APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1986, p. 314–319
0099-2240/86/080314-06$02.00/0

Vol. 52, No. 2

Novel Method of Lactic Acid Production by Electrodialysis Fermentation

MOTOYOSHI HONGO, YOSHIYUKI NOMURA,* AND MASAYOSHI IWAHARA

Department of Applied Microbial Technology, The Kumamoto Institute of Technology, Ikeda 4-22-1, Kumamoto 860, Japan

Received 10 March 1986/Accepted 21 May 1986

In lactic acid fermentation by *Lactobacillus delbrueckii*, the produced lactic acid affected the lactic acid productivity. Therefore, for the purpose of alleviating this inhibitory effect, an electrodialysis fermentation method which can continuously remove produced lactic acid from the fermentation broth was applied to this fermentation process. As a result, the continuation of fermentation activity was obtained, and the productivity was three times higher than in non-pH-controlled fermentation. In electrodialysis fermentation, the amount of produced lactic acid was 82.2 g/liter, which was about 5.5 times greater than that produced in non-pH-controlled fermentation. It was concluded that these good results were obtained on account of alleviating the lactic acid inhibitory effect by electrodialysis fermentation. However, the fouling of anion-exchange membranes by cells was observed in electrodialysis fermentation.

In lactic acid fermentation, there is an inhibitory effect caused by the produced lactic acid (5, 14, 15, 18) on the lactic acid productivity of lactic acid fermentation bacteria. If this inhibitory effect could be alleviated, the continuation of lactic acid production activity and an increase in produced lactic acid would be expected.

Gerhardt and Gallup (7) reported that lactic acid production by *Lactobacillus acidophilus* was increased by dialysis. In a more detailed study, Friedman and Gaden (5) reported a kinetic analysis of lactic acid production by dialysis fermentation. Abbott and Gerhardt (1) also reported that in the production of salicylic acid by *Pseudomonas fluorescens*, dialysis fermentation was a very effective method. Thus, dialysis fermentation has been proven to be very effective. However, to achieve more effective dialysis fermentation, the reservoir volume or volume of the dialysate must be expanded to more than that of the fermentation vessel or fermentation broth (1, 6–8). Therefore, diffusion dialysis fermentation has the limitation of diffusion efficiency.

On the other hand, an electrodialysis method with ion-exchange membranes has been applied to a variety of processes, including desalting of seawater (25), the food industry (25), the recovery of nickel (9), and wastewater treatment in plating factories (16, 17). If an inhibitory product ionizes in the fermentation broth, it is possible to remove the substance continuously from the fermentation broth by using an electrodialyzer with ion-exchange membranes. Therefore, we devised an electrodialysis fermentation (ED-F) method that uses an electrodialyzer. ED-F is defined as a novel fermentation which is able to alleviate the inhibitory effect of a microbial metabolic product on microbial metabolism and to increase the product by this alleviation, in addition to the effect of electroenergizing (10–13) on microbial metabolism.

In industrial lactic acid fermentation, fermentation under conditions of excess $CaCO_3$ to prevent lowering of the pH of the broth is conventional (3). In this fermentation method, $CaCO_3$ reacts with lactic acid to produce calcium lactate; thus, the pH of the fermentation broth is maintained at about 5.0. As the solubility of calcium lactate is low (3), at high concentrations of this substance the broth tends to solidify, complicating following procedures (3). Recently, there have been reports of a continuous dialysis process for ammonium lactate fermentation with neutralization to a constant pH achieved by NH_4OH (4, 14, 21–24) and lactic acid production in a hollow fiber fermentor with neutralization achieved by NaOH (19). In the former experiments, good results were obtained. In this process, ammonium lactate in the dialysate was purer than that in the fermentation broth, but the concentration was low (21, 22, 24). On the other hand, in the latter experiments, the lactic acid production rate was limited by the transport of glucose across the fiber wall. Until now, there has been no report of an ED-F procedure which can control the pH of the fermentation broth without the use of a neutralizer.

Accordingly, in this paper, we describe the application to lactic acid fermentation of electrochemically pH-controlled ED-F in which the pH of the fermentation broth can be kept at a favorable value by continuous removal of lactic acid from the broth.

MATERIALS AND METHODS

Microorganism and culture conditions. *L. delbrueckii* IFO 3534, a homolactic acid fermentation bacterium, was used.

The compositions of the media were as follows. (i) The medium for the refreshing culture contained 2.5 g of yeast extract, 5.0 g of Polypeptone (BBL Microbiology Systems), and 1.0 g of glucose in 100 ml of tap water, pH 7.0 (medium R). (ii) The medium for the seed culture contained 0.5 g of yeast extract, 1.0 g of Polypeptone, 1.0 g of sodium acetate, and 1.0 g of glucose in 100 ml of tap water, pH 6.8 (medium S). (iii) The medium for lactic acid production contained 10.0 g of glucose, 2.0 g of yeast extract, 0.8 g of Polypeptone, 0.2 g of KH_2PO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of NaCl in 100 ml of tap water, pH 7.2 (medium P). If necessary, 10% (wt/vol) $CaCO_3$ was added to medium P after being sterilized at 160°C for more than 2 h.

L. delbrueckii was inoculated twice by use of a platinum needle into 10 ml of medium R from a stock culture (prepared every week) and cultured statically at 45°C for 24 h. The seed culture (100 ml of medium S) was inoculated with

* Corresponding author.

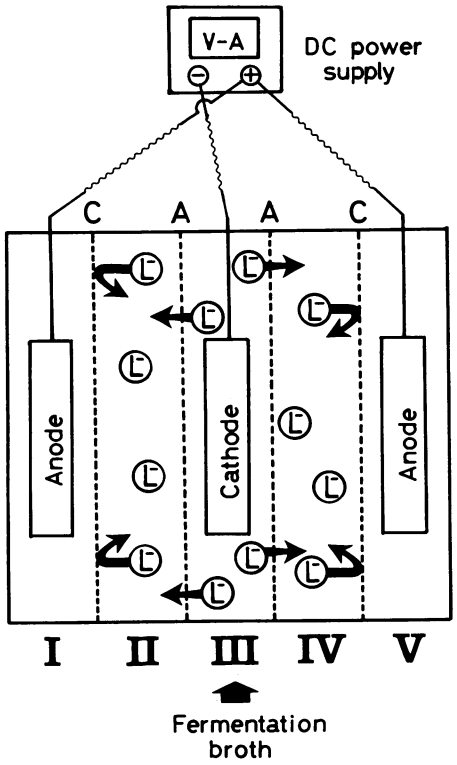

FIG. 1. Schematic diagram of electrodialyzer. L^-, Lactate ion; C, cation-exchange membrane; A, anion-exchange membrane; I and V, anode compartments; II and IV, concentration compartments; III, cathode compartment; DC, direct current; V-A, voltage and ampere meter.

10 ml of the refreshed culture and cultured statically at 45°C for 15 h. In lactic acid fermentation, medium P was inoculated with 10% (vol/vol) seed culture and cultured at 45°C with gentle stirring.

Experimental apparatus for ED-F. The five compartments making up the electrodialyzer are two anode compartments, two concentration compartments, and one cathode compartment (Fig. 1). Each compartment is separated by an anion-exchange membrane (available membrane area, 75 cm^2; Neosepta ACH-45T; Tokuyama Soda Co., Ltd.) or a cation-exchange membrane (available membrane area, 75 cm^2; Neosepta CH-45T; Tokuyama Soda Co., Ltd.). The fermentation broth is passed through the cathode compartment (Fig. 1, III). As lactic acid in the fermentation broth charges negatively, it penetrates the anion-exchange membrane and is attracted to the anode compartments (Fig. 1, I and V) but is unable to penetrate the cation-exchange membrane. Consequently, produced lactic acid accumulates in the concentration compartments (Fig. 1, II and IV).

Schematic diagram of the electrochemically pH-controlled ED-F system. A schematic diagram of the ED-F system is shown in Fig. 2. *L. delbrueckii* is cultured in a 750-ml miniature fermentor (working volume, 550 ml). Lactic acid is produced, and the pH of the fermentation broth falls below the set pH value, causing the direct-current power supply (MPO 35-2; Takasago Seisakusho, Ltd.) connected to the pH controller (PHD II; Oriental Electric Co., Ltd.) to operate, resulting in the flow of electric current. Lactate ion penetrates the anion-exchange membrane and accumulates in the two concentration compartments. As lactate ion moves to these compartments, the pH of the fermentation broth rises above the set pH value.

Consequently, the pH controller operates and the direct-current power supply is switched off. More lactic acid is produced, the pH falls below the set pH value, the pH controller operates, and an electric current flows. Thus, produced lactic acid is continuously excluded to the outside of the fermentation broth, and the pH of the broth is constantly maintained at a favorable value. When the pH of the fermentation broth falls to a lower value, at which it is difficult for other bacteria to grow, that is, after 7 h postinoculation (optical density, 0.10, pH 4.7), the fermentation broth, concentrated fluid (tap water, 500 ml), and anolyte (0.1 N H_2SO_4, 500 ml) are circulated at the rate of 20 ml/min (Microtube pump MP-3; Tokyo Rikakikai Co., Ltd.). An electric current is maintained at 0.2 to 0.8 A. A copper plate (surface area, 34 cm^2) is used as the cathode, and a platinum plate (surface area, 12.5 cm^2) is used as the anode.

Analyses. The lactic acid concentration was estimated by the method of Barker and Summerson (2). The residual glucose concentration was measured by the method of Somogyi (20) or with a new Glucostat (Worthington Diagnostics) in accordance with the instructions of the manufacturer. Cell growth was measured by the optical density at 660 nm after 11-fold dilution of the fermentation broth with 1 N HCl.

RESULTS AND DISCUSSION

Effect of various lactates on cell growth. Lactic acid, calcium lactate, sodium lactate, or ammonium lactate was added to medium P at the time of inoculation. The effect of lactates on cell growth is shown in Fig. 3, and the pH of the culture broth is shown in Table 1. In the case of lactic acid at a concentration of 0.5%, cell growth was recognized as only 20% of cell growth in the absence of lactic acid. Moreover, at a concentration of 1.0%, cell growth entirely ceased. With the addition of 1.0% ammonium lactate, sodium lactate, or calcium lactate, 50, 67, and 72% cell growth was observed, respectively, as compared with that in the absence of additives. The optimum pH of lactic acid bacteria is in the

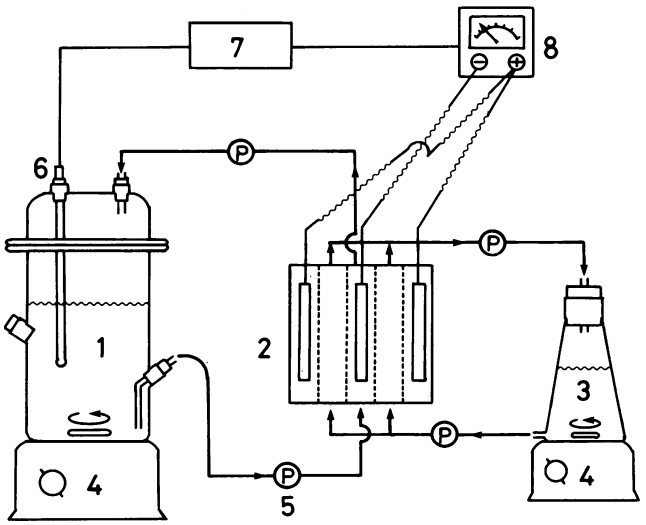

FIG. 2. Schematic diagram of ED-F system. 1, Fermentor; 2, electrodialyzer; 3, concentrated-fluid reservoir; 4, magnetic stirrer; 5, pump; 6, pH electrode; 7, pH controller; 8, direct-current power supply.

vicinity of 5.5 to 6.0 (3). In all cases, the pH of the culture broth after cultivation fell below the favorable value (Table 1). Accordingly, it is considered that lowering of the pH and the presence of lactates inhibit cell growth. In the case of calcium lactate at a concentration of 0.5%, cell growth was inhibited by only 3%, as compared with the control. The pH value of that culture broth was 3.90. On the other hand, the addition of 1.0% calcium lactate inhibited cell growth by 28%, as compared with the control, and the pH value was 4.07. Thus, the higher the concentration of added lactate, the lower the rate of cell growth observed. Conversely, the pH value was gradually higher. In a further detailed experiment (Table 2), the pH value of medium P was varied by the addition of 1 N HCl. Cell growth decreased with a decrease in pH. However, in a comparison of the addition of 1.0% lactic acid in Table 1 (pH 3.85) with the pH adjustment to 3.75 with HCl (Table 2), the former produced almost unrecognizable cell growth. On the other hand, in the latter, approximately 50% cell growth was observed, as compared with the control (corresponding to no addition in Table 1). On the basis of this observation, it is considered that the addition of lactates themselves rather than lowering of the pH has some adverse effect on cell growth. It was concluded that lactic acid showed the most inhibitory effect on cell growth and that even neutralized lactates clearly showed inhibitory effects.

TABLE 1. pH before and after cultivation with the addition of various lactates

Lactate[a] and concn[b]	pH	
	Immediately after inoculation	After cultivation[c]
None	5.36	3.67
Lactic acid		
0.1	5.03	4.09
0.2	4.89	4.16
0.3	4.65	4.17
0.5	4.29	4.29
0.7	4.03	4.31
1.0	3.85	4.07
1.5	3.63	3.87
Calcium lactate		
0.5	4.96	3.90
1.0	4.88	4.07
2.0	4.86	4.31
3.0	4.87	4.45
4.0	4.88	4.56
5.0	4.89	4.61
7.0	4.92	4.72
Sodium lactate		
0.5	5.42	3.94
1.0	5.41	4.11
2.0	5.46	4.40
3.0	5.47	4.61
4.0	5.52	4.83
5.0	5.56	5.09
7.0	5.62	5.40
Ammonium lactate		
0.5	5.11	3.96
1.0	5.01	4.21
2.0	4.93	4.51
3.0	4.88	4.65
4.0	4.86	4.75
5.0	4.86	4.85
7.0	4.88	4.88

[a] Lactates were added aseptically at the time of inoculation.
[b] Concentrations are expressed as percentages of lactic acid.
[c] Culture conditions were the same as those in the legend to Fig. 3.

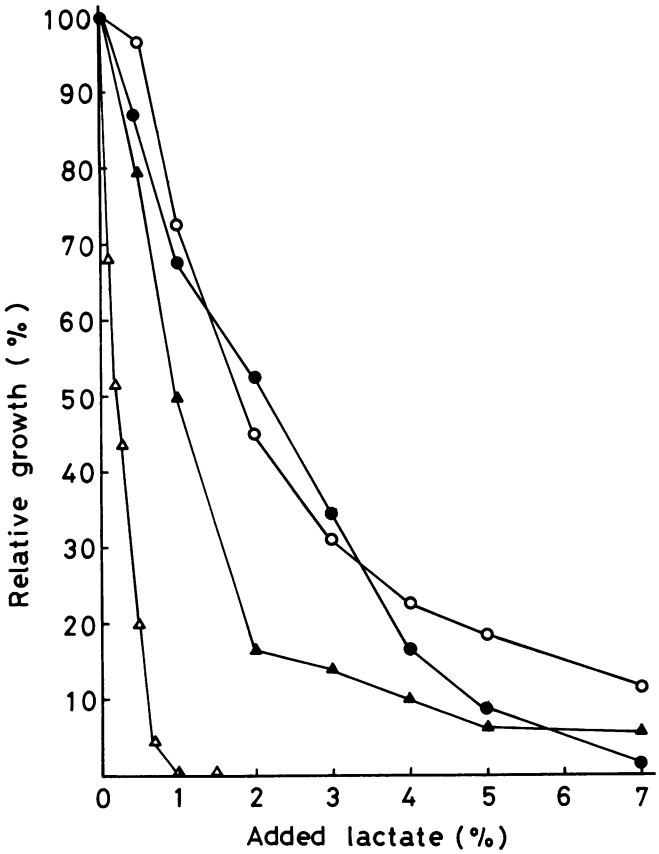

FIG. 3. Effect of various lactates on cell growth. Cells were cultured statically at 45°C for 48 h in screw-capped culture tubes containing 10 ml of medium P. Individual lactates were aseptically added at the time of inoculation. Added lactate is expressed as a percentage of lactic acid. Relative growth is expressed as percent cell growth as compared with the control (without lactate). Symbols: △, lactic acid; ▲, ammonium lactate; ●, sodium lactate; ○, calcium lactate.

Lactic acid production by means of pH control by the

TABLE 2. Effect of various pH values on cell growth

pH[a]		% Relative growth[b]
Immediately after inoculation	After cultivation[c]	
5.36	3.67	100
4.82	3.66	97.2
4.67	3.65	95.3
4.52	3.66	84.7
4.25	3.67	78.9
4.01	3.70	62.5
3.75	3.71	47.2

[a] The pH was adjusted to the indicated values with 1 N HCl.
[b] Relative growth is expressed as percent cell growth as compared with the control; the pH was not adjusted with HCl (corresponding to no addition in Table 1).
[c] Culture conditions were the same as those in the legend to Fig. 3.

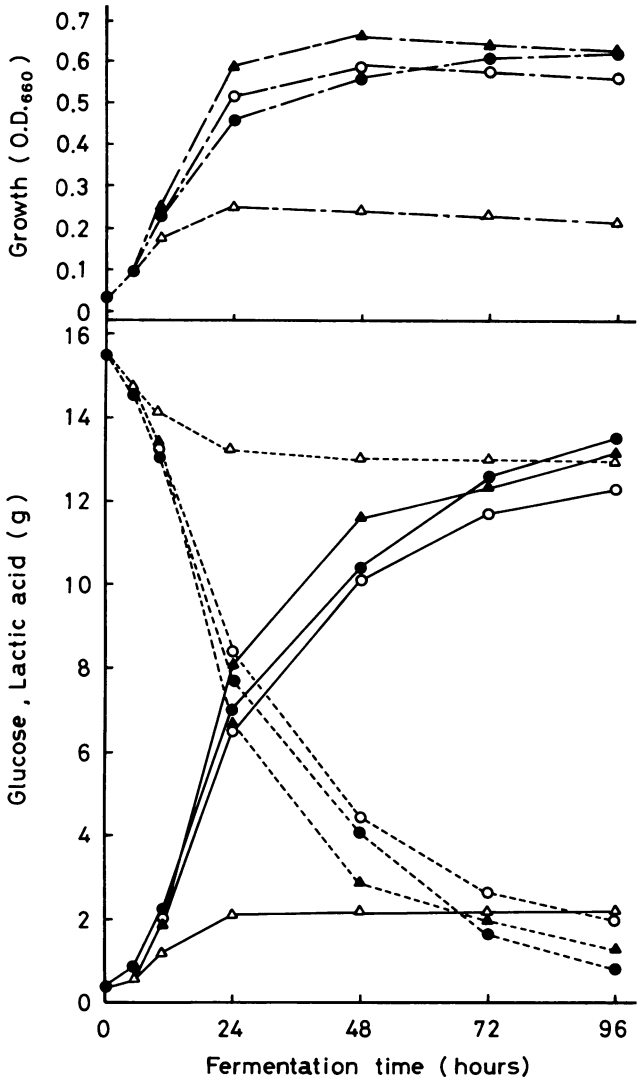

FIG. 4. Time course of lactic acid production by pH control with alkali. Fermentations were conducted in fermentation bottles that contained 150 ml of medium P at 45°C with gentle stirring. The pH value was controlled at 5.0 in all cases except for $CaCO_3$. ——, Lactic acid; -----, residual glucose; —·—, cell growth. Symbols: △, pH not controlled; ▲, pH controlled with 3 N NH_4OH; ○, pH controlled with 3 N NaOH; ●, pH controlled with $CaCO_3$. $O.D._{660}$, Optical density at 660 nm.

addition of an alkali solution. Produced lactic acid was neutralized with $CaCO_3$, 3 N NaOH, or 3 N NH_4OH. In the cases of NaOH and NH_4OH, the pH value was controlled at 5.0, which is almost the same as the pH value resulting from the addition of $CaCO_3$. The time course is shown in Fig. 4. The amount of lactic acid produced is revealed in total quantity, because the working volume is varied by the addition of alkali solution. The non-pH-controlled experiment produced 2.3 g of lactic acid per batch (about 14 mg/ml) in the first 24 h. The fastest rate of lactic acid production and the best cell growth among pH-controlled fermentations was obtained when the pH was controlled with NH_4OH. This observation was different from that in Fig. 3, because added NH_4OH may be utilized not only as a neutralizer but also as a nitrogen source. Consequently, the lactic acid production rate may be increased. After 96 h of fermentation, the amounts of lactic acid produced by the addition of $CaCO_3$, NH_4OH, or NaOH were 13.6, 13.2, or 12.2 g per batch, respectively. The yield per consumed glucose was about 91% in all cases, except for non-pH-controlled fermentation (about 80%).

Optimum pH for lactic acid production by *L. delbrueckii*. It may be more suitable to neutralize produced lactic acid with NH_4OH than with NaOH (Fig. 4). However, 3 N NaOH was selected, because NH_4OH may be utilized as a nitrogen source. The relationship between lactic acid production and controlled pH is shown in Fig. 5. When the pH was kept at 7.0, a long lag phase was observed, and the lactic acid production rate was slow. Keeping the pH at 6.5 also caused a slow lactic acid production rate. After 24 h, the fastest lactic acid production rate was obtained by the addition of $CaCO_3$. After 48 h, however, controlled fermentation at pH 5.5 gave good results. After 96 h, the amounts of lactic acid produced at pH values controlled at 7.0, 6.5, 6.0, 5.5, or 5.0

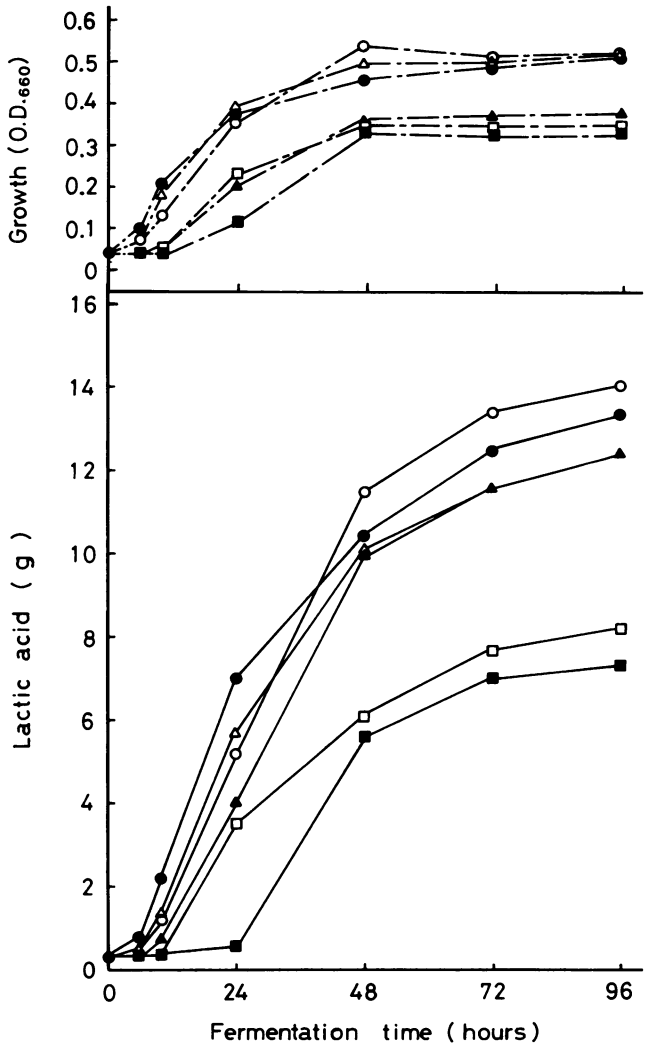

FIG. 5. Effect of pH controlled by the addition of 3 N NaOH on lactic acid production. Experimental conditions were the same as those in the legend to Fig. 4, except for the controlled-pH value. ——, Lactic acid; —·—, cell growth. Symbols: ■, pH 7.0; □, pH 6.5; ▲, pH 6.0; ○, pH 5.5; △, pH 5.0; ●, pH controlled with $CaCO_3$. $O.D._{660}$, Optical density at 660 nm.

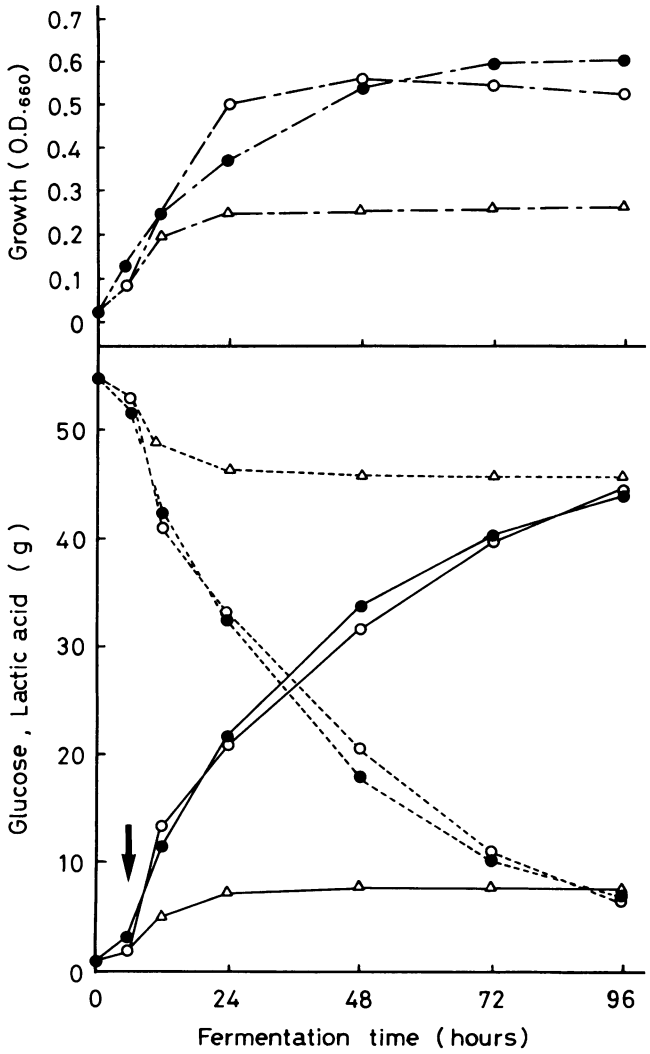

FIG. 6. Time course of lactic acid production in ED-F. Fermentations were conducted in 750-ml miniature fermentors that contained 500 ml of medium P at 45°C with gentle stirring. The pH value was controlled at 5.5. The arrow shows the start of electrodialysis. ——, Lactic acid; -----, residual glucose; —·—, cell growth. Symbols: △, pH not controlled; ●, pH controlled with $CaCO_3$; ○, electrodialysis. $O.D._{660}$, Optical density at 660 nm.

or by $CaCO_3$ were 7.4, 8.1, 12.3, 14.0, 12.3, or 13.4 g per batch, respectively. The yield per consumed glucose was about 91% in all cases. On the basis of these observations, the optimum pH value for lactic acid production by this microbe is about 5.5. Therefore, we determined that the pH in ED-F should be kept at 5.5.

Lactic acid production by the ED-F method. A miniature fermentor was used with the pH in ED-F set at 5.5. The time course of ED-F is shown in Fig. 6. The amount of produced lactic acid is shown as a total quantity, because electrodialysis generates the movement of fluid by electroosmosis of water (25). Actually, after 96 h, the volume of the fermentation broth decreased to 460 ml from 550 ml; conversely, that of the concentrated fluid increased to 630 ml from 500 ml. From 24 h, cell growth was not observed in non-pH-controlled fermentation, and the amount of lactic acid produced was only 14.8 g/liter. On the other hand, the lactic acid production activity of this bacterium in ED-F continued for a long time, as compared with that in non-pH-controlled fermentation. After 96 h, the amount of lactic acid in ED-F was 82.2 g/liter, which was about 5.5 times greater than that in non-pH-controlled fermentation. The productivity of ED-F (1.563 g/liter per h) until 24 h was three times higher than that of non-pH-controlled fermentation (0.513 g/liter per h). The yields per consumed glucose in ED-F and non-pH-controlled fermentation were 91.5 and 83%, respectively. As the lactic acid concentration in the fermentation broth of ED-F was maintained at only about 1% during the fermentation period (data not shown), it is concluded that the above-mentioned results were obtained on account of alleviating the lactic acid inhibitory effect by ED-F. In ED-F and conventional fermentation, produced lactic acid, productivity, and yield per consumed glucose were almost the same. In conventional fermentation, purification of lactic acid is very complicated (3), while in ED-F, lactic acid is almost purely recovered in the concentrated fluid. Moreover, in conventional fermentation, a higher substrate concentration cannot be used because the fermentation broth tends to solidify, owing to the low solubility of calcium lactate. On the other hand, this problem does not occur in ED-F. Therefore, it is expected that the ED-F method is a novel fermentation method which may supplant conventional fermentation.

In ED-F, microbial cells adhered to anion-exchange membranes. Therefore, in ED-F this phenomenon will be the limiting factor. Accordingly, studies to resolve this problem are in progress.

LITERATURE CITED

1. **Abbott, B. J., and P. Gerhardt.** 1970. Dialysis fermentation. I. Enhanced production of salicylic acid from naphthalene by *Pseudomonas fluorescens*. Biotechnol. Bioeng. **12**:577–589.
2. **Barker, S. B., and W. H. Summerson.** 1941. The colorimetric determination of lactic acid in biological materials. J. Biol. Chem. **138**:535–554.
3. **Buchta, K.** 1983. Lactic acid, p. 409–417. *In* H. Dellweg (ed.), Biotechnology, vol. 3. Verlag Chemie, Weinheim, Federal Republic of Germany.
4. **Coulman, G. A., R. W. Stieber, and P. Gerhardt.** 1977. Dialysis continuous process for ammonium-lactate fermentation of whey: mathematical model and computer simulation. Appl. Environ. Microbiol. **34**:725–732.
5. **Friedman, M. R., and E. L. Gaden, Jr.** 1970. Growth and acid production by *Lactobacillus* (*L.*) *delbrueckii* in a dialysis culture system. Biotechnol. Bioeng. **12**:961–974.
6. **Gallup, D. M., and P. Gerhardt.** 1963. Dialysis fermentor systems for concentrated culture of microorganisms. Appl. Microbiol. **11**:506–512.
7. **Gerhardt, P., and D. M. Gallup.** 1963. Dialysis flask for concentrated culture of microorganisms. J. Bacteriol. **86**:919–929.
8. **Gori, G. B.** 1965. Chemostatic concentrated cultures of heteroploid mammalian cell suspensions in dialyzing fermentors. I. Experimental evidence and theoretical considerations. Appl. Microbiol. **13**:93–98.
9. **Heller, H. C., and V. Markovac.** 1983. Identification of a membrane foulant in the electrodialytic recovery of nickel. Anal. Chem. **55**:551A–554A.
10. **Hongo, M., and M. Iwahara.** 1979. Application of electro-energizing method to L-glutamic acid fermentation. Agric. Biol. Chem. **43**:2075–2081.
11. **Hongo, M., and M. Iwahara.** 1979. Determination of electro-energizing conditions for L-glutamic acid fermentation. Agric. Biol. Chem. **43**:2083–2086.
12. **Iwahara, M., and M. Hongo.** 1981. Promotion of reductive amination of α-ketoglutarate to L-glutamate in *Brevibacterium flavum* No. 2247 under electro-energizing conditions. Agric.

Biol. Chem. **45**:2365–2367.
13. **Iwahara, M., and M. Hongo.** 1982. Effect of electro-energizing conditions on cellular permeability toward L-glutamic acid. Agric. Biol. Chem. **46**:183–189.
14. **Keller, A. K., and P. Gerhardt.** 1975. Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. **17**:997–1018.
15. **Longsworth, L. G., and D. A. MacInnes.** 1935. Bacterial growth with automatic pH control. J. Bacteriol. **29**:595–607.
16. **Markovac, V., and H. C. Heller.** 1981. Principles of electrodialysis for nickel-plating rinsewater. Plating Surf. Fin. **68**:66–69.
17. **Markovac, V., and H. C. Heller.** 1982. Engineering aspects of electrodialysis for nickel plating rinsewater. Plating Surf. Finishing **69**:84–87.
18. **Rogers, L. A., and E. O. Whittier.** 1928. Limiting factors in the lactic fermentation. J. Bacteriol. **16**:211–229.
19. **Roy, T. B. V., H. W. Blanch, and C. R. Wilke.** 1982. Lactic acid production by *Lactobacillus delbrueckii* in a hollow fiber fermentor. Biotechnol. Lett. **4**:483–488.
20. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.
21. **Stieber, R. W., G. A. Coulman, and P. Gerhardt.** 1977. Dialysis continuous process for ammonium-lactate fermentation of whey: experimental tests. Appl. Environ. Microbiol. **34**: 733–739.
22. **Stieber, R. W., and P. Gerhardt.** 1979. Dialysis continuous process for ammonium-lactate fermentation: improved mathematical model and use of deproteinized whey. Appl. Environ. Microbiol. **37**:487–495.
23. **Stieber, R. W., and P. Gerhardt.** 1981. Dialysis continuous process for ammonium lactate fermentation: simulated prefermentor and cell-recycling systems. Biotechnol. Bioeng. **23**:523–534.
24. **Stieber, R. W., and P. Gerhardt.** 1981. Dialysis continuous process for ammonium lactate fermentation: simulated and experimental dialysate-feed, immobilized-cell systems. Biotechnol. Bioeng. **23**:535–549.
25. **Yawataya, T.** 1982. Ion exchange membranes, p. 27 and 120. Kyoritsu Shuppan Co., Ltd., Tokyo.