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Production of 2,3-butanediol by *Klebsiella oxytoca*

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Summary. High glucose concentrations result in high levels of 2,3-butanediol, improved yield and productivity, and a decrease in cell growth in batch cultures of *Klebsiella oxytoca*. A maximum of 84.2 g butanediol/l and a yield of 0.5 was obtained with an initial glucose concentration of 262.6 g/l. Adding the substrate in two steps in a modified fed-batch operation resulted in 85.5 g butanediol/l, 6.4 g acetoin/l and 3.4 g ethanol/l with a net yield of 0.5. Increasing the cell density to 60 g/l resulted in productivities as high as 3.22 g/l.h.

Introduction

Solvent production by fermentation usually results in low concentration of the product due to inhibition by substrate, the product or both. This makes recovery costs high, particularly in the case of 2,3-butanediol which has the additional problem of high boiling point and hygroscopicity (Ledingham and Neish 1954; Magee and Kosaric 1987). Thus it is desirable to achieve high butanediol concentrations in the fermentation broth. Using high initial sugar concentrations is not feasible due to substrate inhibition (Jansen et al. 1984a, b; Jansen and Tsao 1983; de Mas et al. 1988).

Fond et al. (1985) suggested, based upon their inhibition studies, that it should be possible to obtain butanediol concentrations as high as 130 g/l. Such a high concentration has not yet been achieved in practice. The objective of this work is to study the factors involved in the fermentation of high sugar concentrations by *Klebsiella oxytoca*, especially its effect on the production of butanediol, cell yields and growth, sugar utilization and specific product formation.

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Materials and methods

Culture and media. Klebsiella oxytoca NRRL B-199 (synonymous with *Klebsiella pneumoniae*) was obtained from U.S. Department of Agriculture, Peoria, Illinois (USA) and was maintained on agar slopes containing 1 g/l glucose, 5 g/l yeast extract (Difco Laboratories, Detroit, Mich., USA), 5 g/l tryptose (Difco), 1 g/l K_2HPO_4 (J. T. Baker, Phillipsburg, NJ, USA) and 15 g/l agar (Difco) in distilled water. This was autoclaved at 121° C for 15 min. The cultures were checked for contamination by microscopic observation.

The media used for the fermentation experiments contained glucose to the desired concentration, 5 g/l yeast extract, 5 g/l tryptose or tryptone and 1 g/l K_2HPO_4. There was no effect on growth or other fermentation parameters whether tryptose or tryptone was used as the nitrogen source. The pH was adjusted to 6.5 before autoclaving at 121° C for 15 min.

Fermentation. Inocula were prepared in 500 ml Erlenmeyer flasks using 150 ml of medium containing 80–100 g/l of glucose. A loopful of inoculum from the agar slopes was used. The flasks were agitated in a reciprocating shaker (80 rpm and 30 mm strokes) at 30° C for growth for 20–24 h. The inocula were transferred as required to shake flasks or to fermentors at levels of 1–3% v/v.

Fermentation studies were conducted in 500 ml Erlenmeyer flasks containing 150 ml media, and in a 4 liter New Brunswick Microferm fermentor. The fermentors containing the media were autoclaved at 121° C for 15 min, unless otherwise specified. The shake flasks were agitated in a rotating shaker at 30° C and the Microferm was agitated and aerated as required. Five ml samples were taken periodically for analyses.

High cell density batch experiments were conducted in 1 liter glass fermentors. Cells were concentrated from a normal batch fermentation to 60 g/l using a hollow fiber ultrafiltration module (Model UFP100-C4, A/G Technology, Needham, Mass., USA). The fermentation mixture was agitated at the maximum possible level using a magnetic stir bar and aerated at 1 vvm. Unless otherwise mentioned, all fermentation experiments were conducted at 30° C.

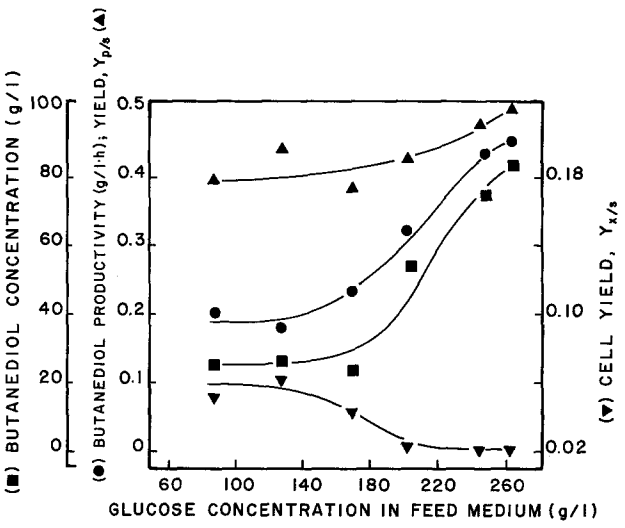

Fig. 1. Effect of initial glucose concentration on fermentation parameters of *Klebsiella oxytoca*. ●, PD; ▲, yield of butanediol; ■, butanediol concentration; ▼, cell yield

Analyses. Concentration of sugars and fermentation products were estimated by HPLC using a BioRad HPX-87H column with a refractive index monitor. The column temperature was 65° C, and solvent (0.01 N H_2SO_4) flow was 0.8 ml/min. Although this method was rapid and reproducible with most of the compounds being analyzed, some difficulties were encountered assaying acetoin, due primarily to the low concentrations that were encountered in this study.

Cell concentration was determined by optical density and a calibration curve with cell dry weight. No attempt was made in this study to account for changes in the calibration curve due to possible changes in cell morphology at different glucose concentrations.

Results and discussion

Figure 1 shows the effect of initial glucose concentration on certain batch fermentation parameters, and Table 1 shows the concentration of by-products produced by *Klebsiella oxytoca*. Increasing the glucose concentration from 88 to 262.6 g/l increased butanediol production from 25 g/l to 84.2 g/l. Sugar utilization varied from 33% to 63%, showing no consistent trends with other parameters. Final cell concentration actually decreased with increased glucose concentration, resulting in a generally declining trend in cell yield (g cells/g glucose). This may be the reason for the slightly higher product yield ($Y_{P/S}$) at higher glucose levels (Fig. 1). Butanediol productivity (PD, defined as butanediol concentration at the end of fermentation divided by time of fermentation) increased with sugar concentration, to a maximum of 0.45 g/l.h at 262.6 g/l sugar.

The by-products obtained in this fermentation study show that, except for a decline in ethanol from 9.7 g/l to 3.4 g/l with increase in initial glucose concentration, no consistent trends could be observed. The results presented here are averages of two experiments. The levels of these by-products are generally the same as what we observed in earlier experiments (Qureshi and Cheryan 1988). Acetic acid and acetoin are typically produced in this fermentation as a result of the aerobic conditions. A certain level of agitation and aeration is necessary, however, to maximize buta-

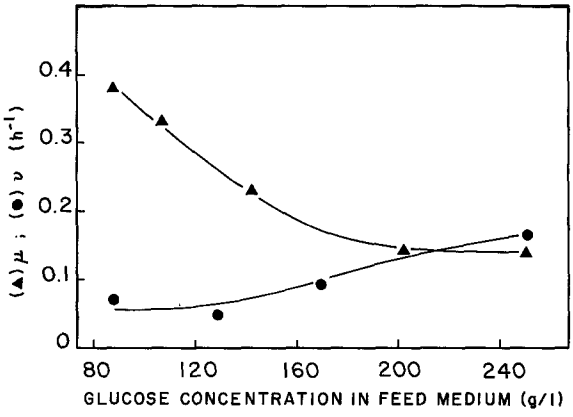

Fig. 2. Effect of initial glucose concentration on specific growth rate and specific rate of butanediol production of *Klebsiella oxytoca*. ▲, specific growth rate (μ); ●, specific rate of product formation (γ)

Table 1. Concentration of by-products and glucose during fermentation by *Klebsiella oxytoca*

Glucose (g/l)		Cells g/l	Acetoin g/l	Ethanol g/l	Acetic acid g/l	Lactic acid g/l
Initial	Final					
87.9	32.7	3.00	2.05	9.7	0.18	0.17
128.1	61.5	3.60	0.83	7.7	0.22	0.18
170.1	110.3	2.60	0.83	5.2	0.22	0.25
202.7	127.3	2.75	trace	3.8	0.22	0.21
246.3	91.7	2.75	4.70	3.4	0.12	0.23
262.6	97.2	2.75	4.70	3.7	0.15	0.22

nediol concentration and yields (Qureshi and Cheryan 1988; Sablayrolles and Goma 1984).

Since high sugar concentration results in low cell concentrations, a study of specific growth rate (μ) and specific product formation rate (γ) was conducted using glucose concentrations ranging from 80 g/l to 250 g/l (Fig. 2). Although growth was inhibited at higher glucose levels, the specific product formation rate increased. Our data suggest that butanediol production by *Klebsiella oxytoca* is not a true growth-associated fermentation.

Fed-batch culture

In an attempt to maximize butanediol concentration while minimizing substrate inhibition, a pseudo fed-batch experiment was conducted (Fig. 3). The batch culture was started with a glucose concentration of 233 g/l. Over the next 5 days, there was a steady increase in butanediol concentration to 43 g/l with a concomitant decrease in glucose concentration to 155 g/l. At this point, additional glucose was added to raise the level to 199 g/l. This resulted in a slight dilution of the reaction mixture, reducing the butanediol concentration to 35 g/l. Fermentation was continued for another 8 days until there was no further apparent utilization of glucose. At this point there was a maximum butanediol concentration of 85.5 g/l and residual glucose concentration of 95 g/l.

In this study, a total of 927 g of glucose was added in two stages to the fermentation vessel, of which 598.5 g was consumed. This represents a net glucose utilization of 65%. There was a net production of 299.3 g butanediol for a product yield ($Y_{P/S}$) of 0.5. This value is calculated based on the amount of glucose consumed; it is possible that the culture also utilized carbon sources from yeast extract and tryptone, which was not accounted for in our calculations.

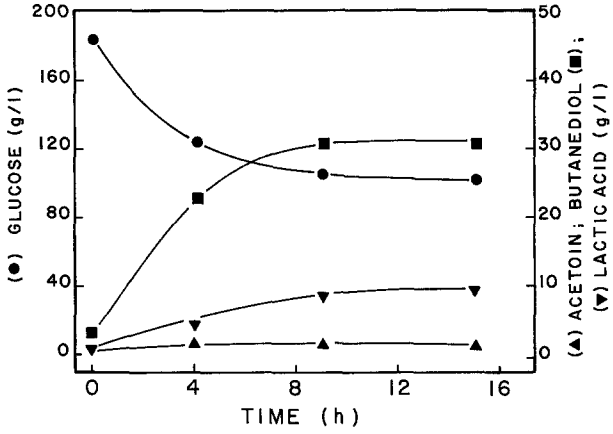

Fig. 4. Production of 2,3-butanediol by *Klebsiella oxytoca* in a high cell density (60 g/l) batch fermentor. ●, glucose; ■, butanediol; ▼, lactic acid; ▲, acetoin

We could not achieve the high final concentration of 130 g butanediol/l suggested by Fond et al. (1985). Our value of 85.5 g/l is comparable to that obtained by Sablayrolles and Goma (1984). A small amount of lactic and acetic acids were produced, and final concentration of acetoin was 6.4 g/l.

High cell density batch culture

In order to maximize productivity, a high initial cell concentration of *Klebsiella oxytoca* (60 g/l) was used in a batch fermentor under aerobic conditions. As shown in Fig. 4, the fermentation was

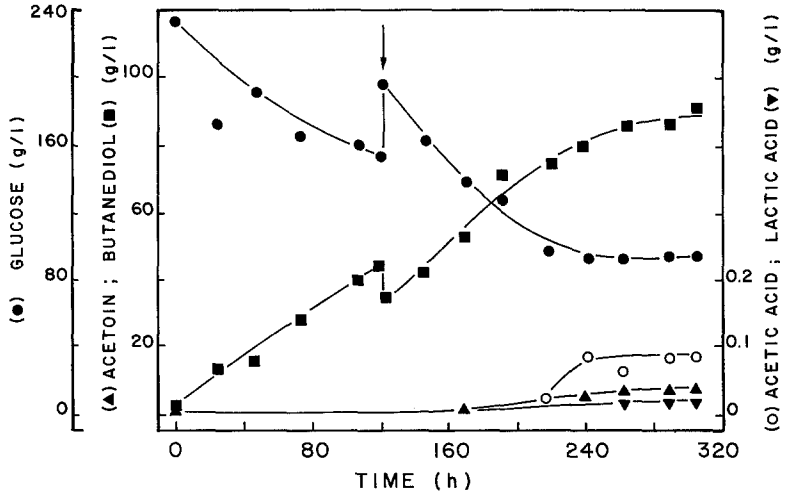

Fig. 3. Production of 2,3-butanediol from glucose by *Klebsiella oxytoca* in fed-batch culture. ●, glucose; ■, butanediol; ○, acetic acid; ▲, acetoin; ▼, lactic acid. *Arrow* indicates addition of glucose

initially quite rapid, producing 29 g butanediol/l in 9 h, which is a productivity of 3.22 g/l.h. However, considerable quantities of lactic acid were produced, which may be the reason for the poor utilization of sugar (43%) and low yield (0.36). Even though the pH was maintained at 5 in this experiment with NaOH, lactate is an especially potent inhibitor of *Klebsiella oxytoca*. Experiments conducted in shake flasks with 15 g/l lactic acid in the fermentation broth showed no growth on prolonged incubation, even when the pH of the medium was adjusted to 6.5 prior to autoclaving as with other experiments. Interestingly, lactic acid at concentrations less than 8 g/l may actually enhance butanediol production (Qureshi and Cheryan 1989).

In conclusion, we have shown that high butanediol productivity (3.22 g/l.h) is possible with *Klebsiella oxytoca* in batch culture if the initial cell concentration is high, and that high butanediol concentrations (85 g/l) can be obtained at high glucose concentrations and with good yield in a fed-batch type of operation. Further studies on optimizing these factors are in progress and will be reported soon.

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References

de Mas C, Jansen NB, Tsao GT (1988) Production of optically active 2,3-butanediol by *Bacillus polymyxa*. Biotechnol Bioeng 31:366–377

Fond O, Jansen NB, Tsao GT (1985) A model of acetic acid and 2,3-butanediol inhibition of the growth and metabolism of *Klebsiella oxytoca*. Biotechnol Lett 7:727–732

Jansen NB, Tsao GT (1983) Bioconversion of pentoses to 2,3-butanediol by *Klebsiella oxytoca*. Adv Biochem Eng Biotechnol 27:85–99

Jansen NB, Flickinger MC, Tsao GT (1984a) Application of bioenergetic to modeling the microbial conversion of D-xylose to 2,3-butanediol. Biotechnol Bioeng 26:573–582

Jansen NB, Flickinger MC, Tsao GT (1984b) Production of 2,3-butanediol from D-xylose by *Klebsiella oxytoca* ATCC 8724. Biotechnol Bioeng 26:362–369

Ledingham GA, Neish AC (1954) In: Underkofler LA, Hickey RJ (eds) Industrial fermentations, vol. 2. Chemical Publishing Co., New York pp 27–91

Magee RJ, Kosaric N (1987) The microbial production of 2,3-butanediol. Adv Appl Microbiol 32:89–161

Qureshi N, Cheryan M (1988) Effect of aeration on 2,3-butanediol production from glucose by *Klebsiella oxytoca*. J Ferment Technol (in press)

Qureshi N, Cheryan M (1989) Effect of lactic acid on the production of 2,3-butanediol by *Klebsiella oxytoca*. J Indust Microbiol (in press)

Sablayrolles JM, Goma G (1984) Butandiol production by *Aerobacter aerogenes* NRRL B-199. Effects of initial substrate concentration and aeration-agitation. Biotechnol Bioeng 26:148–155

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