Acetic Acid Production by an Electrodialysis Fermentation Method with a Computerized Control System

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In acetic acid fermentation by Acetobacter aceti, the acetic acid produced inhibits the production of acetic acid by this microorganism. To alleviate this inhibitory effect, we developed an electrodialysis fermentation method such that acetic acid is continuously removed from the broth. The fermentation unit has a computerized system for the control of the pH and the concentration of ethanol in the fermentation broth. The electrodialysis fermentation system resulted in improved cell growth and higher productivity over an extended period; the productivity exceeded that of non-pH-controlled fermentation. During electrodialysis fermentation in our system, 97.6 g of acetic acid was produced from 86.0 g of ethanol; the amount of acetic acid was about 2.4 times greater than that produced by non-pH-controlled fermentation (40.1 g of acetic acid produced from 33.5 g of ethanol). Maximum productivity of electrodialysis fermentation in our system was 2.13 g/h, a rate which was 1.35 times higher than that of non-pH-controlled fermentation (1.58 g/h).

Acetic acid is an important component in the synthesis of many chemicals, such as cellulose acetate, vinyl acetate, acetic acid esters, terephthalic acid, etc. There are two types of acetic acid fermentation with Acetobacter aceti: surface fermentation and submerged fermentation. The rate of production of acetic acid is slow during surface fermentation but fast during submerged fermentation. Hromatka et al. (6-8) reported that the submerged fermentation had been applied for the first time to the oxidation of alcohol to acetic acid, resulting in an increase in acid formation, and that the cause of damage to the bacteria was not lack of oxygen itself but the toxic effect of alcohol and acetic acid when the oxidative metabolism was interrupted through lack of air. Lopez et al. (9) reported observations on a laboratory method for submerged acetic acid fermentation, and Richardson (15) reported the production of a satisfactory vinegar from waste pineapple juice by submerged fermentation. In acetic acid fermentation by A. aceti, an inhibitory effect is exerted by the acetic acid produced on the production of acetic acid. Mori et al. (10) suggested that undissociated acetic acid is incorporated into cells and lowers the intracellular pH, causing inhibition of respiration via inhibition of respiratory enzymes. Moreover, Mori et al. (11) reported that the acetate ion or undissociated acetic acid has a greater inhibitory effect on the growth of the Acetobacter cells than on respiration of the cells. Muraoka et al. (12) reported in detail that production of acetic acid is inhibited by undissociated acetic acid in the fermentation broth when the pH is above 3.1 and by both undissociated acetic acid and hydrogen ions when the pH is below 3.1. Therefore, if this inhibitory effect could be alleviated, the production of acetic acid by bacteria that produce acetic acid by fermentation would be expected to increase. In a previous paper (5), we reported an electrodialysis fermentation (ED-F) method that was very effective in anaerobic lactic acid fermentation. Therefore, ED-F, which can continuously remove acetic acid produced in the fermentation broth, was used for the aerobic production of acetic acid. With ED-F a favorable broth pH can be maintained without a neutralizer by continuously removing the acetic acid produced from the broth.

In this paper, we describe the application to acetic acid fermentation of ED-F with computerized control of pH and the concentration of ethanol.

MATERIALS AND METHODS

Microorganism, media, and culture conditions. A. aceti IF03281 was used.

The compositions of the media were as follows. (i) The medium for the stock and the refreshing culture contained 0.5 g of beef extract, 0.3 g of yeast extract, 0.15 g of glycerol, 0.5 g of glucose, and 2.0 g of agar in 100 ml of a 20% dilution of a solution of potato extract (pH 7.0) and 1.0 g of CaCO₃ (medium S). (ii) The preparation of the potato extract solution was as follows. Sliced potatoes (20 g) were gently boiled in 80 ml of tap water for 30 min, and the solids were removed by filtration through cloth. The filtrate (made up to 100 ml) was used as the solution of potato extract. CaCO₃ was added after being sterilized at 160°C for more than 2 h. (ii) The medium for the seed culture contained 1.0 g of glucose, 1.0 g of yeast extract, 1.0 g of peptone, and 2.5 g of glyceral in 100 ml of tap water, pH 7.0 (medium S). (iii) The medium for production of acetic acid contained 3.0 g of glucose, 1.0 g of meat extract, and 1.0 g of peptone in 100 ml of tap water (pH 7.0), and 1 to 8% (vol/vol) of 99.5% ethanol (medium P). Ethanol was added after filtration through a membrane filter (pore size, 0.45 μm) at the time of inoculation. A. aceti was inoculated from a stock culture on an agar slant of medium R and cultured at 30°C for 24 h. A loopful of cells grown on the agar slant of medium R was inoculated into a 500-ml flask which contained 50 ml of medium S, and the suspension was cultured at 30°C for 24 h on a reciprocal shaker (120 strokes per min). For acetic acid fermentation, 500-ml flasks containing 100 ml of medium P or 2-liter miniature fermentors (MB type; Iwashiya Bio-Science Co., Ltd.) containing 1 liter of medium P were employed. Medium P in the 500-ml flask or in the miniature fermentor was inoculated with 5% (vol/vol) seed culture and cultured at 30°C.

Experimental apparatus for ED-F. A schematic diagram of

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the electrodialyzer is shown in Fig. 1. The four compartments making up the electrodialyzer (model TS-2; Tokuyama Soda Co., Ltd.) are one anode (platinum-plated titanium; 193 cm²) compartment (Fig. 1, I), one concentration compartment (Fig. 1, II), one dialyzing compartment (Fig. 1, III), and one cathode (SUS-316; 193 cm²) compartment (Fig. 1, IV). Each compartment is separated by an anion-exchange membrane (available area of membrane, 205 cm²; Neosepta ACH-45T; Tokuyama Soda Co.) or a cation-exchange membrane (available area of membrane, 205 cm²; Neosepta CH-45T; Tokuyama Soda Co.). Anions penetrate the anion-exchange membrane but are unable to penetrate the cation-exchange membrane. With cations, the situation is reversed. The fermentation broth is passed through the dialyzing compartment during ED-F. The fermentation broth and the concentrating fluid (tap water, 1,000 ml) were circulated at a rate of 183 ml/min (Master Flex Pump; Cole-Parmer Instrument Co.). An anolyte (0.1 N H₂SO₄, 500 ml) and a catholyte (0.1 N NaOH, 500 ml) were circulated at a rate of 7.0 ml/min (microtube MP-3; Tokyo Rikakikai Co., Ltd.). Since acetate ions are negatively charged, they penetrate the anion-exchange membrane and are attracted to the anode compartment but are unable to penetrate the cation-exchange membrane. Consequently, acetic acid accumulates in the concentration compartment.

System for the control of pH and the concentration of ethanol in the fermentation broth. The system for controlling the pH and the concentration of ethanol in the fermentation broth is illustrated in Fig. 2. A Figaro gas sensor (no. 812; Kyoritsu Electronic Industry Co., Ltd.) was used as a simple ethanol sensor. The gas sensor, which responds to inflammable gas, is a semiconductor sensor. Inflammable gas is burned by the heater in the sensor, giving rise to a change in temperature. The sensor detects the change in temperature as a change in the resistance of the semiconductor. The voltage signals from the pH electrode (GS-8270; Toa Electronics Ltd.) or the gas sensor are amplified by an amplifier, and the amplified signals are converted to digital signals by an amplified-digital signal converter (Remote TE-801S; Taisei Electric Co., Ltd.). The digital signals are fed into a microcomputer (PC-8801 mk II; Nippon Electronics Co.). The information from the computer, which is converted to analog signals by a digital-amplified signal converter (remote TE-801S; Taisei Electric Co.), regulates a relay circuit which controls a direct-current power supply (MPO 35-2; Takasago Seisakusho, Ltd.) used for electrodialysis and the pump (Perista pump SJ 1211; Atto Co.), which supplies the ethanol (0.23 ml/min).

**Schematic diagram of ED-F with the computerized control system.** A schematic diagram of ED-F with the computerized control system is shown in Fig. 3. *A. aceti* is cultured in a 2-liter miniature fermentor (initial volume of medium, 1 liter). Acetic acid is produced, and the pH of the fermentation broth falls below the set value, causing the direct-current power supply connected to the relay circuit to produce electric current. Acetate ions penetrate the anion-exchange membrane and accumulate in the concentration compartment. As acetate ions move to this compartment, the pH of the fermentation broth rises above the set value. Consequently, the relay circuit operates, and the direct-current power supply is switched off. More acetic acid is produced, the pH falls below the set value, the relay circuit operates, and electric current flows again. Thus, the acetic acid produced is continuously removed to the outside of the fermentation broth, and the pH of the broth is constantly maintained at a favorable value. The electric current is maintained at 1 A during ED-F. When the concentration of
ethanol in the fermentation broth falls below the set value, the computer engages the relay circuit, so that the pump which supplies the ethanol starts to operate. Conversely, when the concentration exceeds the set value, the pump is switched off. Thus, the concentration of ethanol is maintained at a constant value. The ethanol sensor cannot measure ethanol directly in the gaseous phase in the fermentor, because the concentration of ethanol in the gaseous phase is beyond the range of this sensor. Therefore, the discharged gaseous phase from the fermentor is diluted 10-fold with air at 30°C, and the concentration of ethanol in the diluted gaseous phase is measured.

Analyses. The concentration of acetic acid was estimated by gas chromatography (GC-7AG chromatograph; Shimazu Co.) or by titration with 0.1 N NaOH, with phenolphthalein as the indicator, after steam distillation under acidic conditions with 1 N H₂SO₄. The concentration of ethanol was estimated by gas chromatography or by an enzymatic method (3). The rate coefficient of oxygen absorption was determined by the sulfite method (2, 4), in a 500-ml flask with a silicone cap (C-40; Shinetco Chemical Co.) or in a 2-liter miniature fermentor.

RESULTS AND DISCUSSION

Effect of acetic acid or acetates on production of acetic acid. Samples of acetic acid, calcium acetate, or sodium acetate were individually added to medium P (100 ml) in 500-ml flasks at the time of inoculation. Ethanol (4 ml) was also added at the same time. The production of acetic acid under these conditions is shown in Fig. 4. At a concentration of 1% acetic acid, sodium acetates, or calcium acetate (Fig. 4A), after 24 h of culture the pH of the broth was 3.12, 4.14, or 4.31, respectively; acetic acid had the least inhibitory effect

FIG. 3. Schematic diagram of ED-F. 1, Miniature fermentor; 2, electrodialyzer; 3, concentrating fluid reservoir; 4, magnetic stirrer; 5, gas sensor; 6, pH electrode; 7, direct-current power supply; 8, control system (see Fig. 2); 9, air pump; 10, pump for supplying ethanol; 11, gas outlet; 12, recirculation pump.

FIG. 4. Effect of acetic acid or acetates on production of acetic acid (A, 1% acetate; B, 1.5% acetate). Fermentations were conducted in 500-ml flasks which contained 100 ml of medium P at 30°C on a reciprocal shaker (120 strokes per min). Concentration is expressed as percentage of acetic acid. Ethanol (4%, vol/vol) was added to medium P at the time of inoculation. Symbols: O, no addition; ●, free acetic acid; △, sodium acetate; A, calcium acetate.
(about 16%) on the production of acetic acid. Sodium acetate and calcium acetate inhibited production of acetic acid by 40 and 58%, respectively, as compared with the control (without acetate). At 48 h after the addition of acetic acid or sodium acetate, the amount of acetic acid produced was the same as that produced by the control. At 24 h after the addition of 1.5% acetic acid, sodium acetate, or calcium acetate (Fig. 4B), about 75% inhibition of the production of acetic acid was observed, and the pH of the broth was 3.40, 4.98, or 4.68, respectively. After 48 h, however, and only in the case of 1.5% acetic acid, 28 mg of acetic acid per ml was produced. In all cases with the addition of 2% acetic acid or acetates (data not shown), no acetic acid was produced. These results show that the inhibition increases along with rise of the pH. In further experiments, the pH of medium P was adjusted to 3.94 (corresponding to the pH measured before inoculation in the medium P added 1% acetic acid) with HCl, and this medium was cultured. After 24 h of culture, amount of acetic acid produced (29.7 mg/ml) was the same as that produced by the control. Therefore, we considered that the presence of acetic acid or acetates themselves rather than the lowering of the pH had some adverse effect on production of acetic acid. As mentioned above, acetic acid or acetates inhibited the production of acetic acid, and at the same concentration (below 1.5%) acetates had a greater inhibitory effect than acetic acid, probably because the added acetates changed the pH from the optimum pH for production of acetic acid (see below). From these observations, it appears that increased production of acetic acid can be achieved by removal of the acetic acid produced from the fermentation broth.

The effect of ethanol concentration on the production of acetic acid was studied with cultures in 500-ml flasks (data not shown). At the time of inoculation, ethanol was added to medium P at various concentrations (1 to 8%, vol/vol). At levels above 5% ethanol, an inhibitory effect of ethanol on the production of acetic acid was observed. At a concentration of 8%, after 15 h only 4 mg of acetic acid per ml had been produced and further production was not observed. However, at concentrations below 4%, ethanol was converted to acetic acid at a conversion rate of about 93%. At concentrations of 1, 2, 3, or 4% ethanol, acetic acid was produced, for the first 8 h at a rate of 0.99, 0.98, 0.88, or 0.83 mg/ml per h, respectively. Therefore, we decided that the concentration of ethanol in the fermentation broth should be maintained at 1% during the controlled supply of ethanol.

**Optimum pH for production of acetic acid.** The rate coefficient of oxygen absorption, \( K_{a1} \), was measured in 500-ml flasks which contained 100 ml of 1 N Na\(_2\)SO\(_4\) on a reciprocal shaker (120 strokes per min; amplitude, 70 mm). The \( K_{a1} \) was 2.55 \( \times \) 10\(^2\) mmol of O\(_2\) per liter per h at atm (1 atm is equal to 101.29 KPa), giving useful criteria for scaled-up fermentation. The culture conditions in 2-liter miniature fermentors containing 1 liter of 1 N Na\(_2\)SO\(_4\) have been studied. Yamada et al. (17) reported that the variation in speed of agitation rather than variation in rate of air flow significantly affects the \( K_{a1} \). In addition, since both ethanol and acetic acid are volatile substances, they are liable to volatilize when the rate of air flow is high. Therefore, the rate of air flow was kept low (200 ml/min). When the rate of air flow was kept constant (200 ml/min) with an agitation speed of 700 rpm, a \( K_{a1} \) equal to that mentioned above was obtained (2.60 \( \times \) 10\(^2\) mmol of O\(_2\) per liter per h atm). Under these conditions, the optimum pH for acetic acid fermentation was examined with the pH being controlled by neutralization with 5 N NaOH (data not shown). Ethanol (4%, vol/vol) was added to medium P at the time of inoculation. When the pH was kept below 4.0, the rates of production of acetic acid were almost identical and stable production of acetic acid was observed. However, when the pH was above 4.5, the rate of production of acetic acid fell rapidly. Enzymes participating in the oxidation of ethanol to acetic acid are the membrane-bound alcohol dehydrogenase and the membrane-bound aldehyde dehydrogenase (16). The optimum pH of this alcohol dehydrogenase from A. aceti is 4.0 (1, 14), whereas the aldehyde dehydrogenase from A. aceti has an optimum pH of 8.0 and is relatively stable at pH 4.0 to 6.0 (13). This optimum pH of the aldehyde dehydrogenase seems to make it unsuitable for acetic acid fermentation. However, after 15 h (when ethanol was still present in the fermentation broth) the amounts of acetic acid produced during pH-controlled fermentation at pH 6.0, 5.0, 4.5, 4.0, 3.8, and 3.5 and during non-pH-controlled fermentation were 12.0, 15.7, 18.2, 31.6, 32.7, 31.4, and 31.4 g, respectively. After 40 h, in all fermentations except for pH-controlled fermentation at 6.0 (87 h), the amounts of acetic acid produced were 37.6, 38.4, 38.6, 39.1, 39.1, 39.0, and 39.0 g, respectively, and the yields of acetic acid in terms of ethanol consumed were 87.8, 91.5, 92.0, 93.0, 93.2, 93.2, and 93.2%, respectively. Therefore the pH in ED-F was fixed at 3.8.

**Production of acetic acid by ED-F.** Ethanol (1%, vol/vol) was added to medium P at the time of inoculation, and during fermentations the concentration of ethanol was kept at 1% (vol/vol) by supplying ethanol via the pump. The time course of ED-F is shown in Fig. 5. In the case of non-pH-controlled fermentation, poor cell growth and acetic acid-producing activity dropped considerably after 24 h. After 48 h, the amount of acetic acid produced was 36.3 mg/ml and the pH of the fermentation broth was 3.1, and further production was not observed. In pH-controlled fermentation at pH 3.8, obtained by the addition of 5 N NaOH, good cell growth was observed and the rate of production of acetic acid increased as compared with non-pH-controlled fermentation. However, the acetic acid-producing activity fell considerably after 24 h, in the same manner as in non-pH-controlled fermentation, probably because of the inhibition by accumulated acetate. After 48 h, the amount of acetic acid produced was 50.4 mg/ml, and further production was not observed. By contrast, in ED-F improved cell growth was observed, acetic acid-producing activity did not fall after 24 h, and production continued for long time as compared with pH-controlled fermentation at pH 3.8. After 72 h of fermentation, the amount of acetic acid produced in the fermentation broth was 24.5 mg/ml and that in the concentrating fluid was 56.6 mg/ml. In non-pH-controlled fermentation, pH-controlled fermentation, and ED-F, the yields of acetic acid in terms of ethanol consumed were about 91, 91, and 87%, respectively. This ED-F value, compared with the yield obtained in the experiments on optimum pH for acetic acid production, was significantly lower, probably because of large losses of ethanol, because pH-controlled fermentation at 3.8 in the experiments of optimum pH was completed in about 18 h, whereas ED-F continued for a longer time.

Ethanol was supplied, and the volumes of fermentation broth and concentrating fluid were changed during ED-F (18). In fact, after fermentation for 72 h, the volumes of the fermentation broth and the concentrating fluid were 910 and 1,330 ml, whereas their initial volumes were 1,060 and 1,000 ml, respectively. Total amounts of acetic acid produced are shown in Table 1. In non-pH-controlled fermentation, 40.1 g of acetic acid was produced from 33.8 g of ethanol; in
TABLE 1. Production of acetic acid by ED-F

<table>
<thead>
<tr>
<th>Fermentation process</th>
<th>Acetic acid produced (g [mg/ml])</th>
<th>Relative production (%)</th>
<th>Maximum productivity (g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fermentation broth Concentrating fluid Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-pH controlled</td>
<td>40.1 (36.3)</td>
<td>40.1c</td>
<td>100</td>
</tr>
<tr>
<td>pH controlled</td>
<td>58.5 (50.4)</td>
<td>58.5d</td>
<td>146</td>
</tr>
<tr>
<td>ED-F</td>
<td>22.3 (24.5)</td>
<td>75.3 (56.6)</td>
<td>97.6f</td>
</tr>
</tbody>
</table>

* Fermentation conditions were as described in the legend to Fig. 5. Values within parentheses are final concentrations of acetic acid.

† Relative production is expressed as amount of acetic acid produced as a percentage of that produced during non-pH-controlled fermentation.

‡ Produced from 33.8 g of ethanol.

§ Produced from 49.3 g of ethanol.

¶ Produced from 86.0 g of ethanol.

Ph-controlled fermentation at 3.8, 58.5 g of acetic acid was produced from 49.3 g of ethanol. However, in ED-F 97.6 g of acetic acid was produced from 86.0 g of ethanol; the amount of acetic acid produced was about 2.4 times greater than that produced by non-pH-controlled fermentation. Moreover, the maximum productivity of ED-F was 2.13 g/h, which was 1.35 times higher than that of non-pH-controlled fermentation (1.58 g/h) and the same as that of pH-controlled fermentation (2.19 g/h). Unlike results with lactic acid fermentation (5), cell mass was low (after 11-fold dilution, the maximum optical density 0.2); by contrast, the maximum optical density was 0.55 in lactic acid fermentation in acetic acid fermentation. When a electric current was maintained at 1 A, voltage was also maintained at 5 V. Therefore, electrodialysis efficiency did not decrease, because electric resistance did not increase. Further studies on ED-F are in progress.

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LITERATURE CITED


