Anaerobic Digestion of Wastewater Including High Concentration of Yeast, *Pichia pastoris*

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Abstract

The anaerobic treatment of wastewater including yeast, *Pichia pastoris*, with a high concentration of 30 g/l was carried out. To digest the high VSS concentration, a two-phase anaerobic digestion process consisting of liquefaction and gasification was used. Performance of thermophilic liquefaction (53 °C) was better than mesophilic liquefaction (37 °C) with respect to VSS digestion, using a upflow anaerobic filter reactor. The digestion efficiency of VSS was about 80 % at an organic matter volumetric loading rate of 1 to 2 g//d. The effluent from the liquefaction process had to be diluted prior to the gasification treatment. The allowable maximum TOC loading rate was only 1 g//d to achieve a TOC removal efficiency of more than 80%. It was shown that thiourea inhibited 70% of the gasification performance even at a low concentration of 10 mg/l, and the reason for the inhibition of gasification seems to be the presence of a thiourea-like compound in the wastewater.

Key words: two-phase anaerobic digestion, wastewater including *Pichia* yeast, thiourea-like compound

INTRODUCTION

Saccharomyces cerevisiae species, which are used for making of wine, Japanese wine (*sake*), beer, *shochu* and whisky *et al.*, are recognized as edible microorganisms. Therefore, the yeast eluted from each making processes are reused as Japanese traditional soap (*sake*-waste soap), supplement and fodder *et al.* However, other microorganisms except for edible microorganisms are not permitted to be reused without safety tests for a long time.

Recently, *Pichia pastoris* has been used for the production of various proteins¹⁾. The amounts of these proteins are larger than those expressed using *Saccharomyces cerevisiae*²⁾. Accordingly, the use of *P. pastoris* for expression is expected to become more common in the future. However, de-coloring

agents including amino group and sterilized milk are generally added to reduce color index and to protect contamination during batchwise cultivation. And also NH_4^+ and PO_4^{3-} were added to medium to enhance the growth. As *Pichia* yeasts are not only recognized as an edible microorganism but also its paste is considered to contain various agents, it should be difficult to effectively treat the wastewater containing high concentration of *P. pastoris*. Although the treatment for the wastewater is just combustion at present, an efficient process substitute for combustion will have to be developed as soon as possible to protect global warming.

In this study, the results of research and development of the treatment of *P. pastoris*-containing wastewater with energy recovery is reported. A two-phase methane fer-

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mentation method was applied since the wastewater contained high concentrations of VSS, which is the rate-limiting factor of liquefaction³⁾.

MATERIALS AND METHODS

Wastewater including recombinant micro-organisms The wastewater including *Pichia pastoris* (hereafter called the wastewater including yeast) was provided by M corporation. Table 1 shows the typical composition of the wastewater. For the gasification of the effluent from the liquefaction, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ were added to concentrations of 13.1 mg/l and 4.1 mg/l, respectively, to enhance gasification speed^{4, 5)}.

Municipal sewage sludge Sewage sludge which consists of primary and surplus sludges was provided by Kumamoto Tobu Sewage Treatment Plant, Kumamoto, Japan. During the acclimation period, the mixed sewage sludge was used.

Seeding sludge Table 2 shows the compositions of thermophilic and mesophilic digested sludges provided from Kumamoto Hokubu Sewage Treatment Plant in Kumamoto, Japan, and Kumamoto Tobu Sewage Treatment Plant in Kumamoto, Japan, respectively. They were acclimated with the municipal sewage sludge at an organic matter loading rate of 3 g/l/d and 2 g/l/d, respectively, for several years by a fill and draw method⁶⁾ in our laboratory before use. The acclimated thermophilic and mesophilic sludges were used for the liquefaction and the gasification treatments, respectively.

Liquefaction of yeast contained in wastewater Figure 1 shows a schematic diagram of the anaerobic liquefaction process using the upflow anaerobic filter (UAF) reactor with a working volume of 0.78 l, which was used in the previous study⁷⁾.

An aliquot of 800 ml of the acclimated thermophilic sludge (VSS, 10,000 mg/l) was added to the reactor. The liquid in the reactor was then circulated overnight at 53°C and a circulating flow rate of 5.2 cm/min by pump P

-2. During acclimation, the sewage sludge was added to the thermophilic liquefaction reactor at an organic matter loading rate of 3 g/l/d by the fill and draw method (once a day) and to the mesophilic liquefaction reactor at an organic matter loading rate of 2 g/l/d. After acclimation, the wastewater containing yeast was added to the liquefaction reactor instead of the sewage sludge in the same manner. The organic matter loading rate was increased stepwisely from 1 to 3 g/l/d at pH 6.

Mesophilic gasification of organic matter in effluent from liquefaction $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ were added to concentrations of 13.1 mg/l and 4.1 mg/l, respectively, to the

Table 1 The typical composition of the wastewater including *Pichia* yeast

TOC	8325 mg/l	SS	31.0 g/l
NH_4^+	420 mg/l	VSS	29.5 g/l
PO_4^{3-}	1433 mg/l	TS	52.6 g/l
CH_3COOH	636 mg/l	TVS	45.6 g/l
Succinic acid	63 mg/l		
I-valeric acid	203 mg/l	pH	6.5

Table 2 The composition of thermophilic- and mesophilic digestion sludges provided from sewage works Unit: mg/l (except for pH)

	Thermophilic sludge	Mesophilic sludge
MLSS	12370	12410
MLVSS	7990	8320
TOC	1043	200
IC	422	375
VFA	1137	N.D.
pH	7.4	7.2

N.D., not detected.

Fig. 1 Schematic diagram of the anaerobic liquefaction process using an upflow anaerobic filter (UAF) reactor

effluent from the reactor (hereafter called liquefied effluent) and was diluted about 3 times or 5 times with tap water. Then the gasification test was done at 37°C using two of the same type of UAF reactors. After acclimation, the effluent from the liquefaction reactor supplemented with Ni^{2+} and Co^{2+} was added into the gasification reactor by the fill and draw. The evolved gas was fed into a gas holder through a vinyl tube and the amount of evolved gas was measured.

Determination of specific gas evolution rate to evaluate negative effect of thiourea

The specific gas evolution rate was determined according to the method of Kida *et al.*⁸⁾ as follows: 0.2 ml of sodium acetate solution (292.85 mg/l) was added into a vial with a working volume of 500 ml containing a stirring bar to a final concentration of 70 mM. To evaluate toxicity effects, thiourea (toxicant) was added to the test vials. The vials were sealed with a rubber stopper and an aluminum cap and then a needle was penetrated into the rubber stopper to allow for N_2 purging to create anaerobic conditions. A 10-ml aliquot of culture broth, which was cultivated continuously with the acetate medium (added as energy and carbon sources)⁸⁾, was transferred to each vial and the vials were then connected using a vinyl tube to a volumetric pipette immersed in a thermostated water-bath. After being immersed for 60 min, the gas evolution rate was measured during incubation at 37°C with agitation at 100 rpm.

Analytical method All parameters, except pH, SS, VSS, TS and TVS, were analyzed for supernatants obtained after centrifugation at 10,000 rpm for 10 minutes. pH, SS, VSS, TS and TVS were determined in accordance with Standard Methods⁹⁾. The TOC concentration was measured with a TOC autoanalyzer (TOC-500, Shimadzu Corporation)⁹⁾. The amount of volatile fatty acids (VFA) was measured by a post-labeled method using an HPLC. For quantification of NH_4^+ and PO_4^{3-} were separated by ion exchange chromatography (DX-AQ, Dionex Corporation) using cation separation columns (CG 12, CS 12, Dionex Corporation) and

anion separation columns (CG 12A, CS 12A, Dionex Corporation), respectively. The evolved gas were analyzed by gas chromatography (KOR-2G, Gasukuro Kogyo Inc.) with packed column (Porapak Q, Gasukuro Kogyo Inc., Tokyo). The heavy metal contents were determined by means of Inductively Coupled Plasma Atomic Emission Spectrometry (IPC-AES). Aromatic compounds were measured by RP-HPLC with a L-column ODS (Chemicals Inspection & Testing Institute).

Thiourea was measured by considering that it acts as a catalyst in the following manner¹⁰⁾.

The reduction rate of I_2 increases in proportion to the concentration of thiourea. Hence the quantity of thiourea was determined by measuring the reduction in the amount of I_2 by absorptiometry based on a blue color change from the iodostarch reaction. Thiourea-like compound was also measured in the same method.

RESULTS AND DISCUSSION

Effects of temperature on liquefaction of the wastewater containing yeast

As shown in Table 1, the wastewater containing yeast had a high VSS concentration, which might have been responsible for limiting the rate of liquefaction. The effect of treatment temperature on liquefaction efficiency was evaluated by a two-phase methane fermentation process using a UAF reactor. The pH was maintained automatically at 6 as the liquefaction efficiency decreased when treating the coffee waste at a pH lower than 6¹¹⁾.

Figure 2 shows the chronological changes of treatment efficiencies during thermophilic liquefaction. At an organic matter loading rate of 2 g/l/d, the VFA level increased rapidly and reached to 22,000 - 24,000 mg/l. During this treatment, the amount of gas evolution decreased to more or less zero, but the VSS digestion efficiency increased to about 80%. The loading rate was then increased to 3 g/l/d and the treatment test was continued. The VFA levels decreased to 16,000 mg/l with a decrease in VSS digestion efficiency. When the loading rate was

decreased to 2 g/l/d, the VFA concentration again increased to 21,000 mg/l with an increase in the VSS digestion efficiency.

Conversely, during mesophilic liquefaction, gas did not evolved during acclimation and VSS digestion efficiency was slightly lower than that in thermophilic liquefaction process. After acclimation, treatment was conducted at an organic matter loading rate of 1 g/l/d and almost the same results as for the thermophilic liquefaction process were obtained. The organic matter loading rate was then increased to 2 g/l/d in the same manner. However, the VFA concentration

and VSS digestion efficiency decreased significantly (data not shown).

Thus it is evident that the performance of thermophilic liquefaction process was better at an organic matter loading rate of 1–2 g/l/d.

Effect of dilution on mesophilic gasification performance of liquefied effluent

The high concentration of VFA in the liquefied effluent may have inhibited methanogenic organisms. There is also a lot of low-strength wastewater such as washing water, thus, to abate the effect of the high VFA levels, the liquefied effluent was diluted 3 or 5 times with tap water and was subjected to mesophilic gasification (37°C) at a TOC loading rate of 1 g/l/d. Figure 3 shows the chronological change of treatment efficiencies during the mesophilic gasification. After acclimation, 3-times diluted liquefied effluent was fed to the UAF reactor and VFA levels increased rapidly to 2500 mg/l while the gas evolution rate decreased to zero during 2-weeks of operation. However, for treatment of 5-times liquefied effluent, VFA increased to 1500 mg/l and then decreased to 600 mg/l during 30 days operation. In addition, biogas was generated at a rate of about 300 ml/d with a methane content of about 50% as shown in Figure 4. When increasing the TOC loading

Fig. 2 Changes of VFA, TOC, VSS digestion efficiency, gas evolution rate and pH during the thermophilic liquefaction treatment

Fig. 3 Changes of VFA, TOC, gas evolution rate and pH when the 3-times diluted effluent from the liquefaction reactor was treated in the anaerobic gasification process

Fig. 4 Changes of VFA, TOC, gas evolution rate and pH when the 5-times diluted effluent from the liquefaction reactor was treated in the anaerobic gasification process

rate to 2 g/l/d, the residual VFA concentration increased, while the gas evolution once increased and gradually decreased. As a result, the maximum TOC loading rate was 1 g/l/d, which is much lower than the maximum loading rate achieved for the methane fermentation of other kinds of wastewater in our laboratory^{4, 5, 12)}. It has been reported that heavy metals and thiourea can inhibit methane fermentation¹³⁾. To investigate the effects of these compounds, the concentrations included in each effluent were measured. As shown in Table 3, the concentrations of heavy metals were below than 1 mg/l. These values were much less than the concentrations giving inhibition of methane fermentation, being more than 10–20 mg/l¹³⁾. However, a thiourea-like compound was present at high concentrations in each effluent. This is probably due to inhibition by the thiourea-like compound. To clarify inhibition by thiourea, the specific gas evolution rate was measured according to the experimental method. As shown in Figure 5, gasification was found to be strongly inhibited by a low thiourea concentration.

In order to develop efficient treatment or recycle processes for the wastewater including high concentration of yeast, researches on methods to remove thiourea-like compound is greatly needed.

CONCLUSIONS

To develop an efficient treatment process for *Pichia pastoris*-containing wastewater with energy recovery, two-phase methane fermentation was investigated. The results are summarized as follows;

- (1) The performance of liquefaction under thermophilic than mesophilic conditions was better with respect to the VSS

digestion. The VSS digestion efficiency was relatively high, being 80% at a organic matter loading rate of 1–2 g/l/d in the thermophilic liquefaction process.

- (2) For the gasification of the liquefied effluent, the effluent had to be diluted. Although the liquefied effluent with 5-times dilution was treated by gasification, the maximum allowable TOC loading rate achieved was only 1 g/l/d.
- (3) It was shown that thiourea strongly inhibited gasification by measurement of the specific gas evolution rate.

ACKNOWLEDGEMENTS

The authors thank Associate Professor Makoto Hirata in the Department of Applied Chemistry, Faculty of Engineering, Oita University for his kind support of the thiourea-like compound analysis. And also wish to thank Mr H. Igara and K. Sato for their technical assistances.

Fig. 5 Effect of thiourea on the activity of acetoclastic methanogens

Table 3 The concentrations of heavy metals and thiourea-like compound in each effluent as toxicants
Unit: mg/l

	Heavy metals					Thiourea-like compound
	Ni	Cu	Zn	Cr	Hg	
Wastewater containing yeast *	–	0.29	4.05	0.04	0.07	202
Liquefied effluent	3.1	0.02	0.05	0.03	0.02	170
Gasified effluent	0.27	–	0.07	–	–	40

* Ni²⁺ was added to the liquefied effluent for gasification treatment.

–, not measured.

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(Submitted 2005. 7. 4)

(Accepted 2005. 9. 1)