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The isolation, identification of sludge-lysing thermophilic bacteria and its utilization in solubilization for excess sludge

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A novel strain of thermophilic bacteria with a highly efficient sludge dissolution performance was isolated from garden soil at 65°C in this study. The colony morphology, physiological and biochemical characteristics of the strain were investigated. The results showed that the strain was Gram-positive, small rod-shaped, sporulating and secreted extracellular enzymes (protease and amylase). The 16S rDNA analysis demonstrated that this strain had not been previously reported. Therefore, it was labelled Bacillus thermophilic bacteria AT07-1 (registration number: FJ231108). To evaluate its capability for excess sludge solubilization, a pure culture of the strain was used in sludge solubilization tests; an enhanced solubilization process was subsequently obtained. After 36 h digestion, the protease activity in the inoculated system reached 0.37 U/ml, an increase of 0.16 U/ml compared with the non-inoculated system (0.21 U/ml). The solubilization rate for volatile suspended solids reached 46.45% in 48 h after inoculation with Bacillus thermophilic bacteria AT07-1, which was 10.24% higher than the non-inoculated system, and which could meet the standard of sludge stability suggested by the US Environmental Protection Agency.

Keywords: excess sludge; extracellular enzyme; protease activity; sludge solubilization; thermophilic bacteria; VSS

1. Introduction

The volume of excess sludge (ES) generated in the sewage treatment process is about 0.15–1% of the treated water. A significant increase in ES production is caused by an increase in the amount of wastewater and the treatment rate. The cost of ES treatment is very high, accounting for up to 60% of the total operational costs of a plant [1,2]. It is known that ES contains many hazardous materials such as pathogens, parasite eggs, unstable organics, etc., and the disposal of untreated ES brings a heavy environmental burden. Hence, the disposal and treatment of ES has been considered to be a serious social problem, and it is essential therefore to develop an effective disposal technique to reduce ES as soon as possible.

In order to reduce ES production, new sludge disintegration processes, which involve mechanical, chemical, thermal and biological methods, have been developed and are now increasingly available on a commercial basis [3–11]. Among these approaches, biological solubilization of organic sludge by thermophilic bacteria is considered to be one of the most efficient means, because of its high sludge disintegration and solubilization capability. Due to their extreme habitat specificity, thermophilic bacteria have a unique enzyme system and defence mechanism adapted to a bio-environment in which most bacteria find it difficult to survive. Their cells and enzymes have a unique heat resistance and corresponding molecular structure, which can help the development and utilization of important biological resources; thus, thermophilic bacteria can be used to produce a variety of enzymes, such as cellulase, protease, amylase enzyme, lipase, inulin enzymes, and so on. Novel industrial enzymes and other bioactive substances, which have been separated and extracted from thermophilic bacteria, have great application value in genetic engineering, protein engineering, fermentation engineering, and development and utilization of mineral resources. Using the extracellular enzymes excreted by thermophilic bacteria to depolymerize and crack sludge quickly and efficiently may improve the degradation of the sludge and enhance methane production in anaerobic digestion [12–14], and finally achieve the aim of 'zero emissions' sludge and resource utilization.

A number of early studies reported that some bacteria could excrete an exoenzyme which lysed the cells of other microorganisms [15–17]. In addition, Yan *et al*. [18] reported that when microorganisms died during the initial stage of thermophilic aerobic digestion they released protease, which was the major reason for the hydrolysis of

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sludge, and that protease activity was high during thermophilic conditions. Mori [19] reported that thermophilic bacteria could solubilize sludge at high temperature under aerobic conditions. Hasegawa *et al*. [20] reported a thermophilic bacteria strain SPT2-1 which could solubilize ES efficiently, and the strain was used to develop a full-scale activated sludge system with low ES production [21,22]. Since over 50% of the organic material in the sludge is protein, protein hydrolysis was determined as the limiting step of sludge digestion [23]. The enzymatic hydrolysis of sludge has been investigated over the last three decades, and enzyme-treated sludge has become more amenable to further treatment and can be easily and rapidly converted to value-added products [24]. Several studies have shown that protease accelerated the solubilization of municipal sludge [14,25–28].

The objective of this research is to isolate a strain of thermophilic bacteria and to investigate its colony morphology, physiological and biochemical characteristics. Furthermore, the strain was inoculated into sludge in thermophilic enzyme tests (S-TE) to test its effect on ES dissolution.

2. Materials and methods

2.1. ES and the culture media used in this study

In this study, the sludge was taken from the first municipal wastewater treatment plant in Changsha, P.R. China. The sludge was left for 24 h and then the supernatant (S) was removed; this sludge was defined as raw sludge (RS). As is typical for ES, V_{RS}:V_S was at 2:1 before total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were adjusted to about 17.36 g/l and 11.06 g/l, respectively. RS and ES were both stored at 4°C.

LB broth comprised the following: tryptone (Oxoid) 10 g, yeast extract (Oxoid) 5 g, NaCl 5 g, distilled water 1 l (20 g agar powder added for nutrient agar). Skimmed milk agar comprised the following: skimmed milk powder 10 g, yeast extract (Oxoid) 3 g, agar powder 20 g, distilled water 1 l. Starch agar comprised the following: soluble starch 5 g, yeast extract (Oxoid) 1 g, tryptone (Oxoid) 2 g, $CaCl_2$ 0.003 g, $MgCl_2$ 0.1 g, KH_2PO_4 0.36 g, Na_2HPO_4 1.3 g, agar powder 20 g, distilled water 1 l.

2.2. Strain isolation and identification

2.2.1. Strain isolation

The thermophilic bacteria were isolated from the school's garden soil. In total, 10 g of garden soil samples was suspended in 100 ml sterilized ES and incubated at 65°C with occasional stirring for 2 days. A volume of 0.1 ml of the dilution was spread on a standard nutrient agar plate, and incubated at 65°C for 2 days. After the growth of bacterial colonies on the agar plate, an isolated colony was picked from the plate and streaked onto another agar plate. This procedure was repeated at least five times to ensure a pure culture. The isolated pure strain of the thermophilic bacteria was designated strain AT07-1. LB broth (100 ml) was added to a 250-ml flask. The isolated colony was transferred from the plate into the flask. The flask culture was incubated at 65°C for 2 days, and then stored at 4°C.

2.2.2. Strain identification

The isolated bacteria were spread on a standard nutrient agar plate, and incubated at 65°C for 2 days. The morphology, size, colour, surface and edge characteristics of the colony were observed. Gram staining and spore staining were performed for the targeted strain. In addition, the shape and size of the cell were observed under an optical microscope.

Protease secretion was investigated as follows. The targeted bacteria were cultivated in skimmed milk agar for 24 h at 65°C. Colonies with haloes of colourless agar due to digested milk were regarded as protease-secreting bacteria. Amylase secretion was also examined using starch agar. Colonies with haloes of colourless agar due to starch breakdown were regarded as amylase-secreting bacteria.

Furthermore, 16S rRNA gene sequence analysis was used to identify the species of the target strain, and the DNA was extracted from 3 ml strain pure culture solution (LB medium, 65°C, 24 h) using a DNA extraction kit (KQ Co., Ltd., Shanghai China). A polymerase chain reaction (PCR) was run with a primer set of 27F (5′-GAGAGTTTG ATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACG A-3′). The cycle program for amplification was as follows: 10 min at 95°C; 30 cycles each of 30 s at 95°C, 30 s at 53°C and 2 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 isolated bacteria was purified, cloned and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

2.3. S-TE experiment

The S-TE device is shown in Figure 1. Two sets of same test module design were made with plexiglass; the effective volume of the device was 2.8 l. Electric mixers were used for mixing, with rotation speed of 140 ± 10 r/min.

Both the inoculated sludge and non-inoculated sludge were used in the batch solubilization experiment. The inoculated sludge was prepared by adding 200 ml bacterial suspension containing standard LB culture medium and AT07-1 into 2000 ml ES, and the non-inoculated sludge was 2000 ml ES plus 200 ml of distilled water. Table 1 shows the concentration parameters. The temperature was adjusted to 65°C, and the ventilation was about 0.16 l/min. In these aerating conditions, dissolved oxygen (DO) levels ranged from 1–3 mg/l. Total running time was 84 h, and parallel samples were analysed every 12 h.

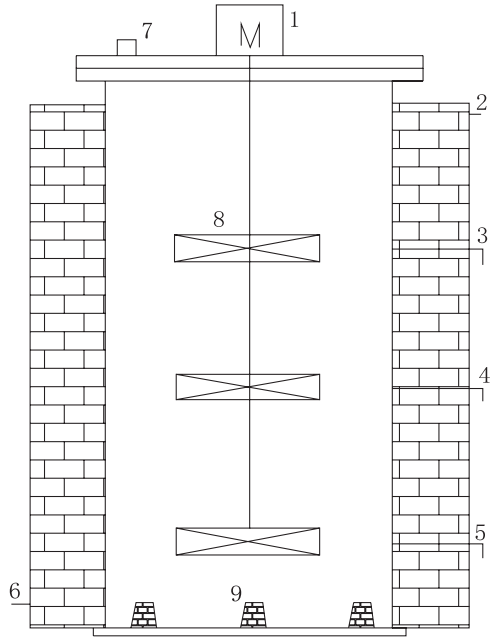

Figure 1. Schematic diagram of S-TE reactor (1: electric stirrer; 2: water exit (water bath layer); 3, 4, 5: sludge exit; 6: water inlet (water bath layer); 7: Sludge inlet; 8: stirrer; 9: air diffuser).

Table 1. Total suspended solids (TSS), volatile suspended solids (VSS) and protease activity in the S-TE test.

	TSS (g/l)	VSS (g/l)	protease activity (U/ml)
non-inoculated	16.88	10.88	0.025
inoculated	25.06	15.88	0.038

2.4. *Analytical methods*

TSS and VSS were measured using the Standard Methods [29]. Protease activity was assayed by the method of Okamura *et al*. [30]. A volume of 0.5 ml of sample and 2% casein solution (2.0 g in 100 ml of 0.4 M of Tris–HCl buffer, pH 8.5) was incubated for 15 min at 37°C. The reaction was stopped when 1 ml of 0.44 M trichloroacetic acid was added, and the mixture was filtered though filter paper after 30 min. Next, 2.5 ml of sodium carbonate (0.4 M) and 0.5 ml of Folin reagent were added to 0.5 ml of the filtrate and absorbance was detected at 660 nm. One unit of absorbance is expressed as 1 U/ml protease activity. pH and DO were detected using a Multi 340i pH/DO meter (Wissenschaftlich-Technische Werkstatten GmbH&Co.KG).

3. Results

3.1. *Strain isolation and identification*

The strain AT07-1 which could hydrolyze ES was isolated from garden soil at 65°C. Physiological and biochemical analysis showed that the strain was a gram-positive, bacillus and was able to form endospores. The scanning electron microscopy image of the thermophilic bacterium AT07-1 is shown in Figure 2. Some early studies had reported that the bacteria which dissolved sludge effectively were mostly thermophilic bacteria Bacillus [20,31]. On the agar plate, AT07-1 produced semi-transparent, edge-irregular, convex, smooth surface and shallow cleft light gray colonies. In the skimmed milk agar and starch agar, AT07-1 formed on the hydrolysis of a transparent or translucent halo. This confirmed that AT07-1 could secrete extracellular enzymes (protease and amylase). According to DNA homology analysis of 16S rRNA gene, there were no similar bacteria in the gene pool. This strain was newly registered with the approved name Bacillus thermophilic bacteria AT07-1 (Registration No.: FJ231108).

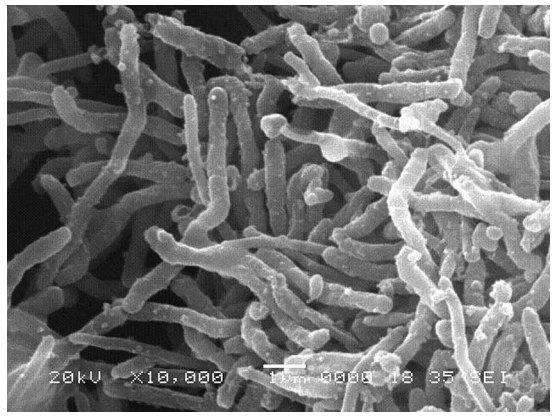

Figure 2. Scanning electron microscopy image of the thermophilic bacterium AT07-1.

3.2. *S-TE experiment*

AT07-1 was inoculated into one of the S-TE processing systems to verify its sludge-solubilization activity. TSS, VSS and protease activity were evaluated in this study.

3.2.1. *Effect of AT07-1 on protease activity*

The protease activities of the inoculated system and control are shown in Figure 3. Throughout the digestion progress, the protease activity of the inoculated system was higher than that of the control. Protease activity increased rapidly for 36 h for all cases, and then maintained a slight increasing trend. Figure 3 shows that protease activity in the inoculated system reached 0.37 U/ml after 36 h, while only 0.21 U/ml was found in the control. After 84 h of digestion, the protease activity in the inoculated system increased to 0.43 U/ml, which was 0.18 U/ml higher than the control system (0.25 U/ml).

3.2.2. *Effect of AT07-1 on TSS and VSS degradations*

During ES digestion, the whole process lasted 84 h and the removal results of TSS and VSS are shown in Figure 4. The

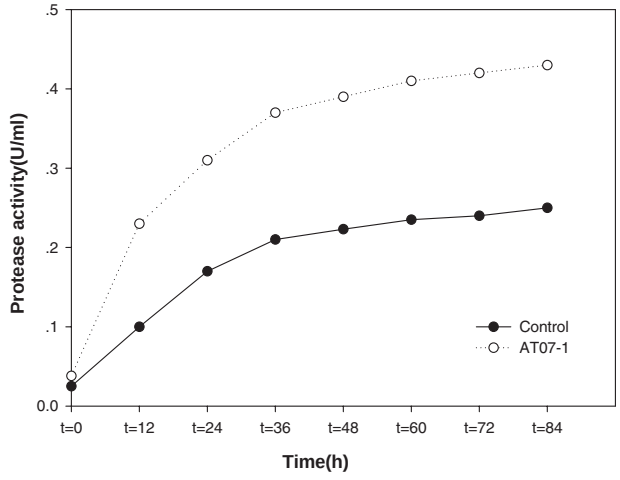

Figure 3. Variation in protease activity.

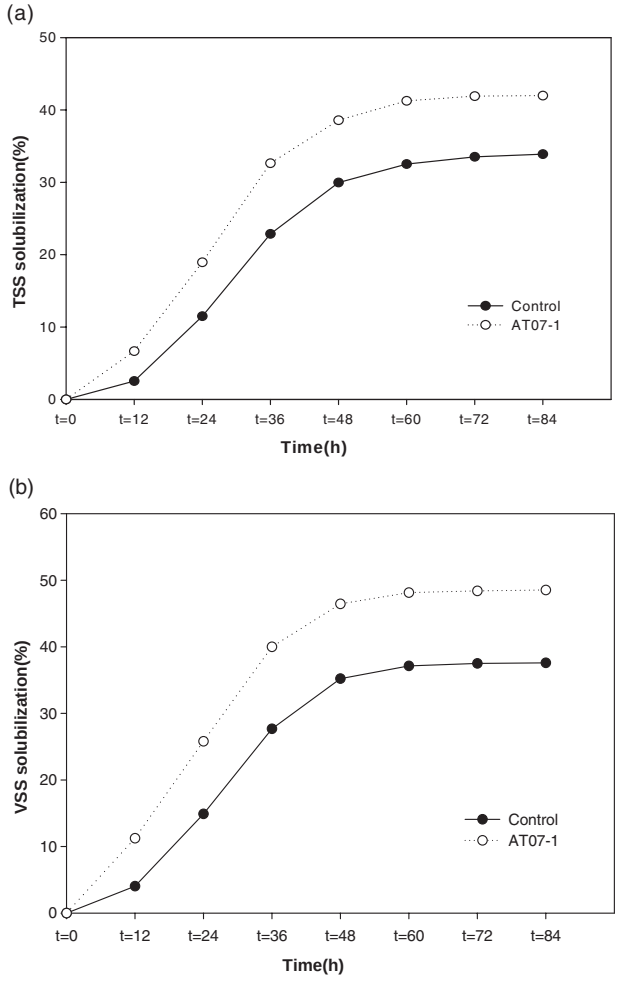

Figure 4. Variations in (a) TSS and (b) VSS solubilization rate.

TSS degradation ratio of the AT07-1-inoculated sludge with was higher than that of the control (Figure 4(a)). Sludge digestion could be divided into two stages: a fast digestion phase within first 48 h, followed by a stable phase. During the first 48 h, both inoculated and control systems had a fast degradation rate; however, degradation of TSS in the inoculated system was higher than that in the control. The maximum digestion ratios in the first 48 h were 38.57% and 29.97% for the inoculated system and the control, respectively. After 48 h, the degradation in both systems began to decrease and then was maintained stably. There was no obvious difference in degradation rate for either system during the stable phase. The variations of VSS solubilization rate in the inoculated system and control system are shown in Figure 4(b), and the trends were almost the same as TSS. The VSS degradation ratio of the AT07-1-inoculated sludge was higher than that of the control. In the inoculated system, a VSS solubilization rate of 46.45% was obtained after 48 h, which was 11.24% higher than the control system (35.21%).

4. Discussion

During the first 36 h, high temperature caused the death of a proportion of the microorganisms in the ES, thus much intracellular enzyme was released to the liquid phase, so the enzyme activity increased rapidly. In the inoculated system in particular, extracellular polymeric substances (EPS) were used by the AT07-1 bacteria as nutrient to form endospores and generate secretions of extracellular enzymes [32], which were distributed swiftly to soluble organics. After 36 h, the thermophilic bacteria in the system reached saturation while the protease activity was maintained as stable. Therefore, compared with the control system, the protease activity increased much more rapidly during the initial phase.

From Figures 3 and 4, it can be seen that during the first 36 h the protease activity, TSS and VSS dissolution rates showed a similar rapid growth trend. The thermal hydrolysis effect is the same due to the same high-temperature conditions. TSS, VSS dissolution rates lie in the strength of the enzyme effect. As enzyme activity increased, the increase in the effects of the enzymes enhanced TSS and VSS dissolution rates, which speeded up rapidly. After 36 h, protease activity maintained this rising trend, but less obviously. However, while high enzyme activity was maintained, TSS and VSS dissolution rates began to decline. Although the system has sufficient enzymes, the effects of the enzymes in the remaining sludge micelle were small. In the initial stage, the enzymes attached to the surface of the sludge flocs, demonstrating enzyme effects; with the disintegration of the sludge, the structure of the sludge flocs became sloppy, and most of the enzymes were adsorbed to, entrapped by, or bound to the sludge structure [14], so no further enzymatic catalytic effect was apparent. A simultaneous decrease of TSS and VSS dissolution rate was observed.

VSS is measured as an indicator of organic solids, which comprise more than 95% of organic materials in sludge. According to the US EPA requirements, the sludge will be stabilized when VSS dissolution rate is higher than 38% [33]. During our experiment, after 84 h of digestion the VSS

degradation ratio increased to 48.52%, which could meet the EPA's sludge stabilization requirements. In the S-TE system, TSS and VSS dissolved first because of the thermal hydrolysis effect [34]. With increasing dissolution time, the thermophilic bacteria formed spores and secreted extracellular enzymes (protease and amylase), and then the enzyme catalytic effect became apparent [35]. Regarding the inoculated system, due to the vaccination of thermophilic strain AT07-1, the enzyme concentration is higher than in the non-inoculated system, revealing a stronger catalytic effect of enzymes; therefore, the dissolution rates of TSS and VSS in the inoculated system are much higher than those in the non-inoculated system.

5. Conclusion

A thermophilic strain was isolated and identified as a new species of Bacillus by 16S rRNA gene sequence analysis, named Bacillus thermophilic bacteria AT07-1. This strain was able to secrete extracellular enzymes (protease and amylase) which could dissolve sludge. In the S-TE system, the protease activity of the inoculated system was higher than that of the control. The inoculation of Bacillus thermophilic bacteria AT07-1 could enhance sludge digestion rate significantly. After 84 h of digestion, the TSS degradation ratio increased to 41.98%, which was 8.09% higher than that of control. In addition, the VSS degradation ratio increased to 48.52%, which could meet the provisions of the EPA's sludge stabilization requirements; in contrast, the control system ratio was only 37.58%. Thus it is evident that Bacillus thermophilic bacteria AT07-1 could enhance sludge degradation.

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