Isolation of Acrylamide-Resistant Facultative Anaerobes from Methane-Fermented Swine Waste-Water and Biodegradation of Acrylamide by Immobilized Cells of Isolated Bacterial Strain

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(Received March 27, 1992)

Abstract The effect of acrylamide (ACR) on methane fermentation was examined. The volume of gas production was found extremely decreased in the presence of more than 3.3 mg/l of ACR. When isolation of ACR-resistant facultative anaerobes from the digested sludge was attempted, *E. coli* SK-1 strain against which ACR had minimum inhibitory concentration (MIC) of 20,000 mg/l was isolated. This bacterial strain was found to utilize ACR as a carbon source. On examining the degradation of ACR by this bacterial strain immobilized by the PVA-boric acid method, 52% was decomposed in four days. These results showed that methane fermentation was affected in the presence of a very small amount of ACR and also suggested that *E. coli* SK-1 isolated from the digested sludge acclimated by addition of ACR can be utilized as an immobilized bioreactor.

Anim. Sci. Technol. (Jpn.) 63 (12) : 1228-1233, 1992

Key words : acrylamide, methane fermentation, biodegradation, *Escherichia coli*, bacterial immobilization

Acrylamide (ACR, $\text{CH}_2=\text{CHCONH}_2$) is used as a powerful paper strengthener, a flocculant in the treatment of water, a soil stabilizer and so on. In the field of biochemistry, it is utilized in electrophoresis²⁾. ACR-polymer of low toxicity is used for the three purposes mentioned above^{8,10)}. Since commercial poly-ACR contains 0.3-5.0% hypertoxic ACR-monomer, scrupulous care is demanded in the use of poly-ACR^{5,7,10)}. In fact, CROLL *et al.*⁴⁾ have measured the concentration of ACR in industrial waste-water from factories which used poly-ACR and detected 0.47-42 $\mu\text{g/l}$ of

hypertoxic ACR-monomer. It is presumed that either ACR-monomer was originally contained in commercial poly-ACR or polymer was decomposed into monomer.

The toxicity of ACR-monomer in animals causes injuries mainly to the central and peripheral nervous systems⁶⁾. LD₅₀ of ACR-monomer in rats, guinear pigs and rabbits has been estimated to range from 100 to 200 mg/kg^{7,10)}. The biodegradation of ACR has been described under an aerobic condition^{1,3,4,11)}. However, the influence of ACR on methane fermentation and the biodegradation of ACR

by facultative anaerobes have not been studied. This study was designed to examine the influence of ACR on methane fermentation of pretreated swine feces. Isolation of ACR-resistant facultative anaerobes from the digested sludge was also attempted. In addition, the biodegradation of ACR was investigated using an ACR-resistant strain isolated and immobilized by the polyvinyl alcohol (PVA)-boric acid method⁵⁾.

Materials and Methods

1. Evaluation of the effect of ACR on methane fermentation

Methane fermentation was carried out under the operating condition shown in Table 1. Swine feces was used as a waste water sample. It was diluted three times and filtered through a 32-mesh filter. It was adjusted to the concentration of biochemical oxygen demand 12,000 mg/l. Digested sludge was grown and acclimated at our Laboratory.

Two 1,000-ml flasks (effective capacity: 600 ml each) were used as fermenters. One was used without ACR as a control, and the other with 0.5–3.0 mg of ACR (Run). During the first one week, 0.5 mg of ACR was added daily and subsequently, the daily amount was increased by 0.5 mg at weekly intervals.

Table 1. Operating condition of methane fermentation

	Control	Run
BOD volume loading, g/l/d	20	20
Influent BOD, mg/l	12,000	12,000
Fermentation tank capacity, ml	600	600
Influent volume, ml	30	30
Detention time, day	20	20
Initial sludge volume, %	20	20
Acrylamide, mg/l	0	0.5–3.0*
Temperature, °C	35±1	35±1

*Acrylamide was added to Run in daily amount of 0.5mg during the first one week. The daily amount was increased by 0.5mg at weekly intervals.

Methane fermentation was continued for six weeks.

ACR used was a 98%-purity product for electrophoresis (Nacalai Tesque, Inc., Japan). The concentration of methane was determined using a Model 373 Thermal-Conductivity Gas Chromatograph (Gasukuro Kogyo Inc., Japan).

2. Isolation of ACR-resistant facultative anaerobes

Isolation of ACR-resistant bacteria was carried out by the following method. The tripticase soy agar medium (TS agar, BBL) containing 2,000 mg/l of ACR was inoculated with sludge obtained after 3–5 weeks of fermentation in the Run. It was subjected to aerobic culture for 24 hours at 37°C. The bacterial colony formed on the agar was again inoculated to TS agar and subjected to anaerobic culture for 24 hours at 37°C using a Gas Pac anaerobic system (BBL). The bacterial strains isolated were stored on TS agar slants.

3. Measurement of the minimum inhibitory concentration (MIC) of ACR against ACR-resistant strains

The bacterial strains isolated were inoculated to a TS broth containing 2.5, 5.0, 7.5, 10, 15 or 20 g/l of ACR and subjected to aerobic culture for 24 hours at 37°C. They were then inoculated to TS agar and incubated under the same condition. The proliferation of bacterial cells was then examined.

4. Biodegradation of ACR

The biodegradation of ACR was tested using free and immobilized cells of the bacterial strain which was found having the highest resistance to ACR. Cells were mass-cultured with shaking in 1,000 ml of TS broth at 37°C for 24 hours. They were then condensed by centrifugation (centrifuge: Kokusan H-103 RLH) at 5,000 rpm for five minutes at 4°C. The condensate was washed twice in phosphate buffer. Free cells for the testing of ACR biodegradation were prepared by inoculating the bacteria to 10 ml each of Broth A and B. The biodegradation of ACR by the free cells

was examined at 30°C for three days. Broth A was composed of 0.2 g of KH_2PO_4 , 1.15 g of Na_2HPO_4 , 0.1 g of ACR and 1,000 ml of water. Broth B contained 0.5 g of L-asparagine additionally.

Immobilized cells were prepared by the polyvinyl alcohol (PVA)-boric acid method of KAKIICHI *et al.*⁵⁾ Immobilized cells were aggregated into 360 beads (mean diameter 3 mm) and were placed into four test tubes to contain 200, 100, 50 and 10 beads respectively. Then 10 ml of broth A were added to the tubes and shaken. The biodegradation of ACR was examined at 30°C for four days.

The concentration of ACR was determined using a Shimadzu LC-6 A High Performance Liquid Chromatograph (HPLC) equipped with a Shim-Pack CLC-ODS column and a UV detector set at 210 nm.

Results

1. Effect of ACR on methane fermentation

Table 2 and Fig. 1 show the changes in the biogas production and the concentration of methane in it, respectively. The gas production in the control ranged from 158 to 175 ml/day. In the Run, almost the same volume of gas was produced as in the control until the third week until when the daily addition of ACR was increased from 0.5 to 1.5 mg. The gas production was significantly decreased,

Table 2. Comparison of biogas production

Week	Gas production*, ml/day	
	Control	Run**
1st	163±13	158±16 (0.5)
2nd	172±16	169±20 (1.0)
3rd	158±11	162±8 (1.5)
4th	167±24	98±22 ^{a)} (2.0)
5th	175±22	47±8 ^{a)} (2.5)
6th	159±26	16±13 ^{a)} (3.0)

* Mean±SD.

** In parentheses are shown the amount (mg/30 ml/day) of acrylamide added.

^{a)} $P<0.01$ as compared with control.

compared to the control, from the fourth week from when the amount of ACR added was increased to 2.0 mg/day or more.

The concentration of methane in the gas produced ranged from 59 to 66% in the control and from 57 to 69% in the Run, thus showing no significant difference.

2. ACR-resistant strains and MIC of ACR

As shown in Table 3, five facultative anaerobic strains were isolated; *E. coli* SK-1, *E. coli* SK-2, *Serratia* sp., *Proteus* sp. and *Micrococcus* sp. MICs of ACR against these strains were 20,000, 15,000, 7,500, 5,000 and 2,500 µg/ml respectively. Thus, *E. coli* SK-1 was found to have the highest resistance to ACR.

3. Biodegradation of ACR by *E. coli* SK-1

Fig. 2 shows the biodegradation of ACR by the free cells of *E. coli* SK-1 in the presence

Fig. 1. Changes of methane concentration in biogas produced.

○: Control ●: Run

Table 3. MIC of ACR against ACR-resistant bacterial strains

Strain	MIC, µg/ml
<i>Escherichia coli</i> SK-1	20,000
<i>Escherichia coli</i> SK-2	15,000
<i>Serratia</i> sp.	7,500
<i>Proteus</i> sp.	5,000
<i>Micrococcus</i> sp.	2,500

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Fig. 2. Effect of L-asparagine as carbon source on ACR degradability by free *E. coli* SK-1 cells.

○: In the broth containing 100 mg/l of ACR and 500 mg/l of L-asparagine
●: In the broth containing 100 mg/l of ACR only

Fig. 3. ACR degradability in relation to the number of beads of immobilized *E. coli* SK-1 cells.

○: 200 beads ●: 100 beads
⊙: 50 beads ⊗: 10 beads

and absence of L-asparagine.

Biodegradation of ACR was not recognized in the broth containing 100 mg/l of ACR and 500 mg/l of L-asparagine. On the other hand, 35% of ACR was degraded in 72 hours in the broth containing 100 mg/l of ACR only.

4. Biodegradation of ACR by immobilized cells of *E. coli* SK-1

Fig. 3 shows the relation between the degradation of ACR and the number of beads of *E. coli* SK-1 cells immobilized by the PVA-boric acid method.

The rate of ACR degradation increased with increase in the number of beads. It was found that 10, 20, 36 and 52% of ACR were degraded in four days in the presence of 10, 50, 100 and 200 beads immobilized *E. coli* SK-1 cells, respectively.

Discussion

The effect of ACR on methane fermentation was investigated in this experiment. Since the biogas production was considerably decreased by daily addition of 2.0 mg/30 ml of ACR to the fermentation tank of the effective capacity of 600 ml, it was presumed that the fermentation was disturbed when the concentration of ACR in the fermenter exceeded 3.3 mg/l. MORIMOTO *et al.*⁹⁾, who detected 220–1,200 mg/l of ACR in well water in Fukuoka Prefecture in 1974, have attributed this finding to the melting-out from the road paved with an acrylamide soil stabilizer. If such ACR-contaminated well water is used for cleaning of animal sheds or as drinking water for domestic animals, it is presumed that methane fermentation is disturbed and animals suffer from poisoning.

The biodegradation of ACR in natural environments has been described^{3,4,11)}. CROLL *et al.*⁴⁾ have found that when river water containing and not containing acclimated microorganisms were treated with 8 μ g/l of ACR and were slightly aerated, the degradation of ACR required more than 100 hours in the river water not containing microorganisms, while ACR was completely degraded in 40 hours in the river water containing microorganisms. ARIMITSU *et al.*¹⁾ have described that when water influent into a water purification plant was treated with 20 μ g/l of ACR and was then

aerated, the concentration of ACR was decreased to 0.5 $\mu\text{g/l}$ in five days. In a degradation experiment of 200 mg/l of ACR using ACR-decomposing bacteria (*Pseudomonas* sp.) isolated from soil, SHANKER¹¹⁾ has found complete degradation of ACR in six days. These studies have estimated the biodegradability of ACR by microbial flora in aerated water with high concentration of dissolved oxygen or by aerobic bacteria. The biodegradation of ACR by facultative anaerobic bacteria has not been studied. In this experiment, we sought ACR-resistant facultative anaerobes in the ACR-added digested sludge and isolated *E. coli* SK-1 against which ACR was found to have MIC of 20,000 mg/l. The degradation experiment was carried out on suspicion that this might be not only an ACR-resistant strain but also a degrader of ACR. It was found that *E. coli* SK-1 did not decompose ACR in the broth containing both ACR and L-asparagine but did in the broth containing ACR alone. This fact suggests that although this bacterial strain utilizes L-asparagine as a carbon source in preference to ACR, obviously it also utilizes ACR as a carbon source. It is at the same time presumed that the biodegradation of ACR by this bacterial strain is hindered when there is another carbon source more preferred by the bacteria to utilize. When the relation between the number of cell beads and biodegradation of ACR was examined using the *E. coli* SK-1 cells immobilized by the PVA-boric acid method, the biodegradation progressed at the highest rate in the presence of 200 beads and the rate of biodegradation tended to decrease with decrease in the number of beads.

From the above results, the inhibitory concentration of ACR against methane fermentation was estimated to be approximately 3.3 mg/l. It was demonstrated that *E. coli* SK-1 which is an ACR-resistant facultative strain utilized ACR as a carbon source. It was also suggested that this bacterial strain can be used as a bioreactor for biodegradation of ACR by

immobilization of its cells by the PVA-boric acid method. We intend to attempt a long-term, comprehensive study on biodegradation of ACR.

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豚舎汚水処理メタン発酵からのアクリルアミド耐性
通性嫌気性菌の分離と固定化菌体による
アクリルアミドの分解性

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アクリルアミド (ACR) のメタン発酵に対する影響試験を試みたところ, ACR 3.3 mg/l 以上で発生ガス量が極度に低下した. このメタン発酵スラッジから ACR 耐性通性嫌気性菌の分離を試みた結果, ACR に対する最小発育阻止濃度 (MIC) が 20,000 mg/l の *E. coli* SK-1 株が分離された. また, 本菌株は ACR を炭素源として利用することが明らかにされた. さらに, 本菌株を PVA-硼酸法により固定化し, ACR の分解を調べたところ, 4 日間で 52% が分解された. 以上より, メタン発酵は微量の ACR の存在で阻害されることが明らかとされた. また, ACR により馴化したメタン発酵スラッジ由来の *E. coli* SK-1 は固定化したバイオリアクターとしての応用の可能性が示唆された.

日畜会報, 63 (12) : 1228-1233, 1992