally good within the range 0.9169–0.9523 (Fig. 4a and Table III). At a temperature 50 °C, the correlation coefficients were in the range from 0.8660–0.9505 (Fig. 4b and Table III). However, the correlation coefficients of 60 °C at different oxygen supply were not high, only 0.7608–0.8460 (Fig. 4c and Table III). This phenomenon may be due to thermophilic bacteria exhibiting higher protease activity at higher temperatures (optimum temperature 60 °C) resulting in faster degradation of the volatile suspended solid, while at high temperatures partially mesophilic bacteria are killed and the organic particles rupture, which results in the sludge hydrolysis that does not follow the first-order kinetic model at the highest temperature (60 °C). The hydrolysis rate constants (k_h) for the anaerobic, microaerobic, and aerobic conditions were 3, 4.8, and 7 times (40 °C), 3.5, 9.8, and 11.8 times (50 °C) and 2.7, 7.2, and 10.3 times (60 °C)

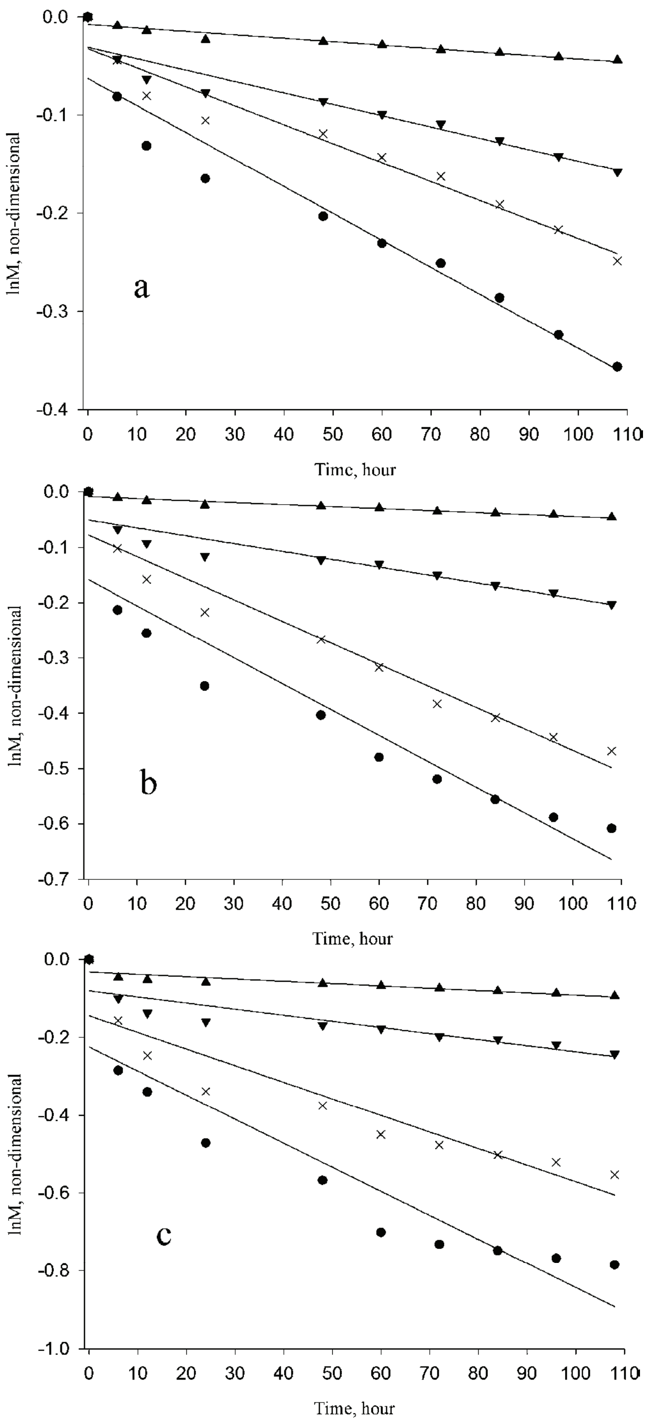

Fig. 4. Relation between ln *M* and time during sludge hydrolysis. a) 40 °C (▲ – control, ▼ – anaerobic, × – microaerobic, ● – aerobic); b) 50 °C (▲ – control, ▼ – anaerobic, × – microaerobic, ● – aerobic); c) 60 °C (▲ – control, ▼ – anaerobic, × – microaerobic, ● – aerobic).

higher, respectively, than that of the control test, indicating that the *Bacillus* sp. Hnu and the oxygen supply helped accelerate the hydrolysis rate.

TABLE III. Hydrolysis rate constants under different temperature and oxygen supply conditions

Temperature, °C	Type of treatment	Dynamic equation	Rate constant k_h / h^{-1}	R^2
40	Control	$y = -0.0004x - 0.0075$	0.0004	0.9344
	Anaerobic	$y = -0.0012x - 0.0308$	0.0012	0.9169
	Microaerobic	$y = -0.0019x - 0.0324$	0.0019	0.9523
	Aerobic	$y = -0.0028x - 0.0626$	0.0028	0.9392
50	Control	$y = -0.0004x - 0.0087$	0.0004	0.9268
	Anaerobic	$y = -0.0014x - 0.0359$	0.0014	0.8660
	Microaerobic	$y = -0.0039x - 0.0778$	0.0039	0.9505
	Aerobic	$y = -0.0047x - 0.1584$	0.0047	0.8839
60	Control	$y = -0.0006x - 0.0324$	0.0006	0.7608
	Anaerobic	$y = -0.0016x - 0.0808$	0.0016	0.7676
	Microaerobic	$y = -0.0043x - 0.1444$	0.0043	0.8640
	Aerobic	$y = -0.0062x - 0.2243$	0.0062	0.8493

CONCLUSION

A thermophilic strain was isolated from excess sludge and identified as a new species of *Bacillus* by 16S rRNA gene sequence analysis, named *Bacillus* sp. Hnu. *Bacillus* sp. Hnu was able to release a protease that could dissolve sludge. The results indicated that temperature and oxygen supply affect the volatile suspended solid removal ratio and protease activity, and higher temperature and greater oxygen supply were advantageous to the volatile suspended solid removal ratio and protease activity. The maximum volatile suspended solid removal ratios of 21.5, 42.5 and 54.4 % were obtained after 108 h digestion at pH 6.9 and 60 °C under anaerobic, microaerobic and aerobic conditions, respectively. Volatile suspended solid removal ratio and protease activity were only slightly affected by the pH. The kinetic study showed that the hydrolysis of sludge with *Bacillus* sp. Hnu and the control test followed the first-order kinetics except at the highest employed temperature (60 °C). The hydrolysis rate constants (k_h) for the anaerobic, microaerobic, and aerobic conditions were 3, 4.8, and 7 times (40 °C), 3.5, 9.8, and 11.8 times (50 °C) and 2.7, 7.2, and 10.3 times (60 °C) higher, respectively, than that of the control test, indicating that *Bacillus* sp. Hnu and oxygen supply helped accelerate the hydrolysis rate.

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ИЗВОД

УБРЗАВАЊЕ ХИДРОЛИЗЕ ОТПАДНИХ НАСЛАГА ПРИМЕНОМ ТЕРМОФИЛНЕ БАКТЕРИЈЕ *Bacillus* sp. Hnu У ПРИСУСТВУ РАЗЛИЧИТИХ КОНЦЕНТРАЦИЈА КИСЕОНИКА

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Термофилна бактерија је изолована из отпадних наслага у води и 16S рДНК анализом је констатовано да припада роду *Bacillus*. Идентификована је као нова и названа је *Bacillus* sp. Hnu. Циљ рада је био да испита ефикасност хидролизе отпадних наслага овом бактеријом у присуству различитих концентрација кисеоника. Резултати су показали да је боље уклањање испарљивих састојака при већим температурама и више кисеоника, као и да је повећана протеазна активност. Највећи удео уклањања испарљивих супстанци суспендованих у чврстом талогу постигнут је после 108 сати дигестије на pH 6,9 и 60 °C и повећавао се за 17,2, 38,0 и 45,4 % у анеробним, микроаеробним и аеробним условима, редом, у односу на контролни тест. Константе брзине хидролизе за анаеробне, микроаеробне и аеробне услове су биле 3,0; 4,8 и 7 пута (40 °C), 3,5; 9,8 и 11,8 пута (50 °C), односно 2,7; 7,2 и 10,3 пута (60 °C) веће од оних у контролним условима. Може се закључити да је *Bacillus* sp. Hnu способна да убрза хидролизу отпадних наслага. Кинетичка испитивања су показала да хидролиза отпада бактеријом *Bacillus* sp. Hnu, као и контролни тест, прате кинетику првог реда, осим на 60 °C.

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