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2,3-Butanediol Production from Hydrolyzed Whey Permeate by Immobilized Cells of *Bacillus polymyxa*

Scientific Note

SARAH B. MARTINEZ* AND RAY A. SPECKMAN

Department of Food Science, University of Illinois, Urbana, IL 61801

Index Entries: *Bacillus polymyxa*; immobilized cells; hydrolyzed whey permeate; 2,3-butanediol; acetoin.

INTRODUCTION

The majority of feedstock chemicals are currently supplied by the petrochemical industry. However, the need for alternate syntheses of these compounds has brought about a lot of interest in the production of these compounds by fermentation of renewable resources such as wastes from food and other agriculturally related industries.

One potentially valuable chemical feedstock is 2,3-butanediol. Its dehydration yields the common industrial solvent, methyl ethyl ketone. Compared to ethanol, methyl ethyl ketone has a higher heat of combustion. It is also a more effective liquid fuel additive than ethanol since it gives a higher octane number when mixed (25%) with gasoline (*1*). 2,3-Butanediol can also be converted to 1,3-butadiene, a monomer used in the production of synthetic rubber, plastics and other consumer oriented polymers. The L-form of the butanediol also has potential use as a substitute for ethylene glycol as an antifreeze (*2*).

There have been a number of attempts in the last few decades of producing 2,3-butanediol by fermentation of different substrates. *Enterobacter aerogenes* was shown to produce this compound from citrus wastes (*3*). *Klebsiella pneumoniae* was able to utilize wood hydrolysates as sub-

*Author to whom all correspondence and reprint requests should be addressed.

strates for 2,3-butanediol production (*4*). Another renewable resource of interest is whey. The first report of whey as a medium for 2,3-butanediol production showed that *Bacillus polymyxa* was a microbe with industrial potential due to its fermentative capabilities, its nonpathogenicity and its genetic stability (*5*). Increased butanediol yields by this organism when hydrolyzed whey was used has also been reported (*6*).

Numerous studies have reported the advantages of using immobilized cell systems over free cells in fermentations (*7*). Cells of *Enterobacter* sp. immobilized in carrageenan or calcium alginate have been shown to produce 2,3-butanediol from glucose or xylose, respectively (*8,9*). A technique has also been described using titanium hydroxide as a matrix for immobilizing *Aeromonas hydrophila* for 2,3-butanediol production from starch (*10*). More recently, immobilized cells of *Klebsiella pneumoniae* were reported to show good productivity using whey permeate as substrate (*11*). The purpose of this study was to compare 2,3-butanediol production by free cells with immobilized cells of *B. polymyxa* using hydrolyzed whey permeate as a growth medium.

METHODS

Microorganism and Culture Conditions

A mutant of *Bacillus polymyxa* ATCC 12321, designated as AlsR$^-$ #11-9-1, was used. This mutant, isolated from a series of spontaneous and nitrosoguanidine-induced mutations, was shown to produce acetoin and 2,3-butanediol earlier and in greater quantities than the wild type strain (*12*). The cells were maintained on brain heart infusion agar slants (Difco). Inocula were prepared by transferring a loopful of cells from the slant into a hydrolyzed whey permeate solution and incubating with agitation at 35°C for 12 h.

To concentrate the cells for immobilization, a biphasic medium of hydrolyzed whey permeate was used. In this system, the culture is confined to a small volume of liquid overlay but has diffusional access to the nutrients in the solidified base and consequently becomes densely concentrated (*13*).

All fermentations were conducted in duplicate shake flasks at 35°C unless otherwise indicated. The ratio of immobilized cell beads to substrate was 1:10 (wt%). For comparison with free cells, the equivalent weight of cells in the immobilized beads were suspended in the same volume of substrate.

Substrate

The substrate used was whey permeate powder (Express Foods Co. Inc., Louisville, KY) reconstituted to 50 g/L lactose, unless otherwise in-

dicated. The whey permeate solution was hydrolyzed with Novo Lactozym 3000L Type HP enzyme (Novo Laboratories, Inc., Wilton, CT) at a dose of 3.5 ml/L at 40°C for 1 h. The pH of this solution is approximately 6.5.

Immobilization

B. polymxa cells were immobilized in calcium alginate beads. Unless otherwise indicated, a cell loading of 14% (g cell dry wt/g alginate) was used. The cells were harvested by centrifugation at 12,000 × g for 15 min and resuspended in a small volume of saline solution and mixed with a sodium alginate solution to give a final alginate concentration of 4%. The alginate-cell slurry was then extruded as drops through a syringe into a 0.15 M $CaCl_2$ solution resulting in beads of about 3 mm in diameter. The beads were incubated in this solution for 1 h at room temperature after which they were rinsed with sterile distilled water prior to use.

Analytical Methods

The fermentation products of interest were 2,3-butanediol and its oxidized form, acetoin. 2,3-Butanediol was determined by the method of Desnuelle and Naudet (*14*) and acetoin by the method of Westerfeld (*15*). Diacetyl and acetaldehyde, two compounds associated with this fermentative pathway, were found not to occur in significant quantities.

Glucose was determined by the hexokinase method using a Sigma diagnostic kit (Product no. 115). Galactose was determined by UV spectrophotometry using a Boehringer Mannheim test kit for lactose and galactose.

Cell concentrations were determined by comparing the optical density (OD) of a solution to a standard curve of cell dry weight and OD at 420 nm. Cell dry weights were determined by harvesting the cells by centrifugation at 12,000 × g for 15 min. The cell pellet was washed twice with buffered saline solution and then dried to constant weight in a 105°C air oven.

Scanning Electron Microscopy

The immobilized cell beads were razor cut to reveal the interior and then fixed with 4% glutaraldehyde for 4 h. This was followed by overnight post-fixation with cold 1% osmium tetroxide solution. Following stepwise dehydration with increasing concentrations of ethanol, the specimen was critical point dried with liquid CO_2. After sputter coating with a thin layer of gold and palladium, the beads were observed in an ISI-40 scanning electron microscope.

RESULTS AND DISCUSSION

Effect of Cell Loading

Immobilized cell beads were prepared with varying cell concentrations to determine the effect of cell density on 2,3-butanediol production. Calcium alginate beads were prepared with cell densities of 1.5, 5.0, 8.0, 14.0, and 20.0% (g cell dry wt/g alginate). The fermentations were conducted with sequential replacement of substrate every 24 h.

As shown in Fig. 1, 2,3-butanediol production increased during the first few hours with the most marked increase at the two lowest cell loadings. The comparable product concentrations obtained for the wide range of cell loadings indicate that diffusional resistance through the bead may not be that critical up to a certain point. Physical studies on calcium alginate gels report that, at least for low molecular weight substrates and products, diffusion in and out of these highly hydrated gels progresses quite easily (*16,17*). As such, higher cell concentrations result in higher product concentrations.

Analysis of variance of duplicate runs showed that the cell loadings of 14 and 20% resulted in significantly better 2,3-butanediol production. However, it can be seen that the one with 20% loading consistently pro-

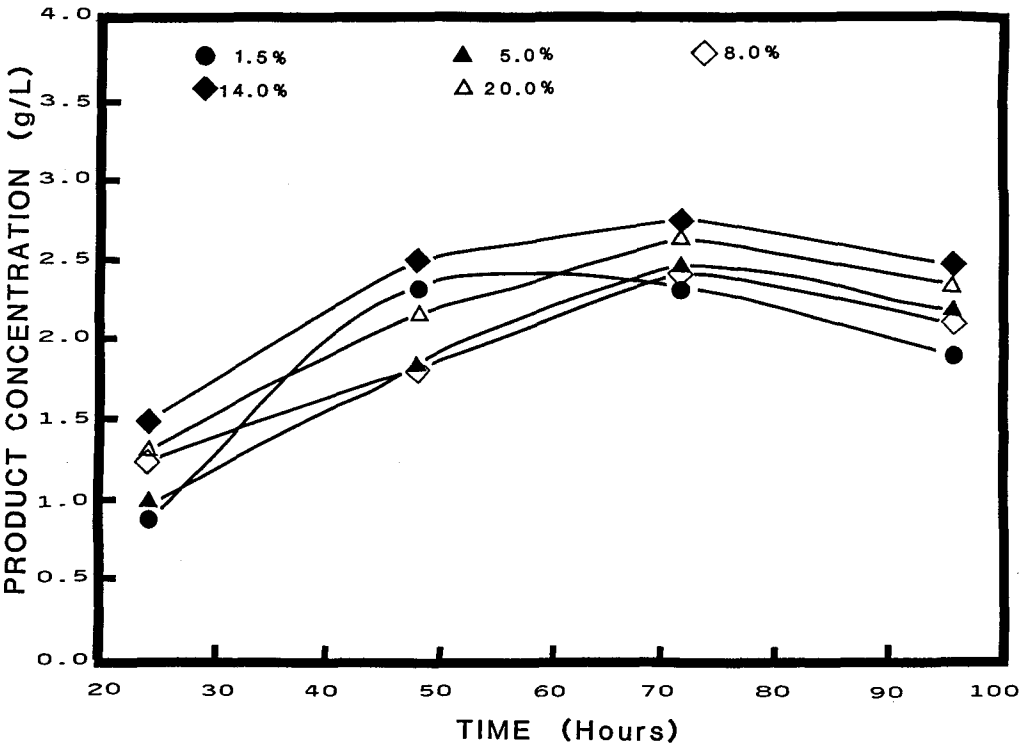

Fig. 1. 2,3-Butanediol production by immobilized cells of *B. polymyxa* at different cell loadings. Cell loading is expressed in g cell dry wt/g of alginate.

duced a lesser amount of 2,3-butanediol than did the one with 14% loading. This could indicate that at higher cell densities, the porous structure of the gel decreases and diffusional restrictions become more evident. With regard to this, an important kinetic parameter to be considered in immobilized cell systems is the effectiveness factor which is the ratio of the observed reaction rate to that achieved in the absence of diffusional limitations. Microscopic observation of the beads shows very dense growth of the bacteria within the calcium alginate matrix (Fig. 2).

Effect of Temperature

2,3-Butanediol production by free and immobilized cells was determined at different incubation temperatures, namely 25, 30, 35, and 40°C. This experiment was done because differences in the optimum temperatures for free and immobilized cells are known to exist such as in the production of ethanol by yeast (*18*).

Figure 3 shows that the trend in butanediol production was similar for both the free and immobilized cells, the optimum being 35°C. At

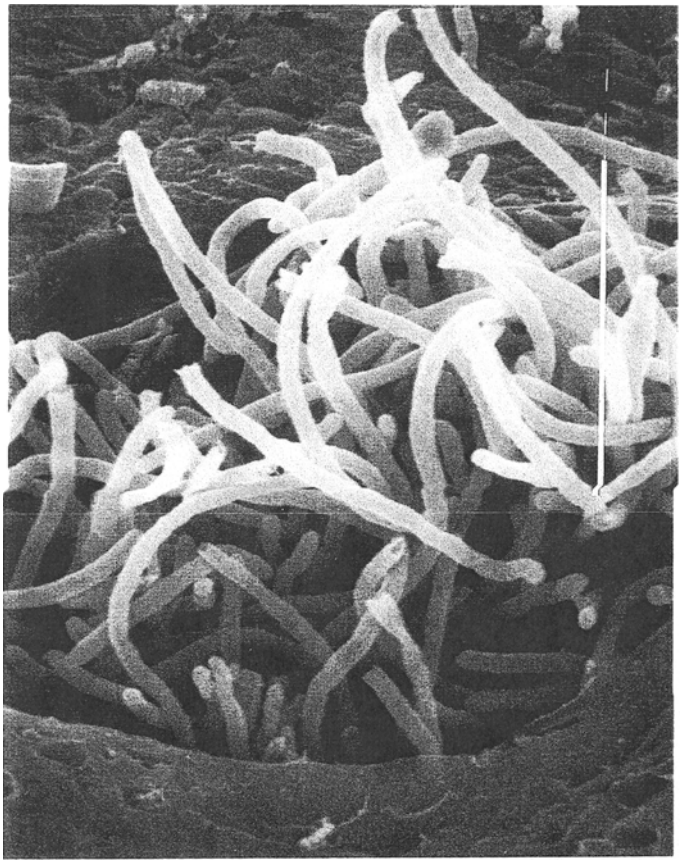

Fig. 2. Scanning electron micrograph of immobilized cells of *Bacillus polymyxa*. Line scale equals 10 μm.

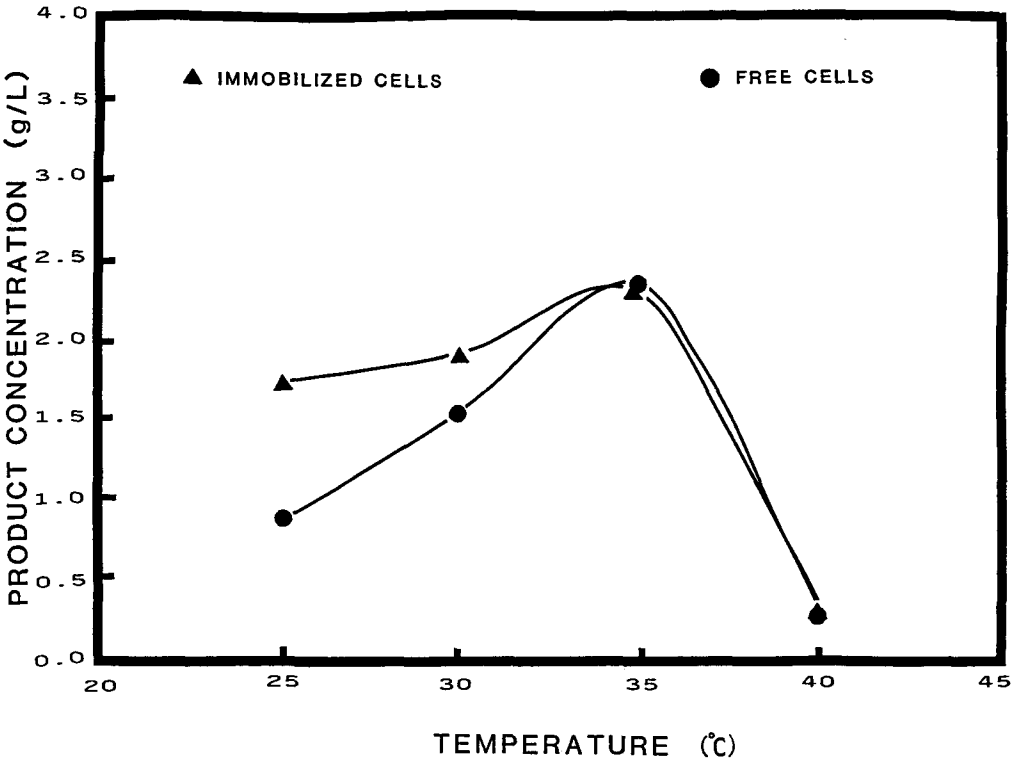

Fig. 3. Effect of incubation temperature on 2,3-butanediol production. Results were obtained after 72 h of fermentation.

40°C, there was considerable reduction in 2,3-butanediol concentrations. This could be directly related to the effect of temperature on the growth of the cells. In a study of 2,3-butanediol production by *Aerobacter aerogenes*, the growth rate was shown to fall to nearly zero at temperatures above 37°C (*19*). Slightly higher product concentrations were obtained for the immobilized cells.

Effect of pH

The effect of the pH of the substrate on 2,3-butanediol production was also determined. As with temperature, the same trend was observed for both free and immobilized cells, with product concentrations increasing with increasing pH (Fig. 4). The higher level of product concentration in the immobilized system may be attributed to a pH gradient formed within the bead. In the microenvironment of the immobilized cell system, partitioning effects may occur that cause shifts in pH as a result of the negative charge on the bacterial cell (*20*). One would reason that these interactions would be greater with the available hydrogen ions than with the uncharged substrate and product molecules.

Although the improved product concentrations at lower pH for the immobilized cell system is not great enough to justify its use in acid

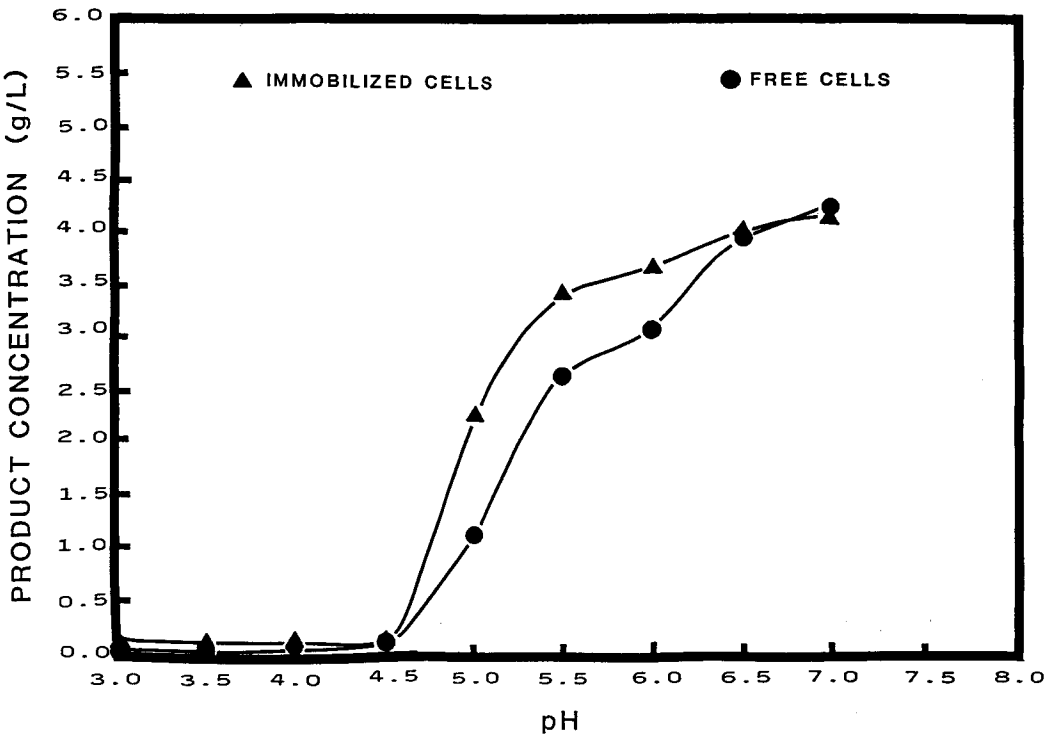

Fig. 4. Effect of pH of substrate on 2,3-butanediol production. Results were obtained after 96 h of fermentation.

whey, this property could offer some protective effect to the cells during increased acid production during fermentation and could possibly extend the fermentation life.

Effect of Agitation

There are numerous conflicting reports in the literature on the effect of aeration on 2,3-butanediol production by free cells of *Bacillus polymyxa* (*21*). We have not found a study that considered the effects of aeration on the production of 2,3-butanediol by immobilized cells of *Bacillus polymyxa*. Therefore, free and immobilized cells were incubated under quiescent and agitated conditions to determine the effects of aeration on 2,3-
butanediol production. The agitated free and immobilized cells resulted in higher product concentrations and had higher yields than the static cultures (Fig. 5). Although it is generally known that a certain degree of aeration is required for the initial stages of the fermentation, it has been reported that aeration also favors the production of biomass at the expense of 2,3-butanediol (22). Results opposite to those obtained would then be expected. It should be noted, however, that under more anaerobic conditions, there is a greater buildup of acetate in the cells. Although acetate is known to induce the enzymes associated with 2,3-butanediol

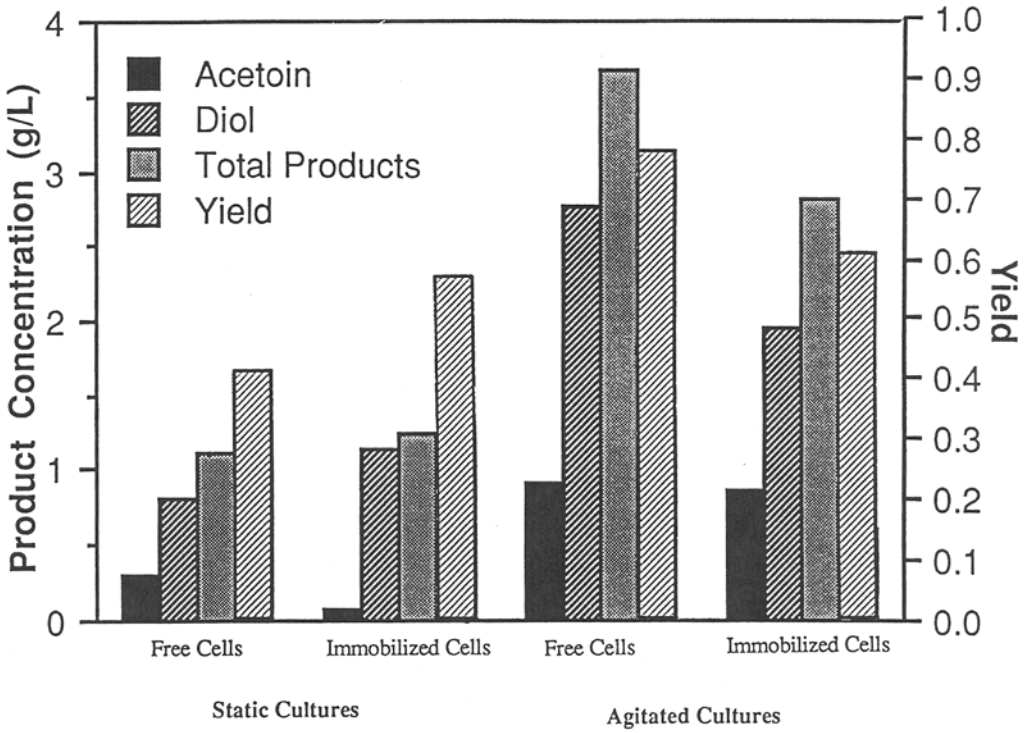

Fig. 5. 2,3-Butanediol concentration and yield of free and immobilized cells incubated in static and agitated cultures. Results were obtained after 72 h of fermentation. Yield is expressed as mol 2,3-butanediol produced/mol of glucose or galactose utilized.

production, it does so only up to a certain optimum level, above which it is toxic to the microbial cell (*23*). This might have been the case in the static cultures even if complete anaerobiosis was not attained. It should also be noted that for static cultures, there could be external diffusional restrictions on substrate and product transfer in the unstirred boundary layer around each cell or particle of immobilized cells.

The higher level of product concentration in the agitated free cells than in the agitated immobilized cells may be attributed to the more aerobic environment of the free cells since the entrapping matrix of the immobilized cells could offer some resistance to the diffusion of oxygen to the cells. (*24*).

Effect of Initial Substrate Concentration

The influence of initial substrate concentration on the fermentation was also studied. The reconstitution of the whey permeate powder to different concentrations was done prior to hydrolysis and thus was based on the lactose concentration. The concentrations studied were 25, 50, 100, and 150 g/L. It should be noted that after complete hydrolysis, the combined concentrations of glucose and galactose, calculated on a mole

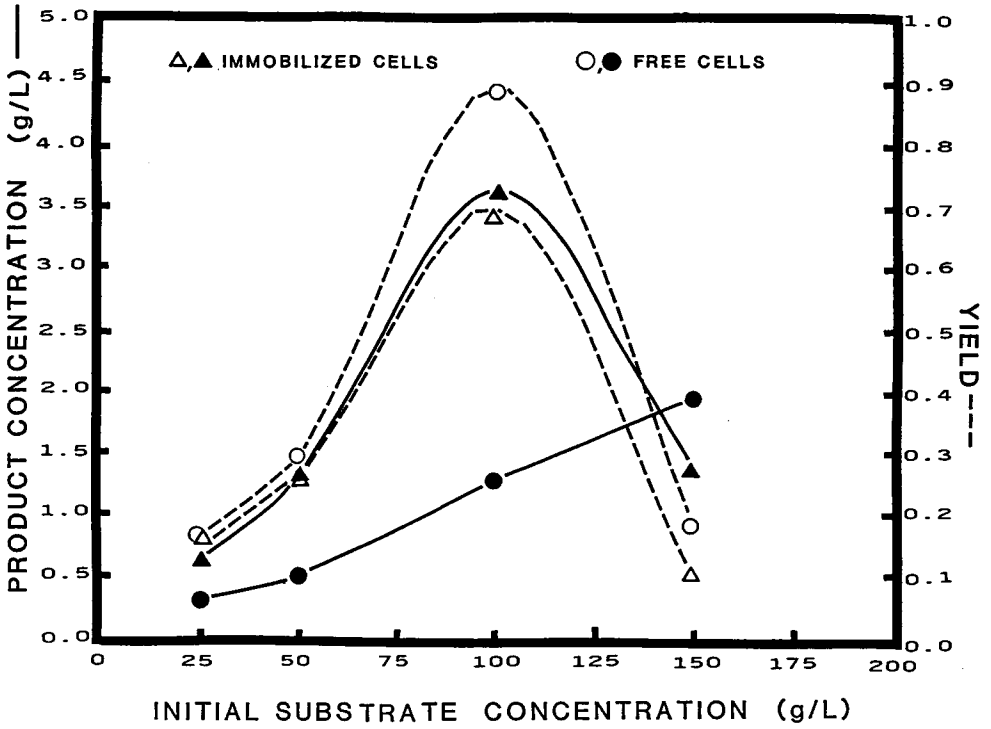

Fig. 6. 2,3-Butanediol concentration and yield at various initial substrate concentrations. Results were obtained after 60 h of fermentation. Yield is expressed as mol of 2,3-butanediol produced/mol of glucose or galactose utilized.

basis, were 26, 53, 106, and 158 g/L, respectively. These data were also verified by chemical analyses.

Figure 6 shows that the highest product concentration and yield were obtained when the initial substrate concentration was 106 g/L. The increase in product concentration up to initial substrate concentrations of 106 g/L could be a result of a proportional increase in the total cell mass produced. Yields, however, are not constant and also increase with increasing initial substrate concentrations. As in the case of *Klebsiella oxytoca*, the differences in yield may be related to the specific oxygen supply (25). These experiments were conducted under the same degree of agitation and so the oxygen supply would be consistent for all the flasks. With a low total cell mass, as in those from the low substrate concentration, the oxygen supply available per cell would be greater. This might have caused more of the substrate to be metabolized through the respiratory pathway.

As in all fermentations, there is an optimum initial substrate concentration for the maximum product yield in a fixed batch time. In this study, the optimum initial substrate concentration was 106 g/L. The sharp decrease in yields beyond this concentration for both free and immobilized cells could be related to the decreased water activity at high

solute concentrations. Under conditions of decreased water activity, the cells spend a significant part of their energy requirements to maintain concentration gradients and viability (*26*).

With increasing initial substrate concentration, the product concentration for immobilized cells followed the same trend as yield whereas with free cells, the product concentration showed an increasing trend up to the highest substrate concentration. Gel entrapped immobilized cell preparations, in themselves, have reduced water activity because of the presence of a polymer (*27*). The effect of reduced water activity at lower substrate concentrations was probably not great enough to affect the metabolic activity of the organism. Higher substrate concentrations, as mentioned earlier, may have compounded this effect so that there was a lower product concentration for the immobilized cells.

In conclusion, immobilized cells of *Bacillus polymyxa* produced 2,3-butanediol in equal or greater amounts than the free cells under the different culture conditions studied. This would prove useful for continuous fermentations in the future.

ACKNOWLEDGMENT

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REFERENCES

1. Tran, A. V., and Chambers, R. P. (1987), *Biotech. Bioeng.* **29,** 343.
2. Ledingham, G. A., and Neish, A. C. (1954), in *Industrial Fermentations,* vol. 2, Underkofler, L. A., and Hickey, R. J., eds., Chemical, New York.
3. Long, S. K., and Patrick, R. (1963), in *Advances in Applied Microbiology,* Umbreit, W. W., ed., Academic, New York.
4. Yu, E. K. C., and Saddler, J. N. (1982), *Biotechnol. Lett.* **4,** 121.
5. Speckman, R. A., and Collins, E. B. (1982), *Appl. Environ. Microbiol.* **43,** 1216.
6. Shazer, W. H., (1983), M. S. thesis, University of Illinois, Urbana, IL.
7. Linko, P., and Linko, Y. Y. (1983), in *Applied Biochemistry and Bioengineering,* vol. 4, Wingard, L. B., Goldstein, L., Katchalski-Katzir, E., eds., Academic, New York.
8. Chua, J. W., Erarslan, A., Kinoshita, S., and Taguchi, H. (1980), *J. Ferment. Technol.* **58,** 123.
9. Kautola, H., Linko, Y. Y., and Linko, P. (1983), in *Annals of the New York Academy of Science,* vol. 434, Laskin, A. I., Tsao, G. T., Wingard, L. B., eds., New York Academy of Science, New York.
10. Willets, A. (1984), *Biotechnol. Lett.* **6,** 263.
11. Lee, H. K., and Maddox, I. S. (1986), *Enzyme Microb. Technol.* **8,** 409.
12. Mallonee, D. H., (1985), Ph.D. thesis, University of Illinois, Urbana, IL.
13. Tyrell, E. A., MacDonald, R. E., and Gerhardt, P., (1958), *J. Bacteriol.* **75,** 1.
14. Desnuelle, P., and Naudet, M., (1945), *Bull. Soc. Chim. Fr.* **12,** 871.
15. Westerfeld, W. W., (1945), *J. Biol. Chem.* **161,** 495.

16. Tanaka, H., Matsumura, M., and Veliky, I. A. (1984), *Biotechnol. Bioeng.* **26,** 53.
17. Cheetham, P., (1979) *Biotechnol. Bioeng.* **21,** 2155.
18. Williams, D., and Munnecke, D. M. (1981), *Biotechnol. Bioeng.* **23,** 1813.
19. Pirt, S. J., and Callow, D. S. (1958), *J. Appl. Bacteriol.* **21,** 188.
20. Cheetham, P. J., (1980), in *Topics in Enzyme and Fermentation Biotechnology,* Wiseman, A., ed., Ellis Horwood, Sussex, England.
21. Groleau, D., Laube, V. M., and Martin, S. M. (1985), *Biotechnol. Lett.* **7,** 53–58.
22. Sablayrolles, J. M., and Goma, G. (1984), *Biotechnol. Bioeng.* **26,** 148.
23. Shazer, W. H., (1985), Ph.D. thesis, University of Illinois, Urbana, IL.
24. Enfors, S. O., and Mattiason, B. (1983), in *Immobilized Cells and Organelles,* Mattiason, B., ed., CRC Press, OH.
25. Jansen, N. B., Flickinger, M. C., and Tsao, G. T. (1984), *Biotechnol. Bioeng.* **26,** 362.
26. Esener, A. A., Bol, G., Kossen, N. W. F., and Roels, J. A., (1981), in *Advances in Biotechnology,* vol. 1, Pergamon, Toronto, Canada.
27. Mattiason, B., (1983), in *Immobilized Cells and Organelles,* Mattiason, B., ed., CRC Press, OH.