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DISINTEGRATION OF SLUDGE BY A TWO-STAGE TREATMENT WITH HYDROGEN PEROXIDE AND SOLID-STATE FERMENTATION BY *ASPERGILLUS ORYZAE* CGMCC5992

A novel and safe method has been reported for wastewater sludge treatment, the first step of which is H_2O_2 pretreatment at 150 cm^3/kg for 80 min. In this step, the majority of organic substrate was degraded, and the shear stress diminished significantly. In the second step, *Aspergillus oryzae* further decomposed the organic substrate of sludge for 8 days, and the activities of manganese peroxidase and lignin peroxidase kept increasing. By the methods, the residual chemical oxygen demand (COD) in the sludge was much lower than that in the sludge treated with high doses of H_2O_2. These results indicate that the present study provides a feasible method to safely dispose sludge from the wastewater treatment plant.

1. INTRODUCTION

In the process of wastewater purification, an excessive amount of waste sludge is generated in the wastewater treatment plant. To safely dispose sludge has recently become a serious issue. The currently commonly used disposal measures include incineration, landfilling and application. However, these methods have been limited by the safety issue and secondary pollution. Several new methods, such as physical- mechanical treatment using ultrasounds [1, 2], chemical treatment using ozone [2], oxidation with H_2O_2 [4, 5], and treatment with acid or alkali [6], have also been explored. The two crucial drawbacks of these methods are the too high disposal cost and secondary pollution. Therefore, ever-increasing interests were concentrated on the biological treatment of sludge with or without enzyme addition [7]. To date, there are two modes of sludge

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biodecomposition, aerobic and anaerobic. However, two critical issues, the odors and bacterial colloid of sludge, hinder the development of biological treatment. The odors of sludge are comprised of a mixture of various gases mainly including hydrogen sulfide (H_2S), mercaptans, and dimethyl sulfide. The heterogeneous nature of these odorous emissions makes odor treatment a difficult task, while the bacterial colloid from anaerobic and aerobic microorganisms brings difficulty to the interchange of substance among microorganism, sludge, and air during the process of solid state fermentation (SSF).

SSF can be simply defined as a process whereby insoluble substrates in the composts, with sufficient moisture but no or nearly no free water, are degraded by various microorganisms. Because of its higher volumetric productivity, simplicity, lower energy requirement, and lack of wastewater producing, SSF has been extensively applied in the biotreatment of solid waste or agricultural waste [8, 9]. However, to date, few studies reported the application of SSF in the biotreatment of sludge.

A. oryzae, a deuteromycotina fungus that secretes many kinds of enzymes including protease, amylase, cellulase, and phytase, is generally recognized as safe by the Food and Drug Administration (FDA) and Association of American Feed Control Officials [10], and extensively used in wastewater disposal and bioremediation in the fermentation industries of food, feed, kojic acid, and brewery. Tung et al. [11] used *A. oryzae* to treat the wastewater from cassava starch processing (CSP), and 90% COD of wastewater was finally removed under optimized treatment conditions. Bhalerao and Puranik [12] studied *A. oryzae* ARIFCC1054 degradation of monocrotophos (MCP) and found that this strain possessed phosphatase activity and could be used to renovate MCP--contaminated soil and treat aqueous wastes. Meng et al. [13] isolated *A. oryzae* 112822 from tobacco leaves that availably degraded nicotine, and firstly elucidated a pathway to degrade nicotine in fungi. After the straw in exploded stover was treated with *A. oryzae*, the contents of cellulose and hemicellulose in the exploded and fermented corn stover (EFCS) were decreased by 24.36% and 69.90%, respectively compared with the untreated one and by 17.35% and 38.59% ($P < 0.05$), respectively, compared with the exploded one ($P < 0.05$) [2]. *A. oryzae* has also been widely applied in removing dyes via biodegradation or heavy metal ions by biosorption [14]. Researches also showed that cetyl dimethyl ethyl ammonium bromide (CDAB) significantly modified the biomass of *A. oryzae* and enhanced its biosorption capacity to dyes in single and binary systems [14]. These results indicate that *A. oryzae* has the potential to degrade organic materials, especially organic recalcitrant compounds.

In previous study [15], *A. oryzae* strain has been isolated that could degrade organic substances including difficult-to-degrade chemicals and reduced the COD of vinasse. The present study reports a new method to treat sludge with H_2O_2 and *A. oryzae*. After treatment by the method described, the microorganisms in the sludge were killed; the bacterial colloid was removed by the chemical action of H_2O_2 during the process of H_2O_2 pretreatment; the COD was further degraded; water was evaporated, and the volume of sludge was reduced with the growth and respiration of *A. oryza*e during the SSF process.

2. EXPERIMENTAL

Sludge. A sludge sample was collected from the secondary settling tank of a municipal wastewater treatment plant located in Jiangdu city (Jiangsu, China) after an activated sludge treatment process. The moisture of sludge was 84.5%. The samples were immediately stored at 4 °C in a refrigerator, then transported to the laboratory within 24 h, and kept in the freezer at –20 °C in order to avoid changes in the characteristics [5, 16].

Isolation and identification of A. oryzae CGMCC5992. The fungus was isolated from the sludge in Yudai River of Jiangsu University (Zhenjiang, China). After sampling, the sludge was diluted 10-fold with aseptic water, spread onto a potato dextrose agar (PDA) plate, and incubated at 32 °C for 48 h. The representative strains of all colony types were isolated by sub-culturing under the same condition until the formation of a single colony. The isolated typical strains were inoculated onto PDA slants, incubated at 32 °C for 72 h, and maintained in refrigerator at 4 °C.

After culture growth on PDA plate at 32 °C for 5–7 days, the strain's configuration and color were observed, and the morphological characters including hyphae, spores, sporangium, etc., were also recorded under microscope.

A total of 10^6 spores from the strain slants were aseptically inoculated into a 250 cm^3 Erlenmeyer flask containing 100 cm^3 of potato dextrose (PD) medium and cultured at 28 °C, 150 rpm for 4 days. The mycelial sphere was transferred into a mortar and ground into powder under the addition of liquid nitrogen. The genomic DNA of the strain was then extracted with a genomic DNA extraction kit (TaKaRa, China) according to the manufacturer's instructions. The primers for amplification of 18s-RNA regions were as follows: forward, 5′-CCTGGTTGATCCTGCCAGTA-3′ and reverse, 5′-GCTTGAT CCTTCTGCAGGTT-3′. PCR amplifications were performed following the manual of a fungi identification PCR kit (TaKaRa, China). Briefly, the reactions were denatured at 94 °C for 5 min, and the PCR was run for 30 cycles with 94 °C denaturation for 1 min, 50–55 °C annealing for 1 min, and 72 °C extension for 1 min. The PCR was ended after final extension at 72 °C for 5 min. Gel electrophoresis was used to detect the presence and size of the amplified DNA products from CGMCC5992. The bands were excised from the gel; the DNA was recovered using a gel extraction kit (TaKaRa, China), cloned into a PMD18-T vector, and transformed into *E. coli* DH5αMCR competent cells according to manufacturer's instructions (TaKaRa, China). Positive colonies were blue/white screened on LB agar. The plasmid DNA was extracted with a plasmid extraction kit (TaKaRa, China) and sequenced by Shanghai Bioasia Biotechnology Co., Ltd. (Shanghai, China). The sequence was analyzed using the BLAST program (http://www.ncbi. nlm.nih.gov/ BLAST/). The resultant sequence was submitted (Accession Number: KC291246-KC291247) to the GenBank database (http://www.ncbi.nlm.nih.gov).

Inoculation and degradation of sludge. A total of 10^6 spores from the strain slants were inoculated aseptically into a 250 cm^3 Erlenmeyer flask containing corncob, 15 g

of bran, and 30 cm^3 of H_2O (sterilized at 121 °C for 60 min) and incubated at 28 °C for 5 days. This culture was later used as seed to treat sludge.

Two kg of sludge and 1.2 kg corncob were fully mixed and treated in batch with 100, 200, 300 and 400 cm^3 of 30% H_2O_2. These treatments were performed for 4 h at ambient temperature. Then the seed culture medium were respectively inoculated into a plastic dish containing 60 g of H_2O_2-treated sludge at the inoculating dose of 6 g and mixed thoroughly. The dishes were sealed up with preservation film to maintain the humidity. The whole SSF was conducted at 32 °C for 15 days under static condition.

Extraction of enzyme. Lignin peroxidase (LiP) and manganese peroxidase (MnP) were extracted from the samples according to Bollag et al. [17] with minor modifications. The entire soil sample from a Petri dish was transferred to a 20 cm^3 capped glass tube, mixed with 1:1 (w/w) sterilized distilled water, and shaken for 30 min at 50 rpm. The solid phase was separated by centrifugation, and the supernatant was frozen, thawed, and centrifuged to remove particles of high-molecular-weight polysaccharide slime. The supernatant was sequentially filtrated through a Whatman No. 1 filter paper, a 0.45 μm membrane filter, and an Amicon YM10 membrane. The filtrate was then analyzed for enzyme activities.

Determination of COD. A total of respective 20 g treated and untreated sludge plus 500 cm^3 of distilled water were mixed thoroughly. The mixtures were then filtered, and 10 cm^3 of filtrate were placed into a 500 cm^3 grinding mouth flask, containing 10 cm^3 of distilled water, 10 cm^3 of 0.25 mol/dm^3 $K_2Cr_2O_7$ solute, and 30 cm^3 of 1% H_2SO_4 Ag_2SO_4 solute. After 2 h heating at 105 °C, the COD was determined by back titration and addition of an ammonium and ferro iron solution (0.1 M Mohr's salt) at a volumetric dose to the excessive potassium dichromate. The COD was calculated from:

$$\mathrm{COD}_{\mathrm{Cr}} = 20\,000\,(V_0 - V_1)C \tag{1}$$

where V_0 is the consumption of ferro iron solution in blank titration, V_1 is the consumption of ferro iron solution in sample titration, and C indicates the concentration of ferro--iron solution.

The removing rate of COD in the SSF was calculated from:

$$\mu = \frac{\mathrm{COD}_{t-2} - \mathrm{COD}_t}{2} \tag{2}$$

where μ is the removing rate of COD on day t (mg/g of dry substance/day), COD_t is the COD value on day t, and COD_{t-2} represents the COD value on the day $t - 2$.

Rheological behavior of activated sludge. A total of 20 g sample and 50 cm^3 distilled water were fully mixed and filtered with filter paper (Xinhua1$^{\#}$). The filtrate was

then used to analyze for rheological behavior, which was determined on a Brookfield LVDV-II rotating viscometer. During the course of measuring, the temperature was kept in the range of 29–30 °C, and the speed of the rotor was in the range of 5–100 rpm. Rheological model for *A. oryza*e fermentation broth was fitted according to the power-law model, i.e., $\tau = Kr^n$, where τ is the shear stress, r is the shear rate, K is the consistency coefficient, and n represents the flow behavior index. Apparent viscosity was calculated as $\eta_a = Kr^{n-1}$. For the case of flask culture, r_{max} represented the maximum shear rate in 250 cm^3 Erlenmeyer flasks, $r_{max} = 2\pi R/60$, where R represents the rotational speed. In the trial, R was 150 rpm, and thus $\eta_a = 15.7^{n-1}K$.

Fungal biomass estimation. Fungal biomass was detected according to the methods described by Zeng et al. [18]. 1 g of SSF substrate was weighed and dispersed in 5 cm^3 sterile distilled water. The samples were completely homogenized to separate the whole fungal biomass from lignocellulose material. The homogenized samples stood undisturbed to settle the higher molecular weight lignocellulose material, while the fungal biomass in the supernatant was collected separately by centrifugation at 8000 rpm for 10 min. The fungal biomass was then dried under vacuum and weighed.

LiP and MnP activity assay. LiP activity was determined spectrophotometrically according to Tien and Kirk [19]. The activity of MnP was measured at 240 nm using Mn(II) as substrate [20]. One enzymatic unit (U) was defined as the amount of enzyme needed to oxidize 1 μmol of substrate per minute under the test condition.

Data analysis. All the experiments were performed at least in triplicate, and the results were presented as mean.

3. RESULTS AND DISCUSSION

3.1. IDENTIFICATION OF THE STRAIN

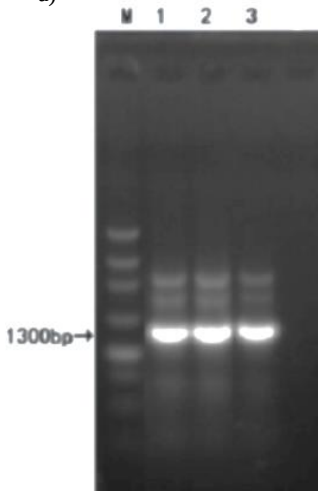
The color of strain CGMCC5992 cultured on PDA plate gradually became white in the center and greenish yellow in the periphery. The color on the bottom of PDA plate within 120 h of incubation at 30 °C was creamy and did not change after additional incubation period for one week. When the colony was observed under a light microscope, the hyphae were septate, and the conidia were in a string shape, spherical on the swollen round-shape acrocyst in the conidiophores head. All the macroscopic characteristics of the isolates were identical with those of the standard strain of *A. oryza*e (Table 1), indicating that the isolates are most likely strains of *A. oryza*e. Therefore, based on these comparable characteristics, the isolates were identified as strains of *A. oryza*e. An approximately 610 bp fragment of the 18S rDNA gene was amplified from the strain and sequenced. The size of the PCR band was confirmed by electrophoresis (Fig. 1a). The gene sequence of the strain is shown in Fig. 1b.

Table 1

Macroscopic characteristics of the isolates
after 5 days of incubation at 30 °C on a PDA plate

Characteristic feature	Isolates
Colony diameter	56 mm
Colony color	white centre and greenish yellow periphery
Colony reverse	pale yellow
Colony texture	wet
Conidial color	creamy
Nature of spores	wet

a)



b)

GGTCCGTGTTTCAAGACGGGTCGTTTACGACCATT
ATGCCAGCGTCCGTGCCGAAGCGCGTTCCTCGGTC
CAGGCTGGCCGCATTGCACTCCCGGCTATAAGGTG
CCCCGGAGGGCACTACATTCCGGGAGCCTTTGACC
GGCCGCCCAAACCGACGCTGGCCCGCCCCCAGGG
AAGTACACCGGCACGAATGCCGGCTGAACCCTGG
AGGCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACA
ATTTCACGTGCTTTTTAACTCTCTTTTCAAAGTGCT
TTTCATCTTTCGATCACTCTACTTGTGCGCTATCGG
TCTCCGGCCAGTATTTAGCTTTAGATGAAATTTAC
CACCCATTTAGAGCTGCATTCCCAAACAACTCGAC
TCGTCGAAGGAGCTTCACACGGGCGCGGACACCC
CATCCCAGACGGGATTCTCACCCTCTCTGACGGCC
CGTTCCAGGGCACTTAGACAGGGGCCGCACCCGA
AGCATCCTCTGCAAATTACAATGCGGACCCCGAA
GGAGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTC
ACTCGCCGTTACTGAGGCAATCCCGGTTGGTTTCT
TTTCCTCCGCTTATTGATATG

Fig. 1. Result of electrophoresis of products (a) and gene sequence (b)
of PCR for 18S-rDNA genes of *A. oryzae* CGMCC5992

The 18S rDNA sequence of strain CGMCC5992 exhibited 99% identity with that of *A. oryzae* RIB40 (GenBank accession no. XM001818169.2), *A. flavus* NRRL3357 (GenBank accession no. XM002373389.1), and *A. oryzae* RIB40 (GenBank accession no. AP007151.1), respectively, by BLAST sequence comparison.

Based on above results of morphological characterization and sequence comparison, the isolated strain was finally identified as *A. oryzae* CGMCC5992.

3.2. THE EFFECT OF H_2O_2 TREATMENT ON TEMPERATURE, COD, AND RHEOLOGICAL BEHAVIOR OF THE SLUDGE

The temperature, COD, and rheological behavior were analyzed at the intervals under natural pH during H_2O_2 treatment of the sludge. Figure 2a shows that the sludge

temperature sharply increased from 17 °C in the first 40 min after the addition of H_2O_2. However, it stopped increasing after 40 min of treatment. The increasing rate and range increased with the increasing amount of H_2O_2 below 300 cm^3/2 kg of sludge. Such temperature increase is caused by addition of H_2O_2 which, releases a large amount of energy when reacting with the substance in the sludge [21].

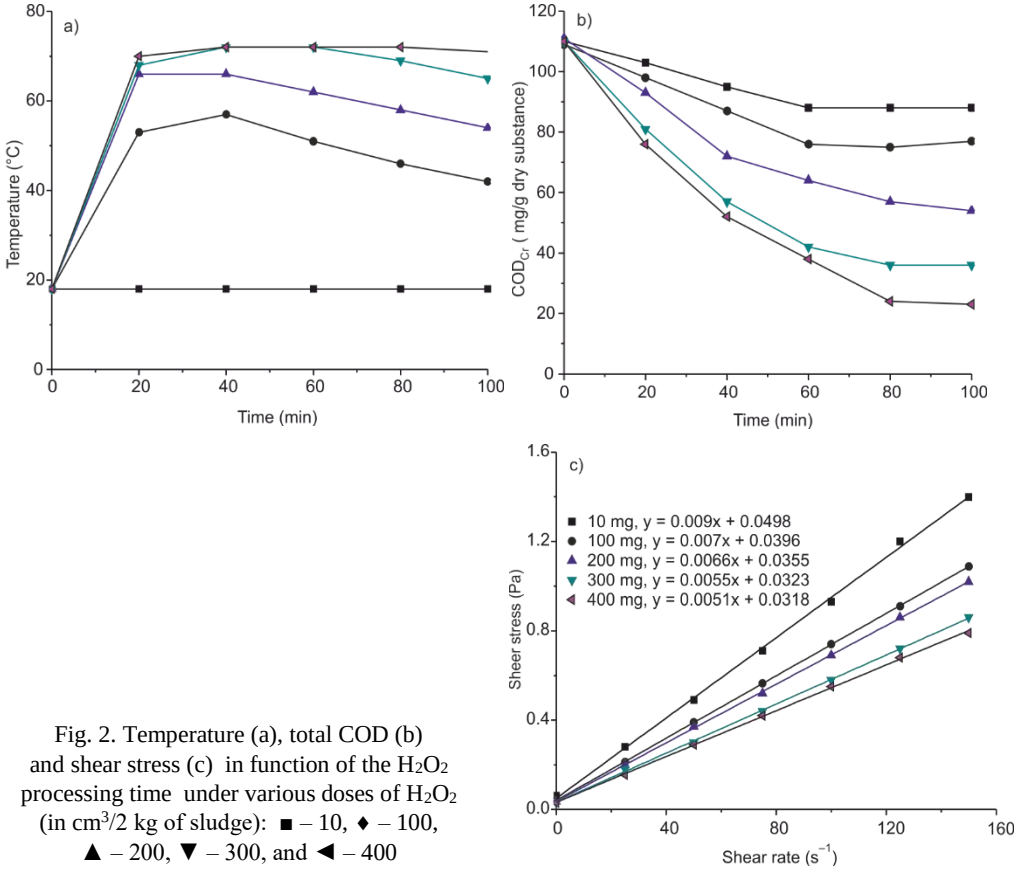


Fig. 2. Temperature (a), total COD (b) and shear stress (c) in function of the H_2O_2 processing time under various doses of H_2O_2 (in cm^3/2 kg of sludge): ■ – 10, ♦ – 100, ▲ – 200, ▼ – 300, and ◄ – 400

COD value in a sample was commonly used to quantify the pollution. Therefore, the COD of samples was analyzed in this study. It is clearly observed that the COD in the sludge decreased from initial 110 mg/g of sludge to 90, 75, 60, 36, and 22 mg/g of sludge respectively, with the increase of H_2O_2 dose from 50 cm^3/kg of sludge to 200 cm^3/kg of sludge in the first 80 min (Fig. 2b), but the COD was nearly stable thereafter. A possible explanation is the presence of mineral ions, such as Fe^{2+}, Cu^{2+}, Mn^{2+}, etc., and enzymes, such as MnP and LiP, secreted by some bacteria and fungi. These ions and enzymes catalyzed the hydrogen peroxide to generate hydroxyl radical, which may attack the complex structure in recalcitrant substrates and degrade many other chemical compounds, including

dyes, chlorinated aromatics, polycyclic aromatic hydrocarbons (PAHs), etc. The above analysis was also applicable to the correlation between shear stress and dose of H_2O_2.

The dependences of the shear stress τ on the shear rate γ at various concentrations of H_2O_2 (Fig. 2c) clearly show that the filtrate from the mixture of 20 g of the sample and 50 cm^3 of distilled water presented a non-Newtonian behavior. Therefore, the experimental data were fitted to a classical Bingham model:

$$\tau = \tau_B + \eta_B \gamma \tag{3}$$

where τ_B and η_B are the Bingham shear stress and the Bingham viscosity, respectively.

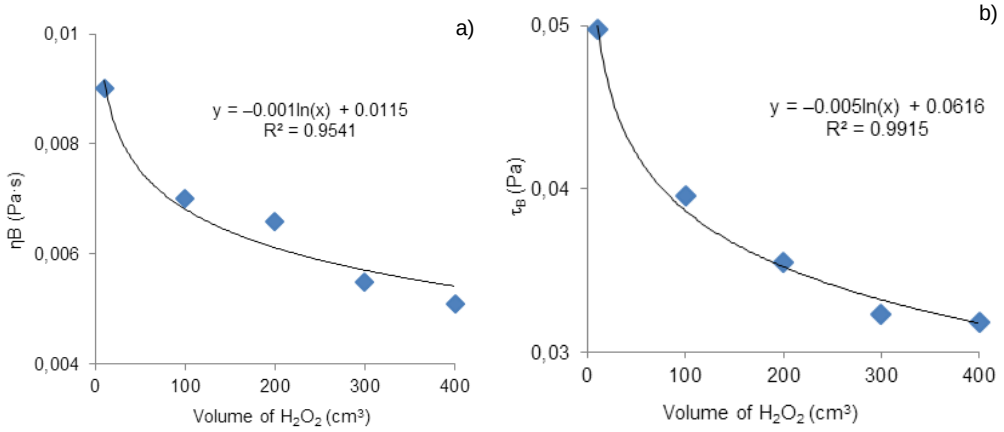


Fig. 3. Yield stress (a) and the Bingham viscosity (b) in function of the dose of H_2O_2

Figures 3a and 3b reveal the dependence of τ_B and η_B on the dose of H_2O_2 which both showed logarithmic decrease upon increasing the dose of H_2O_2. This might be explained by the degradation of the solubilized exopolymers, especially high viscosity organic compounds such as bacterial colloid. Hammadi et al. [22] studied the effect of H_2O_2 on liquid waste sludge and proved that H_2O_2 could reduce the content of organic matter in the final sludge. The change in apparent viscosity of the sludge further demonstrated the above conclusion that H_2O_2 could modify the sludge structure, decrease the bacterial colloid, and hence facilitate the substance exchange during the SSF of sludge.

3.3. THE PROFILE OF BIOMASS AND COD CHANGE OF THE SLUDGE IN THE PROCESS OF SOLID STATE FERMENTATION

To study the impact of pretreatment with various doses of H_2O_2 on the SSF of *A. oryzae*, 6 g of seed culture medium were inoculated into a plastic dish containing 60 g of pretreated sludge with various doses of H_2O_2 for 80 min. Then the samples were analyzed for biomass and COD at the intervals of 48 h.

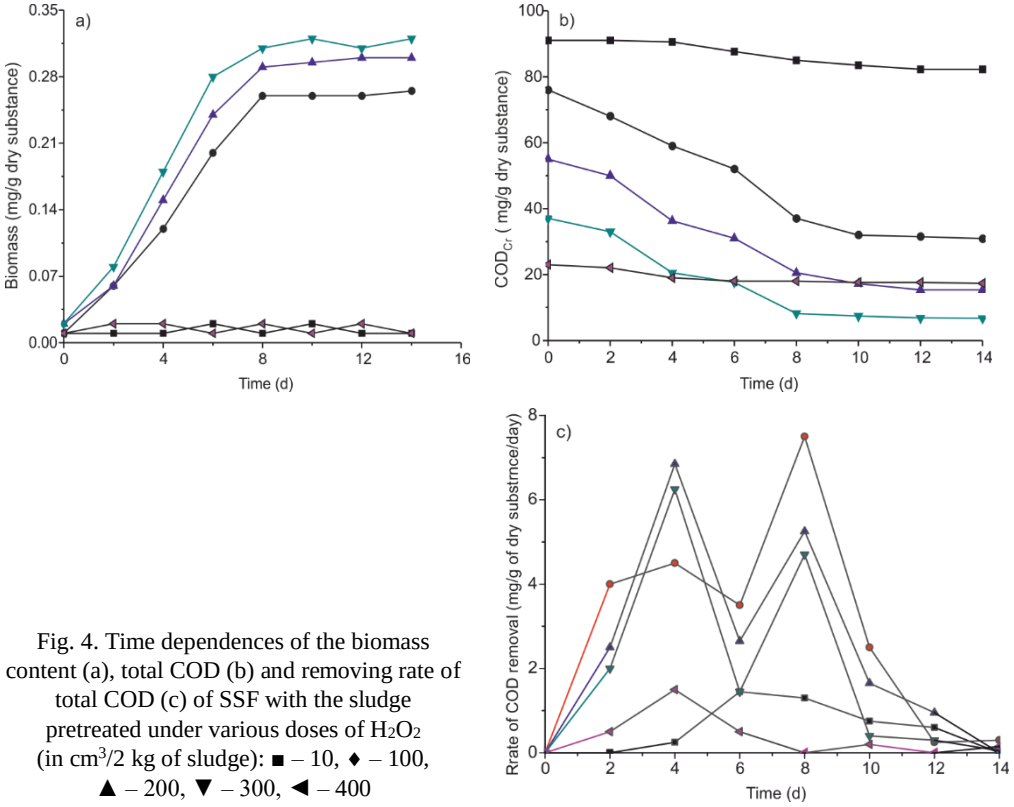


Fig. 4. Time dependences of the biomass content (a), total COD (b) and removing rate of total COD (c) of SSF with the sludge pretreated under various doses of H_2O_2 (in cm^3/2 kg of sludge): ■ – 10, ♦ – 100, ▲ – 200, ▼ – 300, ◄ – 400

The results indicate that *A. oryzae* failed to grow in the sludge pretreated with 5 or 200 $cm^3$of H_2O_2/kg of sludge (Fig. 4a). Similarly, malodor was emitted from the sludge pretreated by H_2O_2 at 5 cm^3/kg of sludge, while the sludge was smoother. In previous studies, malodor was reported to be composed of H_2S, mercaptans, and dimethyl sulfide. It is believed that the amount of bacterial colloid removal did not meet the growth requirement of *A. oryzae* when H_2O_2 was supplied at 5 cm^3/kg of sludge; therefore, the strain could not grow under such conditions. Although H_2O_2 is an oxidant, it cannot inhibit the growth of microorganism under too low concentration. When supplied at the dose of 200 cm^3/kg of sludge, excessive H_2O_2 also inhibited the growth of *A. oryzae*, and the mycelia growth was not observed too. When the dose of H_2O_2 increased from 50 cm^3/kg of sludge to 150 cm^3/kg of sludge, the amount of bacterial colloid removed from the sludge gradually increased, the material transfer and growth of *A. oryzae* mycelia in the SSF were facilitated. The strain biomass in the process of SSF was found to fast increase from 2nd to 8th day (Fig. 4a). COD analysis also showed that when the dose of H_2O_2 was lower than 150 cm^3/kg of sludge (Fig. 4b), the amount of COD removal was sharply increased and correlated with the dose of H_2O_2, and the COD was

removed from 2nd to 8th day of SSF (Fig. 4c). Therefore, the optimal condition to dispose sludge with H_2O_2 and *A. oryzae* was determined as pretreating the sludge with H_2O_2 for 80 min at the dose of 150 cm^3/kg of sludge, and further degrading the organic substance in the sludge by SSF with *A. oryzae* for 8 days. When the dose of H_2O_2 increased from 50 to 150 cm^3/kg of sludge, the removal rate of COD exhibited 2 maxima on days 3 and 8, respectively (Fig. 4c).

3.4. ENZYMATIC ACTIVITY IN THE SLUDGE IN THE PROCESS OF SSF

Recalcitrant substrates were the major organic substances in the sludge which cannot be degraded by the common microorganisms. Their degradation requires special enzymes such as MnP and LiP, two groups of peroxidases involved in the degradation of lignin and recalcitrant compounds. Most studies indicated that LiP and MnP were simultaneously synthesized by a strain. But in this study, the synthesis of MnP and LiP was found to be with different time frame (Fig. 5).

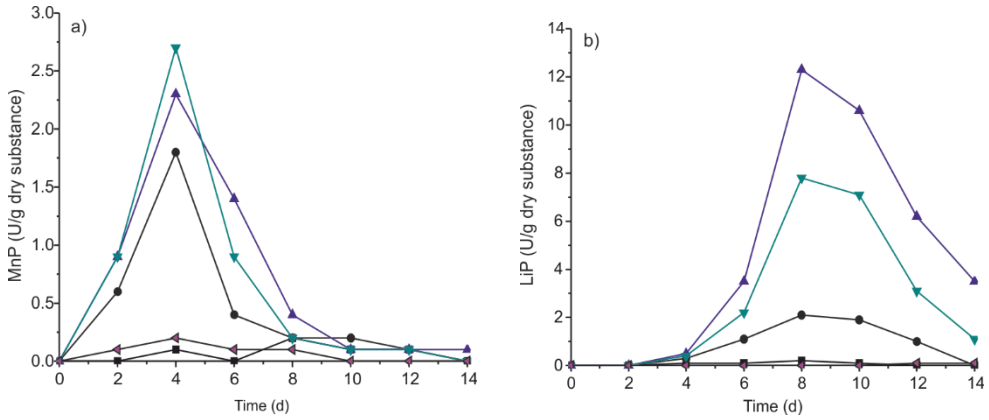


Fig. 5. Time dependences of MnP (a) and LiP (b) activities of SSF with the sludge pretreated under various doses of H_2O_2 (in cm^3/2 kg of sludge): ■ – 10, ♦ – 100, ▲ – 200, ▼ – 300, and ◄– 400

The strain began to synthesize MnP from the start of SSF which reached peak value on the third day (Fig. 5a) and ceased completely before 10th day, whereas LiP was synthesized between the 4th and 10th day, reached peak value on the 8th day and ceased completely till 14th day (Fig. 5b). The timing of synthesis of the two enzymes was consistent with the two peaks of COD removal rate on days 3 and 8 during SSF, and accompanied with the fast growth of the strain. We believe that *A. oryza*e obtained certain nutrients to sustain its growth requirement by degrading substrates in the sludge with MnP and LiP. Primary metabolite is defined as a metabolite that is directly involved in normal growth, development, and reproduction, and usually performs physiological functions [23]. We concluded that MnP and LiP involved in the production of primary,

not secondary metabolites in *A. oryza*e, though they were assumed to be parts of the secondary metabolism in many previous studies [19, 20]. *A. oryza*e cannot grow in sludge without the addition of carbohydrate or other easy carbon source because carbohydrates in the sludge are easily degradable by microorganisms, while the difficult to degrade compounds in the sludge cannot be used in the absence of ligninolytic enzyme. In many previous studies, a slight amount of glucose was added into the medium which initiated the synthesis of ligninlyotic enzymes by corresponding microorganisms.

4. CONCLUSION

A novel strategy has been established to dispose the sludge from wastewater treatment plant. In the first stage, H_2O_2 pretreatment increased the temperature, significantly decreased the COD and apparent viscosity of the sludge, and facilitated the substance interchange during the process of SSF. In the second stage of SSF, *A. oryza*e grew well in the sludge pretreated by H_2O_2 (50–150 cm^3/kg of sludge) and further degraded the COD of substance. The removal rate of COD displayed 2 maxima on days 3 and 8, respectively. In the process of treatment sludge, *A. oryzae* produced LiP and MnP, which is close related with disintegration of sludge. Taking into account such advantages as no secondary pollution, and low disposal cost, the strategy is promising to eliminate organic matter in sludge.

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