0273-2289/90/2425—0755$02.00

Continuous Acetone–Butanol Fermentation

The Phenomenon of Cell Adhesion on a Glass Reactor

B. K. SONI

Departement de Genie Biochimique et Alimentaire, INSA, 31077 Avenue de Rangueil, Toulouse Cedex, France; Present address: Michigan Biotechnology Institute, 3900 Collins Road, Lansing, MI 48909

ABSTRACT

Continuous acetone–butanol fermentation was investigated for solvent production. After approximately 11–15 d of continuous operation, the nutrient composition was changed, and an alteration in cell morphology was observed. This led to the aggregation of cells, followed by cell adhesion on the glass reactor. Under these conditions of continuous operation, there was a many-fold increase in solvent productvity. A calculated solvent productivity of 4.1 g/L/h could be attained under these conditions. The continuous operation could be sustained for more than 250 reactor vols.

Index Entries: *Clostridium acetobutylicum;* cell adhesion; aggregation; chemostat culture; dilution rate.

INTRODUCTION

In recent years, much attention has been focused on continuous acetone–butanol fermentation with cell-recycle systems (*1–6*). Although it appears very attractive for solvent productivity (ranging from 3.0 to 6.5 g/L/h), it is associated with increased operational problems, such as membrane plugging and energy-intensive broth pumping through the membrane. These problems are compounded on scale-up. Moreover, the increase in biomass concentration may not give a proportional increase in solvent productivity, because of diffusional limitations or retarded metabolic activity. As an alternative, immobilized-cell-reactor systems can be used to improve solvent productivity. Acetone–butanol fermentation has

been studied extensively by various researchers using immobilized-cell reactor systems (*7–15*).

Recently, significant improvements in solvent productivity have been achieved in continuous fermentation on both synthetic and complex media (*16–20*). Solvent productivity of 2.54 g/L/h was achieved using synthetic media (*20*). However, one of the limitations of this process was long-term stability. The active solvent production lasted for only about 55–70 reactor vols. The use of nutrient limitation is one proposed approach to improving the stability of chemostat cultures. It was noted that phosphate, potassium, or nitrogen limitation might be used for improved stability of chemostat cultures (*21–23*). However, this improvement would be associated with low solvent productivity.

From the foregoing discussion, it appears that a different approach might be required for a further increase in solvent productivity with improved stability. The emphasis of the current investigations is to modify the nutrient conditions in such a way that cells could be adhered on the glass reactor used for continuous operation. This adsorption by electrostatic interaction, reviewed in the literature (*24*), has been successfully applied for ethanol fermentation with improved productivity.

MATERIALS AND METHODS

Organism and growth conditions: *Clostridium acetobutylicum* ATCC 824 was used throughout these studies. The medium used in these investigations contained the following per liter of water: glucose·H_2O, 51.7 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NH_4Cl, 1.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; *p*-aminobenzoic acid, 8 mg; and biotin, 0.04 mg. However, the concentrations of $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ were varied. In addition, calcium and zinc were also supplemented in some studies, as described. Silicone (100 ppm) was also added to the feed vessel to control the foam in the reactor. The continuous fermentor set-up was the same as described earlier (*17*). A schematic diagram of the continuous operation is shown in Fig. 1. The fermentor was operated at 35°C with pH controlled at 4.4 by the addition of NH_4OH. The enzymatic glucose assay and analyses of liquid samples for solvents and acids were carried out by the method described earlier (*1*).

RESULTS AND DISCUSSION

Influence of Increased Concentration of Mg^{2+} on Performance of Chemostat Cultures

The current investigations have been examined in view of the development of chemostat cultures with improved solvent productivity and stability. The normal chemostat-culture experiments could last for only

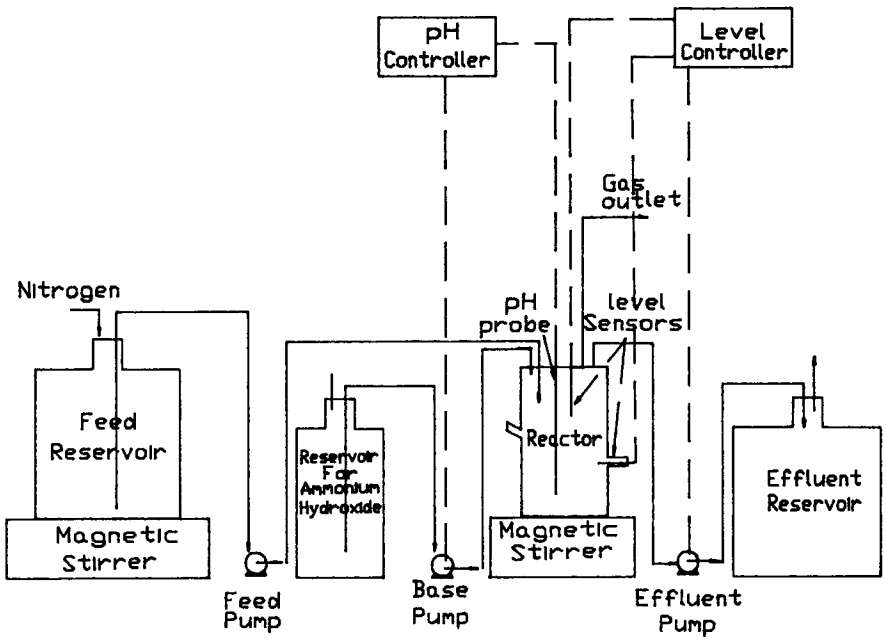

Fig. 1. Schematic diagram for continuous reactor system.

Table 1
Influence of Increased Concentration of Magnesium on the Performance of Chemostat Cultures Growing at the Fixed Dilution Rate of $0.229 \pm 0.005\ h^{-1}$[a]

Day	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
14	1.7	0.38	0.27	2.26
15	6.9	1.54	1.03	5.21
16	8.7	2.04	1.36	6.26
17	9.3	2.13	1.47	6.64
18	9.0	2.05	1.39	6.33
19	8.6	1.97	1.33	6.18
20	9.1	2.08	1.42	6.52

[a]Magnesium concentration was increased from 0.8m*M* to 8m*M* on the fourteenth day.

about 11–15 d at the dilution rate of $0.2 \pm 0.02\ h^{-1}$. Thereafter, degeneration start, resulting in the total loss of solvent-producing capabilities and a change in the acid type of fermentation. In the initial experiments, the concentration of Mg^{2+} was increased to 8m*M* (in the feed vessel) for the continuous culture on the fourteenth day. As a consequence, cells started aggregating, and fewer cells adhered on the glass reactor on the fifteenth day. The results (Table 1) indicated a significant improvement in solvent

Table 2
Influence of Different Concentrations of Magnesium on the Performance of Chemostat Cultures Growing at the Fixed Dilution Rate of $0.229 \pm 0.005\ h^{-1}$[a]

Day	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
21	8.5	1.92	1.33	6.39
22	8.7	1.99	1.40	6.52
23	8.6	1.99	1.39	6.58
24	8.4	1.91	1.34	6.47
25	8.5	1.97	1.39	6.47
26	7.3	1.66	1.11	5.61
27	6.4	1.43	0.87	4.37
28	5.6	1.25	0.76	4.00
29	8.7	1.98	1.32	6.30
30	7.8	1.76	1.08	5.20
31	7.0	1.59	1.04	4.88

[a]Magnesium concentration was increased to 12m*M* on the twentieth day and to 16m*M* on the twenty-fourth day. Finally, on the twenty-eighth day, it was reduced to the initial level of 0.8m*M*.

productivity. The chemostat culture continued to operate under this increased concentration until the twentieth day. During this period, the global solvent production and glucose consumption altered significantly, as a fivefold increase in solvent production rate was noted and a threefold increase in the glucose consumption rate was recorded. Interestingly, the butyrate concentration remained at 1.0 ± 0.1 g/L. This indicated that cells were not of the degenerating type. Another feature of this culture was an increase in the butanol-to-acetone ratio to 3.0 ± 0.2 from 2.0 ± 0.2, as a result of sudden increased magnesium concentration. This indicated that the physiological change of cell aggregation was associated with the change in electron flow pattern for higher butanol production.

To clarify the role of magnesium, the concentration was further altered on the twentieth day, from 8m*M* to 12m*M*. The solvent production and glucose consumption pattern remained unaltered (Table 2). However, a further increase in Mg^{2+} concentration, to 16m*M* (Table 2), led to significant inhibition of solvent production and glucose consumption. A 30% decrease in the rate of solvent production was observed on the twenty-eighth day (compared to the rate before increasing the concentration of Mg^{2+} on the twenty-fourth day). Cell aggregation and adhesion were also reduced signficantly during this period. The feed was changed to normal conditions (i.e., 0.8 m*M* of Mg^{2+}). The results (Table 2) indicated that, although the global activity increased initially on the twenty-ninth day, it could not be continued for an extended period for high production of sol-

Table 3
Influence of Different Concentrations of Magnesium and Zinc on the Performance of Chemostat Cultures of *Clostridium acetobutylicum* Growing at the Fixed Dilution Rate of $0.37 \pm 0.03\ h^{-1}$[a]

Day	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
14	1.5	0.50	0.31	3.47
15	2.2	0.82	0.56	4.93
16	6.8	2.65	1.72	8.75
17	5.6	2.24	1.48	7.80
18	6.8	2.72	1.72	8.80

[a]The chemostat was started at the high concentration of magnesium (2.5m*M*). On the fourteenth day, magnesium was replaced by zinc (1.7m*M*). However, magnesium was also supplemented in the medium at a concentration of 0.8m*M*. On the sixteenth day, the feed medium was again switched over to the earlier concentration of magnesium (2.5m*M*).

vents. This experiment was continued until the fiftieth day. Total solvents could still be produced at the concentration of 3.5 ± 0.2 g/L. The increased concentration of Mg^{2+} was used in the present investigations because earlier studies showed significant improvement in the solvent production rate at an increased concentration of divalent cations, such as Mg^{2+}, Ca^{2+} Zn^{2+}, and Fe^{2+} (*25*). These results indicated that cell adhesion could be induced by the sudden increased concentration of divalent cations; however, the reasons for this were not clear.

Influence of Zn^{2+} on the Performance of Chemostat Cultures

A new chemostat-culture experiment was started to confirm the reproducibility of cell aggregation and adhesion. The feed medium was supplemented with a comparatively higher concentration (2.5m*M*) of Mg^{2+}. The chemostat cultures were operated initially at the dilution rate of $0.34\ h^{-1}$. There was no aggregation of cells until the thirteenth day. The feed was modulated by supplementing it with Zn^{2+} at 1.7m*M*. However, Mg^{2+} was also added at 0.8m*M* because of nutritional requirements for different metabolic functions. Once again the cell responded to this sudden change, because the aggregation was noticed on the fifteenth day, followed by adhesion on the glass surface. As shown in Table 3, solvent production and glucose consumption rates improved significantly.

After continuing with Zn^{2+} for 48 h, the feed pattern was again changed to preexisting conditions (i.e., 2.5m*M* of Mg^{2+}). The results indicated no specific alteration of the global performance for solvent production and glucose consumption. The feed pattern was continued for another 48 h

Table 4
Influence of Calcium on the Chemostat Culture of *Clostridium acetobutylicum* Growing at the Fixed Dilution Rate of $0.40 \pm 0.01\ h^{-1}$[a]

Time, h	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
24	7.2	2.95	2.05	10.15
36	7.4	2.89	2.00	10.04
48	7.6	3.04	2.08	10.60
60	7.5	2.92	1.99	10.10
72	7.5	2.93	1.99	10.02

[a] Calcium was added as calcium chloride on the eighteenth day and the time-course was followed until the twenty-first day.

with Mg^{2+} (Table 3). High solvent productivity could be achieved under these conditions.

Effects of Ca^{2+} and Fe^{2+} on the Performance of Chemostat Cultures

Because Ca^{2+} and Fe^{2+} showed favorable effects on solvent production, the present investigations were continued with these divalent cations. The feed medium with a high concentration of Ca^{2+} (1.7m*M*) was started on the eighteenth day and continued until the twenty-first day. Solvent production rates of 2.95 ± 0.1 g/L/h could be attained under these conditions (Table 4). These values were about sixfold higher than those at the beginning of the changeover (i.e., on the fourteenth day). The feed pattern was changed on the twenty-first day to a high concentration of Fe^{2+}, and this was continued until the twenty-fourth day. The results (Table 5) indicated that high solvent-production and glucose-consumption rates could still be maintained. From these results, it can be inferred that an appropriate concentration of divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}) could be used to attain and maintain cell aggregation for long durations without any destabilization of global performance.

However, to verify the necessity of a high concentration of divalent cations for maintaining high solvent productivity, the feed pattern was changed again on the twenty-fourth day to a normal medium (i.e., 0.8m*M* of Mg^{2+}) and continued until the thirtieth day under these conditions. It was very interesting to note that high solvent-production rates (4.1 g/L/h) could be maintained (Table 6). The concentration of butyric acid remained at 0.7 ± 0.2 g/L throughout these investigations. This low value again indicated that there was no cell degeneration. Because it was still not clear if the divalent ions were essential for cell aggregation, the chemostat-culture experiment was restarted. The above results were confirmed in

Table 5
Influence of Iron on the Chemostat Culture of *Clostridium acetobutylicum* Growing at the Fixed Dilution Rate of $0.423 \pm 0.004\ h^{-1}$[a]

Time, h	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
24	6.6	2.80	1.94	9.06
36	6.8	2.90	1.96	9.48
48	6.6	2.80	1.90	9.48
60	6.5	2.77	1.92	9.35
72	6.5	2.77	1.93	9.24

[a]Iron was added at a concentration of 1.7m*M* on the twenty-first day, and the time-course was continued until the twenty-fourth day.

Table 6
Influence of Normal Medium Condition on the Chemstat Culture of *Clostridium acetobutylicum* Growing at the Fixed Dilution Rate of $0.41 \pm 0.02\ h^{-1}$[a]

Time, h	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
24	7.2	2.96	2.02	10.10
48	8.4	3.60	2.49	11.10
72	9.2	3.96	2.84	12.20
96	9.8	4.03	2.80	13.00
120	10.6	4.14	2.89	13.50
144	10.2	4.10	2.86	13.10

[a]The culture was previously operated at a high concentration of divalent cations; on the twenty-fourth day, normal feed medium was started, and the time-course was followed until the thirtieth day.

another independent experiment (Table 7). From these results, it can be inferred that, although higher concentrations of divalent cations are not necessary for cell aggregation (Tables 6 and 7), these higher concentrations will allow the stable operation of fermentation for longer durations.

A comparison of our results with the literature (Table 8) indicated that solvent productivity similar to that of cell-recycle systems could be achieved by cell adhesion. Another interesting observation was the increase in stability. Further investigation of these findings with the goal of developing a stable aggregating culture could lead to a breakthrough in acetone-butanol fermentation. Based on a multistage continuous process, a recent economic analysis indicated that the solvent production is

Table 7
Influence of High Dilution Rates
on the Performance of Chemostat Cultures of *Clostridium acetobutylicum*[a]

Time, h	Total solvents, g/L	Solvent productivity, g/L/h	Butanol productivity, g/L/h	Glucose consumption rate, g/L/h
0	0.5	0.28	0.11	2.58
12	0.9	0.55	0.31	4.21
24	3.1	1.83	1.24	8.00
36	6.2	3.70	2.49	12.40

[a]The chemostat was started at the dilution rate of 0.20 h^{-} during 10 d. On the tenth day, dilution was further increased to 0.60 ± 0.01 h, and the time-course was followed, considering this increase as time zero.

Table 8
Comparison of Performances of Continuous Fermentation
Under the Condition of Maximum Solvent Productivity
Achieved by Different Groups Using *Clostridium acetobutylicum*

	Largier et al., 1985	Frick and Schugerl, 1986	Asfchar et al., 1985	Ennis and Maddox, 1989	Current work
Fermentation system	Two-stage immobilized cells	Two-stage immobilized cells	Two-stage cell recycle	Single stage cell recycle	Single-stage continuous system
Total solvents, g/L	15.4	4.0	7.0	7.1	10.6
Butanol productivity, g/L/h	1.6	-	-	-	2.9
Solvent productivity, g/L/h	3.0	4.0	4.5	2.9	4.1
References	*10*	*13*	*2*	*4*	-

again very close to chemical routes (*26*). It was noted that the rational price of butanol ranges from \$0.276 to 0.357/lb for fermentation-based process, whereas the price ranges from \$0.269 to 0.312/lb for petrochemical processes. The current investigation has the potential to further reduce the cost of fermentation routes. However, the implementation of the fermentation process would depend on the demand for these solvents and the availability of cheap raw materials.

CONCLUSIONS

Several novel conclusions can be drawn from our investigations: First, although continuous cultures are associated with low stability, this stability can be improved by nutrient limitation at the reduced solvent productivity. However, if a sudden change is introduced in the continuous process, cell aggregation could be obtained, followed by adhesion on the glass reactor. In addition, appropriate concentrations of divalent cations could be added to the feed medium to improve the stability of chemostat cultures. The continuous operation could be sustained for more than 250 reactor vols without any loss of culture activity. The electron flow could be altered by aggregating cells for high production of butanol. A butanol-to-acetone ratio of 3:1 was noted. Finally, a high solvent productivity of 4.1 g/L/h could be attained and maintained for long continuous operation.

REFERENCES

1. Ferras, E., Minier, M., and Goma, G. (1986), *Biotechnol. Bioeng.* **28,** 523.
2. Afschar, A. S., Biebl, H., Schaller, K., and Schugerl, K. (1985), *Appl. Microbiol. Biotechnol.* **22,** 394.
3. Pierrot, P., Fick, M., and Engasser, J. M. (1986), *Biotechnol. Lett.* **8,** 253.
4. Ennis, B. M. and Maddox, I. S. (1989), *Bioprocess Eng.* **4,** 27.
5. Meyer, C. L. and Papoutsakis, E. T. (1989), *Bioprocess Eng.* **4,** 1.
6. Meyer, C. L. and Papoutsakis, E. T. (1989), *Bioprocess Eng.* **4,** 49.
7. Krouwel, P. G., Groot, W. J., Van der Laan, W. F. M., and Kossen, N. W. F. (1983), *Enz. Microbiol. Technol.* **5,** 46.
8. Kolot, F. B. (1984), *Process Biochem.* **19,** 7.
9. Krouwel, P. G., Groot, W. J., and Kossen, N. W. F. (1983), *Biotechnol. Bioeng.* **25,** 281.
10. Largier, S. T., Long, S., Santangelo, J. D., Jones, D. and Woods, D. R. (1985), *Appl. Environ. Microbiol.* **50,** 487.
11. Ennis, B. M., Maddox, I. S., and Schoutens, G. H. (1986), *New Zealand Dairy Sci. Technol.* **21,** 99.
12. Schoutens, G. H., Nieuwenhuizen, M. C. H., and Kossen, N. W. F. (1986), *Appl. Microbiol. Biotechnol.* **21,** 282.
13. Frick, C. and Schugerl, K. (1986), *Appl. Microbiol. Biotechnol.* **25,** 186.
14. Quereshi, N. and Maddox, I. S. (1987), *Enz. Microbiol. Technol.* **9,** 668.
15. Forberg, C. and Haggstrom, L. (1985), *Enz. Microbiol. Technol.* **7,** 230.
16. Leung, J. C. Y. and Wang, D. I. C. (1981), *2nd World Congress of Chem. Eng.*, October 4–9, Montreal, pp. 348.
17. Soni, B. K., Das, K., Soucaille, P., and Goma, G. (1986), *Biotechnol. Bioeng. Symp.* **17,** 591.
18. Soni, B. K., Soucaille, P., and Goma, G. (1986), *Biotechnol. Bioeng. Symp.* **17,** 239.
19. Soni, B. K., Soucaille, P., and Goma, G. (1987), *Appl. Microbiol. Biotechnol.* **23,** 317.

20. Soni, B. K., Soucaille, P., and Goma, G. (1987), *Appl. Microbiol. Biotechnol.* **27,** 1.
21. Bahl, H., Andersch, W., and Gottchalk, G. (1982), *Eur. J. Appl. Microbiol. Biotechnol.* **15,** 201.
22. Soni, B. K. and Soucaille, P. (1988), *8th International Biotechnol. Symposium,* July 17–22, Paris.
23. Barbeau, J. Y., Marchal, R., and Vandecasteele, (1988), *Appl. Microbiol. Biotechnol.* **29,** 447.
24. Godia, F., Casas, C., and Sola, C. (1987), *Process Biochem.* **22,** 43.
25. Soni, B. K., Soucaille, P., and Goma, G. (1987), *4th European Congress on Biotechnol.,* June 14–19, Amsterdam, pp. 335.
26. Marlatt, J. A., and Datta, R. (1986), *Biotechnol. Prog.* **2,** 23.