

# Isolation of extremely acid-resistant acetic acid bacterium and its application to production of high-acid vinegar in mesophilic condition

## 高度耐酸性酢酸菌の分離と中温条件下での高酸度酢生産への利用

Shintaro Tajiri<sup>1)</sup>Katsuki Matsumoto<sup>1)</sup>Tomohiro Kuratomi<sup>1)</sup>Kenji Tanaka<sup>2)</sup>Hideo Iwai<sup>3)</sup>Hiro Ueno<sup>4)</sup>Munehiro Hoshino<sup>4)</sup>

**概要：**福岡県内のお酢メーカーで受け継がれてきた高酸度酢醸造用種酢から酸に対して高度に耐性な酢酸菌を分離した。16SrRNA遺伝子の相同性その他の形質より本菌をグルコンアセトバクター属細菌と推定し、*Gluconacetobacter* sp. Munehiroと命名した。高酸度酢の生産効率化を目的として、発酵中に基質エタノールを連続的に供給補充する流加培養法において、発酵槽内のエタノール濃度が酢酸生産速度と酢酸生産量に及ぼす影響を調べた。その結果をもとに、発酵槽内のエタノール濃度が40 g・L<sup>-1</sup>から20 g・L<sup>-1</sup>、10 g・L<sup>-1</sup>へと段階的に下がるようエタノール供給を調整することによって、酢酸の阻害作用を受けやすい中温域（29℃）で発酵槽を冷却することなく205 g・L<sup>-1</sup>という従来に無い高酢酸濃度のお酢生産に成功した。

**Abstract :** A new acid-resistant acetic acid bacterium was isolated from the seed vinegar which has been used for the production of high-acid vinegar. The bacterium was gram-negative, rod-shaped and motile ; it occurred singly and did not over-oxidize acetic acid. Cellulose formation was not detected. We named the bacterium *Gluconacetobacter* sp. Munehiro because the sequence of the 16S rRNA gene had 99.9% similarity to that of *G. europaeus*. The strain is extremely resistant to acetic acid and produces high-concentration acetic acid under mesophilic culture condition. The effects of maintaining the ethanol concentration at different levels on the production of high-acid vinegar were investigated. The acetic acid concentration reached 205 g・L<sup>-1</sup> by the stepwise lowering of the ethanol concentration from 40 g・L<sup>-1</sup>, 20 g・L<sup>-1</sup> to 10 g・L<sup>-1</sup> at 29°C without cooling the fermenter. However, the cell concentration, the specific growth rate and the acetic acid production rate were very low because the cell culture suffered by long-time oxygen limitation.

**キーワード：** *Gluconacetobacter*、酢酸菌、高酸度酢、流加培養、酢酸発酵

**Key words :** *Gluconacetobacter*, acetic acid bacteria, high-acid vinegar, fed-batch culture, acetate fermentation

## 1. Introduction

Vinegar is manufactured by one of two different culture methods employing acetic acid bacteria: traditional surface culture or submerged culture using modern reactors equipped with aeration and/or agitation. Industrially, acetic acid production using submerged culture is roughly classified into two groups according to the concentration of acetic acid produced. One is the medium-acid-producing submerged culture (acetic acid is produced at a concentration of 5–10%), in which strains of *Gluconacetobacter xylinus* that do not produce cellulose play an important role. The other is the high-acid-producing submerged culture (acetic acid is produced at concentrations >10%). Vinegar with high acidity made in submerged culture is used industrially in food processing.

Organic acids are inhibitory to microorganisms, especially at low pH, and therefore they are widely used in food preservation. Particularly, acetic acid has an intense antibacterial effect. It inhibits the growth of most microorganisms at concentrations as low as 0.5% <sup>1)</sup>, and pathogenic bacteria that cause food poisoning at a concentration of 0.1% <sup>2)</sup>. Acetic acid bacteria are much more resistant to acetic acid than other types of bacteria ; however, they too are inhibited under highly acidity. Ethanol, the substrate in acetate fermentation, also inhibits cell growth and acid production of acetic acid bacteria, especially under highly acidic conditions. In addition, the inhibitory effects of acetic acid and ethanol are enhanced as the culture temperature increases. In the production of high-acid vinegar, therefore, it is very

1) 近畿大学大学院産業技術研究科物質工学専攻博士前期課程 tajishin19911002@yahoo.co.jp

2) 近畿大学産業理工学部生物環境化学科 教授 tanaka@fuk.kindai.ac.jp

3) 株式会社アスキー

4) マルボシ酢株式会社

difficult to increase the acetic acid concentration up to 20% . Entani et al. <sup>3)</sup> managed to produce 21.75% acetic acid using *A. polyoxogenes*, and Higashiide et al. reported the details of the process <sup>4)</sup>. However, it was necessary to lower the culture temperature to 21°C to weaken the inhibitory effect of acetic acid. Refrigerating the fermentation tank to such a low temperature seriously increases the production costs.

In this study, we isolated an acetic acid bacterium, *Gluconacetobacter* sp. Munehiro, which can grow and produce acetic acid at mesophilic temperatures in an extremely acidic condition.

## Materials and methods

### Isolation of acetic acid bacterium from high-acid vinegar

The acetic acid bacterium used in this study was isolated from the seed of high-acid vinegar which has been used for several decade years in Maruboshi Vinegar Co., Ltd., Japan. Isolation was achieved using the double-layer agar plate method reported by Entani et al. <sup>3)</sup>. The agar plates used in this study were made with ML medium with the following composition per 1000 mL of distilled water: glucose, 2 g ; (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.34 g ; KH<sub>2</sub>PO<sub>4</sub>, 0.18 g; MgSO<sub>4</sub>, 13 mg ; yeast extract, 0.1 g ; polypeptone, 0.1 g ; CaCO<sub>3</sub>, 65 mg ; calcium pantothenate, 30 mg ; inositol, 83 mg ; agar, 10 g. ML agar medium was autoclaved at 121°C for 15 min, and then it was augmented with 1 mL of vitamin complex solution, 40 g·L<sup>-1</sup> ethanol and 40 g·L<sup>-1</sup> acetic acid (the final pH was about 2.7). The composition of the vitamin complex solution was folic acid, 66.6 mg; calcium pantothenate, 532.9 mg ; thiamine hydrochloride, 66.6 mg ; riboflavin, 66.6 mg ; pyridoxine hydrochloride, 133.2 mg ; cyanocobalamin, 666 µg ; and nicotinamide, 113.2 mg per 1000 mL of distilled water. The ML agar medium was poured onto 5.0 g·L<sup>-1</sup> agar that was already solidified on a Petri dish. The seed vinegar was spread on the surface layer of the ML agar plates, and they were incubated at 30°C for 30 days at a relative humidity greater than 90% .

Only one colony with a diameter of 1 mm was obtained after several hundred isolation trials. The colony was transferred in liquid culture and stored at 10°C, then batch culture was repeated every month for subculture. The bacterium requires very acidic conditions with a

pH range from 2.2 to 3.5 and an acetic acid concentration higher than 40 g·L<sup>-1</sup> for cell growth and fermentation.

### Fermentation apparatus and culture conditions

All the fermentation tests were carried out by fed-batch culture using an aerobic fermentation system (BMS-13PI, ABLE, Tokyo, Japan) equipped with a glass jar fermenter (total volume, 13 L). Seed was prepared as follows. The cells grown on AE agar plates were inoculated to 1 mL of ML medium containing 40 g·L<sup>-1</sup> acetic acid and 40 g·L<sup>-1</sup> ethanol in a test tube. Cultivation was carried out at 30°C with shaking at 180 strokes·min<sup>-1</sup> for 48 h. The culture broth was added to 100 mL of ML medium in a shake flask (total volume, 500 mL) and cultivated with shaking at 180 strokes·min<sup>-1</sup> for 48 h. In each fermentation test, the batch stage was started by inoculating the seed culture into 7.9 L of ML liquid medium with the addition of 70 g·L<sup>-1</sup> acetic acid and 50 g·L<sup>-1</sup> ethanol. The temperature was kept at 30°C. The agitation speed and the aeration rate were maintained at 600 rpm and 0.8 L·min<sup>-1</sup>, respectively. During the fed-batch culture stage, the aeration rate was reduced to 0.05 L·min<sup>-1</sup> until acetic acid production started and then it was kept at 0.14 L·min<sup>-1</sup>.

### Effect of culture temperature on acid production

The effect of culture temperature on acetic acid production at high acetic acid concentrations was investigated using the method reported by Higashiide et al. <sup>4)</sup>. When the acetic acid concentration in the batch stage increased to 120 g·L<sup>-1</sup>, 40% of the culture broth was removed from the fermenter and the same volume of fresh medium containing 15 g·L<sup>-1</sup> acetic acid and 110 g·L<sup>-1</sup> ethanol was added. After the acetic acid concentration exceeded 120 g·L<sup>-1</sup>, 500 g·L<sup>-1</sup> ethanol solution was fed so as to maintain the ethanol concentration in the fermenter at around 15g·L<sup>-1</sup>. The fed-batch culture was repeated in the same way at different temperatures. The culture temperatures tested were 24, 25, 26, 27, 28, 29, 30, 31, 32 and 33°C. The highest acetic acid production rate was obtained at 29°C until the acetic acid concentration increased to 120 g·L<sup>-1</sup> (the data are not shown). After the acetic acid concentration reached 120 g·L<sup>-1</sup>, the acetic acid production rate decreased as the temperature increased.

### Investigation of the effect of ethanol concentration on acid production

The effect of ethanol concentration on acid production

in fed-batch culture was investigated with maintaining the ethanol concentration in the culture system at three different levels. When the ethanol concentration in the batch stage had decreased to  $15 \text{ g}\cdot\text{L}^{-1}$ , feeding of  $950 \text{ g}\cdot\text{L}^{-1}$  ethanol was started with a tube pump to maintain the ethanol concentration around  $10 \text{ g}\cdot\text{L}^{-1}$ ,  $20 \text{ g}\cdot\text{L}^{-1}$  or  $40 \text{ g}\cdot\text{L}^{-1}$ . Flow rate of the air into the culture medium was changed according the DO and the medium foaming. Fed-batch culture was repeated three times at each ethanol level. The ethanol concentration was lowered stepwise from  $40 \text{ g}\cdot\text{L}^{-1}$  to  $20 \text{ g}\cdot\text{L}^{-1}$  to  $10 \text{ g}\cdot\text{L}^{-1}$  to investigate the effect of ethanol concentration on acid production rate.

#### Analyses of acetic acid, ethanol and cell growth

Acetic acid concentration was determined by titration with  $1 \text{ M}$  NaOH and by HPLC with a 30-cm Aminex HPX-87 ion-exchange column (Bio-rad Laboratories, Hercules, CA, USA). Ethanol was determined by a gas chromatograph (GC-6APF, Shimadzu) using a column packed with 10% PEG and Shimalite TPA 60/80 mesh. Cell growth was monitored by measuring the optical absorbance of culture broth at  $600 \text{ nm}$ .

#### Sequencing of 16S rDNA

Genomic DNA of the bacterium was extracted by the freezing-thawing method and purified with a DNA purification kit, Mag Extractor - Genome (Toyobo, Osaka, Japan). The 16S rDNA fragment was amplified with the following primers: upstream primer (8UA) 5'-AGAGTTTGATCCTGGCTTA-3'; downstream primer (1492R) 5'-GGTTACCTTGTTACGACTT-3'. The polymerase chain reaction (PCR) was performed using the Gene Amp PCR System 2700 (Applied Biosystems, Carlsbad, USA) and the product was purified with the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, Basel, USA). The cycle sequencing of the whole region of the purified 16S rDNA fragment was done by Hokkaido System Science Co., Ltd., (Sapporo, Japan). A database search of the determined 16S rDNA sequence was conducted by the BLAST program using the GenBank database.

## Results and discussion

### Identification of the isolated acetic acid bacterium

Our isolate is gram-negative, rod-shaped and motile and it occurs singly. Its metabolism is respiratory, never fermentative, and it does not over-oxidize acetic acid.

Cellulose formation has not been detected. The bacterium can grow only in acidic ML medium containing acetic acid and ethanol. Therefore, most of the taxonomic characteristics of the bacterium have not yet been identified. However, the homology search for the 16S rDNA sequence indicated 99.9% similarity between our bacterium and *G. europaeus*. Consequently, we named our acetic acid bacterium *Gluconacetobacter* sp. Munehiro. The nucleotide sequence of the 16S rRNA gene was deposited in the DNA Data Bank of Japan under the accession number AB557644.

### Effect of ethanol concentration on acid production in fed-batch culture

Figure 1 shows the result for the fed-batch culture with maintaining the ethanol concentration at  $40 \text{ g}\cdot\text{L}^{-1}$ . The initial concentrations of acetic acid and ethanol were  $77.3 \text{ g}\cdot\text{L}^{-1}$  and  $46.8 \text{ g}\cdot\text{L}^{-1}$ , respectively. The number of cells at the start of cultivation was estimated to be  $1.4 \times 10^7 \text{ cells}\cdot\text{mL}^{-1}$  by counting under a microscope. At 366 h of cultivation, feeding with  $950 \text{ g}\cdot\text{L}^{-1}$  ethanol was started. The DO decreased to 0.3 ppm after 450 h and cell growth stopped. The maximum cell concentration was  $0.456$  in ABS  $600 \text{ nm}$  ( $4.19 \times 10^8 \text{ cells}\cdot\text{mL}^{-1}$ ). The specific growth rate in the exponential phase (from 312 h to 447 h) was  $0.0168 \text{ h}^{-1}$ . The acetic acid production rate drastically increased after 350 h and approached the maximum level ( $0.259 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ). However, after 450 h, the DO decreased to almost zero then the acetic acid production rate decreased. The final concentration of acetic acid was  $139.1 \text{ g}\cdot\text{L}^{-1}$ .

In the same manner as described above, the fed-batch cultures with maintaining ethanol at  $20 \text{ g}\cdot\text{L}^{-1}$  and  $10 \text{ g}\cdot\text{L}^{-1}$  were carried out. The results were summarized in Table 1 and Table 2.

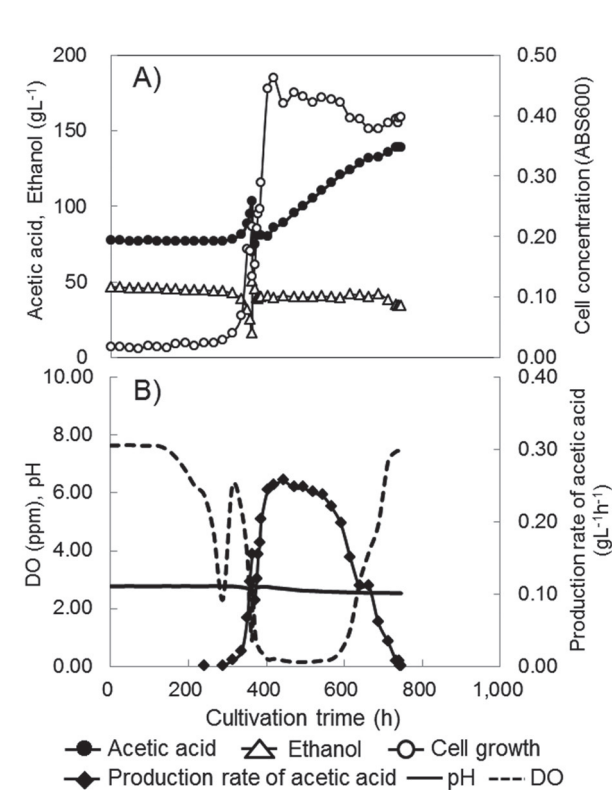


Fig.1 Fermentation profiles for a fed-batch culture of *Gluconacetobacter* sp. Munehiro with the ethanol concentration maintained at 40 g·L<sup>-1</sup>. Culture temperature was maintained at 29°C. Feeding with 950 g·L<sup>-1</sup> ethanol was started at 366 h. A) Concentrations of acetic acid and ethanol, and cell growth. B) pH and DO levels and acetic acid production rate.

The maximum cell concentration was almost the same for the different ethanol concentrations. The specific growth rate was the highest in the culture at 10 g·L<sup>-1</sup> ethanol. Among the three ethanol conditions, acetic acid

concentration was the highest at 20 g·L<sup>-1</sup> ethanol and acetic acid production rate was the highest at 40 g·L<sup>-1</sup> ethanol. For each culture experiment, the average for acetic acid production was calculated for three different periods of each fermentation test (Table 2). From the start of fed-batch stage to the end, acetic acid production was the highest at 40 g·L<sup>-1</sup> ethanol and the lowest at 10 g·L<sup>-1</sup> ethanol. However, it was the highest at 20 g·L<sup>-1</sup> ethanol after the acetic acid concentration exceeded 120 g·L<sup>-1</sup>, and the highest at 10 g·L<sup>-1</sup> ethanol after the acetic acid exceeded 150 g·L<sup>-1</sup>. From these results, we expected that the acetic acid production may be increased by maintaining the ethanol concentration at 40 g·L<sup>-1</sup> for a while after the cultivation started, and lowering it to 20 g·L<sup>-1</sup> then to 10 g·L<sup>-1</sup> as the concentration of acetic acid increased.

#### Enhancement of acid production by stepwise lowering of the ethanol concentration

A fed-batch culture was carried out to investigate the effects of stepwise lowering the ethanol concentration on the acetic acid production (Fig.2). Temperature was maintained at 29°C throughout cultivation. The ethanol concentration in the culture system was maintained at 40 g·L<sup>-1</sup> in the early stage of cultivation. After 440 h of cultivation, the acetic acid production rate increased to 0.253 g·L<sup>-1</sup>·h<sup>-1</sup> and then gradually decreased. At 604 h, the acetic acid concentration increased to 120.1 g·L<sup>-1</sup> and the acetic acid production rate decreased to 0.20 g·L<sup>-1</sup>·h<sup>-1</sup>.

Table 1 Cell growth and production of acetic acid in fed-batch culture of *Gluconacetobacter* sp. Munehiro with maintaining the ethanol concentration at 40, 20 and 10 g·L<sup>-1</sup>

Ethanol concentration (g·L <sup>-1</sup> )	Specific growth rate (h <sup>-1</sup> )	Maximum cell concentration (× 10 <sup>8</sup> cells·mL <sup>-1</sup> )	Maximum concentration of acetic acid (g·L <sup>-1</sup> )	Maximum acetic acid production rate (g·L <sup>-1</sup> ·h <sup>-1</sup> )
40	0.0168 ± 0.0009	4.19 ± 0.12	139.1 ± 3.9	0.259 ± 0.008
20	0.0210 ± 0.0008	4.60 ± 0.06	176.8 ± 3.3	0.243 ± 0.008
10	0.0290 ± 0.0010	4.19 ± 0.15	173.0 ± 5.2	0.233 ± 0.007

Table 2 The average values for acetic acid production in fed-batch culture of *Gluconacetobacter* sp. Munehiro with maintaining the ethanol concentration maintained at 40, 20 and 10 g·L<sup>-1</sup>

Ethanol concentration (g·L <sup>-1</sup> )	Production of acetic acid (g·L <sup>-1</sup> ·h <sup>-1</sup> )		
	After fed-batch stage started	After acetic acid exceeded 120 g·L <sup>-1</sup>	After acetic acid exceeded 150 g·L <sup>-1</sup>
40	0.169 ± 0.007	0.128 ± 0.006	—
20	0.159 ± 0.006	0.148 ± 0.005	0.118 ± 0.004
10	0.158 ± 0.005	0.146 ± 0.005	0.124 ± 0.004

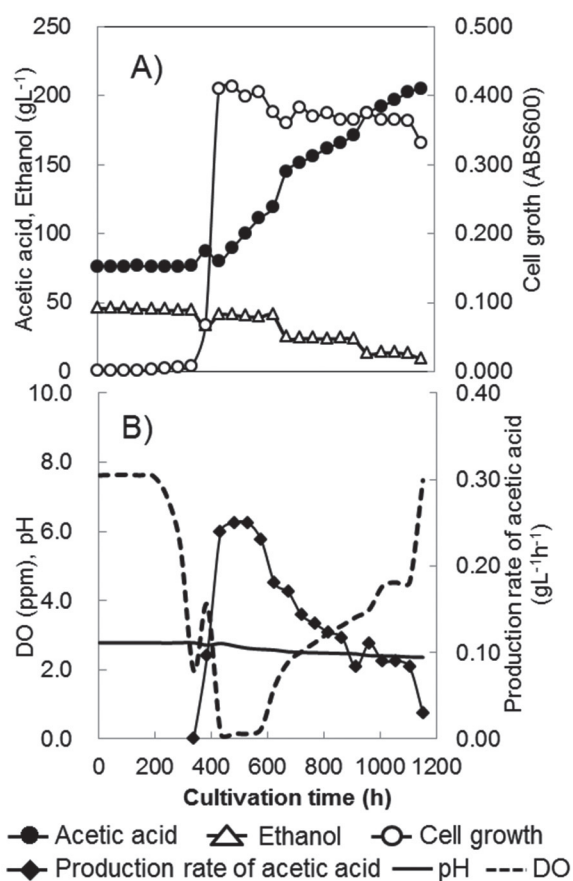


Fig.2 Fermentation profiles for a fed-batch culture of *Gluconacetobacter* sp. Munehiro with stepwise lowering of the controlled level of ethanol concentration from 40 to 20 to 10  $\text{g}\cdot\text{L}^{-1}$  at 29°C. A) Concentrations of acetic acid and ethanol, and cell growth. B) pH and DO levels and acetic acid production rate.

From 604 h to 923 h, the ethanol concentration was maintained at 20  $\text{g}\cdot\text{L}^{-1}$ . At 923 h, the acetic acid concentration increased to 174.4  $\text{g}\cdot\text{L}^{-1}$  and the acetic acid production rate decreased to 0.10  $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ . From 923 h to 1136 h, the ethanol concentration was maintained at 10  $\text{g}\cdot\text{L}^{-1}$ . As a result, the acetic acid concentration reached 205  $\text{g}\cdot\text{L}^{-1}$  at 1136 h. This result shows that the maintenance of ethanol at low concentrations is very available to reduce its inhibitory effect and to increase the acetic acid concentration in the production of high-acid vinegar without lowering the culture temperature if our extremely acid-resistant strain was used. We tried other schemes for lowering the ethanol concentration during cultivation, for example, lowering it from 20  $\text{g}\cdot\text{L}^{-1}$  to 10  $\text{g}\cdot\text{L}^{-1}$ , but that was not very available to increase the highest acetic acid concentration (the data are not shown). Several high-acid-producing acetic acid bacteria were reported, e.g., *G. xylinus*, *A. polyoxogenes*<sup>3, 4)</sup>, *G.*

*europaeus*<sup>5)</sup> and *G. entanii*<sup>6)</sup>. Among these bacteria, *A. polyoxogenes* showed the highest acid production (217.5  $\text{g}\cdot\text{L}^{-1}$  acetic acid). This high acidity was achieved by lowering the culture temperature to 21°C after the acetic acid concentration reached 125  $\text{g}\cdot\text{L}^{-1}$ . Most strains used in industrial vinegar production are mesophilic. However, they can neither grow at the temperature above 30°C nor produce acetic acid above 32°C<sup>7)</sup>. Lu et al. isolated a thermotolerant high acetic acid-producing bacterium, *Acetobacter* sp. I14-2, and they compared the cell growth and acetic acid production at culture temperatures from 30°C to 37°C against those of other *Acetobacter* strains<sup>8)</sup>.

The productivity of acetic acid by *Acetobacter* sp. I14-2 was almost two and three times higher than that by *A. aceti* IFO3283 and *Acetobacter* sp. CCRC12326, and it retained 97% and 68% of its acetic acid-producing activity after 3 days incubation at 35°C and 37°C, respectively, compared with that when incubated at 30°C. However, the maximum amount of acetic acid produced by strain I14-2 was 50  $\text{g}\cdot\text{L}^{-1}$  at 30°C and about 40  $\text{g}\cdot\text{L}^{-1}$  at 37°C. Our strain Munehiro is extremely resistant to acetic acid and it produced 205  $\text{g}\cdot\text{L}^{-1}$  of acetic acid at 29°C. However, the lag phase was very long and the specific growth rate was very low. The production rate of acetic acid was lower than that for submerged fermentation by *Acetobacter* strains. The production rates of acetic acid for *A. rancens* strain S3 and strain F11, and *Acetobacter* sp. 249-1<sup>9)</sup> were 0.23  $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ , 0.26  $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$  and 0.23  $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ , respectively. The strain Munehiro grows and produces acetic acid only in highly acidic conditions with the acetic acid concentration higher than 40  $\text{g}\cdot\text{L}^{-1}$  and the pH below 3.5. In addition, every fermentation test was suffered by oxygen limitation for long time more than 100 h. The cells easily aggregated and flocculated at the acetic acid concentrations higher than 100  $\text{g}\cdot\text{L}^{-1}$ , which seriously suppressed acid production. To avoid the flocculation, it was necessary to reduce the aeration rate to the very low level of less than 0.014 vvm, which caused a great extension of the fermentation time. In contrast, *A. polyoxogenes* produced almost the same amount of acetic acid after about 130 h<sup>3, 4)</sup>. However, it is known that acetic acid bacteria are seriously damaged by very short time oxygen limitation of 30 – 60 s while the strain Munehiro continued to produce acetic acid under such very long oxygen limitation. It is expected that the production rate of acetic acid may be improved



dramatically if the aeration and agitation conditions are optimized.

## References

1. Conner DE ; Kotrola JS, Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Appl. Environ. Microbiol.*, vol.61, 382–385, 1995
2. Entani E ; Asai M ; Tsujihata S ; Tsukamoto Y ; Ohta M, Antibacterial action of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157:H7 (part 1). Examination of bacteriostatic and bactericidal activities (in Japanese with English abstract), *Jpn. J. Assoc. Inf Dis* (Kansenshogakuzasshi), vol.71, 443–450, 1997
3. Entani E ; Ohmori S ; Masai H ; Suzuki K, *Acetobacter polyoxogenes* sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity, *J. Gen. Appl Microbiol.*, vol.31, 475–490, 1985
4. Higashide T ; Okamura H ; Kawamura Y ; Hisamatsu M ; Yamada T, Production of vinegar with high acidity by modified submerged culture, *Nippon Shokuhin Kogyo Gakkaishi*, vol.41, 913–920, 1994 (in Japanese)
5. Sievers M ; Sellmer S ; Teuber M, *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in Central Europe, *Syst. Appl. Microbiol.*, vol.15, 386–392, 1992
6. Schüller G ; Hertel C ; Hammes WP, *Gluconacetobacter entanii* sp. nov., isolated from submerged high-acid industrial vinegar fermentations. *Int. J. Syst. Evol. Microbiol.*, vol.50, 2013–2020, 2000
7. Nakayama T, Studies on acetic acid bacteria. III. Purification and properties of coenzyme-independent aldehyde dehydrogenase, *J. Biochem.*, vol.49, 158–163, 1961
8. Lu SF ; Lee FL ; Chen HK, A thermotolerant and high acetic acid-producing bacterium *Acetobacter* sp. I14-2, *J. Appl. Microbiol.*, vol.86, 55–62, 1999
9. Lotong N ; Malapan W ; Boongorsrang A ; Yongmanitchai W, Production of vinegar by *Acetobacter* cells fixed on a rotating disc reactor, *Appl. Microbiol. Biotechnol.*, vol.32, 27–31, 1989