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Effect of lactic acid on growth and butanediol production by *Klebsiella oxytoca*

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SUMMARY

Production of 2,3-butanediol by *Klebsiella oxytoca* was enhanced in the presence of low levels (< 8 g/l) of added sodium lactate. Cell growth was inhibited, however, and essentially stopped above 15 g/l added lactate. Levels of by-products (acetic acid and ethanol) were also higher. With 3 g/l lactate and an initial glucose level of 98 g/l, butanediol concentration and productivity increased 164% with 98% utilization of glucose. With high glucose concentration (219 g/l), addition of 2.64 g/l lactate after the growth phase resulted in 81 g/l butanediol, with a productivity of 0.65 g/l/h and 71% glucose utilization.

INTRODUCTION

Fermentation of glucose to 2,3-butanediol by *Klebsiella oxytoca* results in several by-products such as ethanol, acetic acid, lactic acid, succinic acid and formic acid [2]. Acetic acid [1,8] and ethanol [8] are known inhibitors of growth and butanediol production, although addition of small amounts of acetic acid to cultures of *Klebsiella pneumoniae* are apparently beneficial in increasing butanediol yields and concentration. Higher butanediol yields have also been reported with low concentrations of acetic acid in cultures of *Aeromonas hydrophila* [7].

Recently, we identified lactic acid as a by-product of the butanediol fermentation with *Klebsiella oxytoca*. Anaerobic fermentation resulted in significant amounts of lactic acid which inhibited cell growth and butanediol production [4,5]. Lesser amounts were produced under aerobic conditions. The objective of this study was to investigate the effect of lactic acid on cell growth, butanediol production and yield, and production of other by-products during fermentation with *Klebsiella oxytoca*.

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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 (U.S.A.) and was maintained on agar slopes containing 1 g/l glucose, 5 g/l yeast extract (Difco Laboratories, Detroit, MI, USA), 5 g/l tryptose (Difco), 1 g/l K_2HPO_4 (J.T. Baker, Phillipsberg, NJ, U.S.A.) and 15 g/l agar (Difco) in distilled water. This was autoclaved at 121°C for 15 min.

The media for the fermentation experiments contained glucose at the specified concentration, 5 g/l yeast extract, 5 g/l tryptone and 1 g/l K_2HPO_4. Lactic acid (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.) was added at the required concentration to the media, and the pH adjusted to 6.5 with 1 N NaOH prior to autoclaving at 121°C for 15 min.

Fermentation

A loopful of inoculum from the agar slopes was transferred to 500-ml Erlenmeyer flasks containing 150 ml of medium with 80–100 g/l glucose. The flasks were agitated in a reciprocating shaker (80 rpm and 30 mm strokes) at 30°C for 20–24 h for growth of the culture. The inocula were transferred as required to shake flasks at levels of 1–3% (v/v). Fermentation studies were conducted in 500 ml Erlenmeyer flasks containing 150 ml media. The shake flasks were agitated at 200 rpm on a rotating shaker at 32°C in an incubator. Five ml samples were withdrawn periodically for analysis.

Analysis

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. The cell concentration was determined by optical density, using a calibration curve with cell dry weight. Productivity was calculated as the product concentration divided by total fermentation time. Specific productivity is based on the final cell concentration.

RESULTS

The effect of added sodium lactate on cell growth of *Klebsiella oxytoca* was measured at 6.5 h and 24 h (Fig. 1). Lactate is a potent inhibitor of cell growth even at concentrations as low as 3 g/l. A concentration of 15 g/l stops growth completely. On the other hand, sugar utilization and butanediol and ethanol concentrations increased in the presence of low levels of lactate. Butanediol concentration increased from 28 g/l with no added lactate to 46 g/l at a lactate concentration of 3 g/l.

Sugar utilization increased from 70% to 98% in 72 h of fermentation (Fig. 2). Productivity was 0.64 g/l·h and yield ($Y_{P/S}$) was 0.47, which are much higher than the corresponding values without added lactate (0.39 g/l·h and 0.37 respectively). Lactate had a beneficial effect on these parameters up to 8 g/l. It is interesting to observe that these increases were obtained in spite of poor growth rates. Specific rate of butanediol production was 2.23 times higher in the presence of 3 g/l added lactate.

Lactate also enhances the production of by-products ethanol and acetic acid (Table 1). Fig. 3 shows that lactate is utilized by *Klebsiella oxytoca* in the later stages of fermentation.

Since supplementation of the media with lactic acid results in an increase of butanediol concentra-

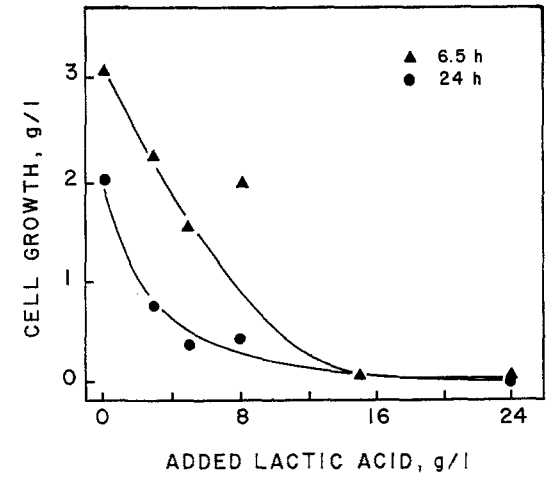

Fig. 1. Effect of added lactic acid (followed by pH adjustment to 6.5 prior to autoclaving) on growth of *Klebsiella oxytoca* in batch culture.

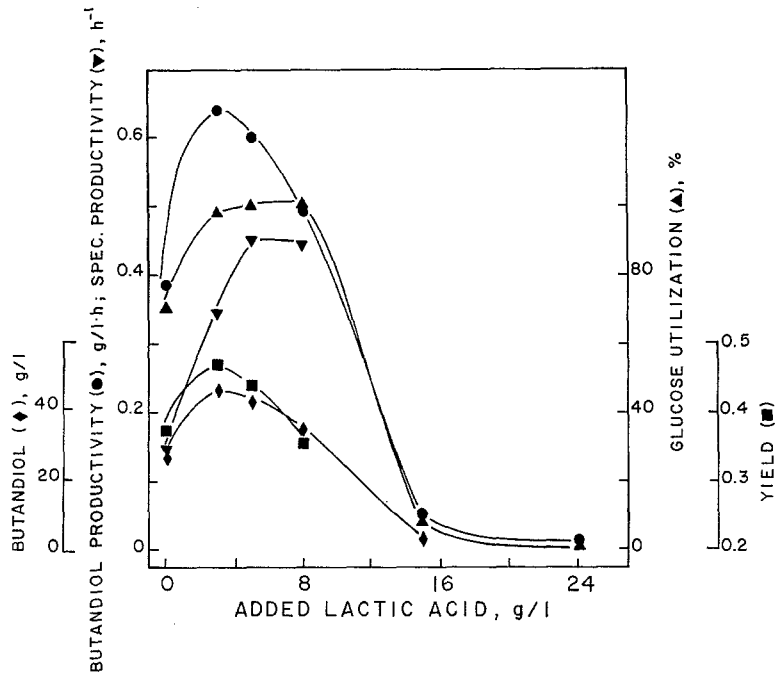

Fig. 2. Effect of added lactate on fermentation parameters of *Klebsiella oxytoca* grown in batch culture. ● Butanediol productivity; ■ Butanediol yield; ◆ Butanediol concentration; ▲ Glucose utilization; ▼ Specific rate of solvent (butanediol + ethanol) production.

tion and productivity, a batch culture experiment was conducted at high initial glucose concentration (219 g/l) to maximize these parameters. To avoid growth inhibition, the lactate was added after cell growth had occurred. The culture was allowed to grow in the normal manner for 24 h, at which time lactic acid was added to a concentration of 2.64 g/l. The pH was immediately adjusted to 4.82 with 1 N NaOH. The culture was incubated further under

Table 1

Effect of added lactic acid on production of by-products in batch cultures of *Klebsiella oxytoca*

Lactic acid added (g/l)	By-products (g/l)		
	Ethanol	Acetic acid	Acetoin
0	3.2	0.30	ND
3	8.7	0.30	ND
5	7.7	2.1	ND
8	8.5	2.4	ND
15	ND	ND	ND

ND = None detected.

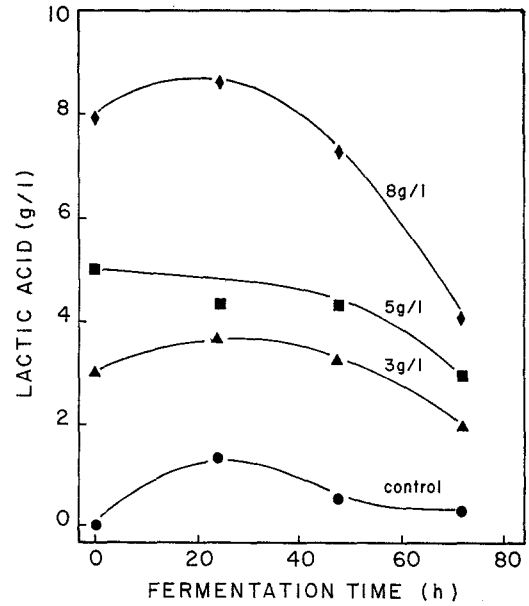

Fig. 3. Lactic acid production and utilization by *Klebsiella oxytoca* in batch cultures with various initial lactic acid concentrations. The initial pH of the medium after addition of lactic acid was adjusted to 6.5 with 1 N NaOH. ● No lactic acid; ▲ 3 g/l lactic acid; ■ 5 g/l lactic acid; ◆ 8 g/l lactic acid.

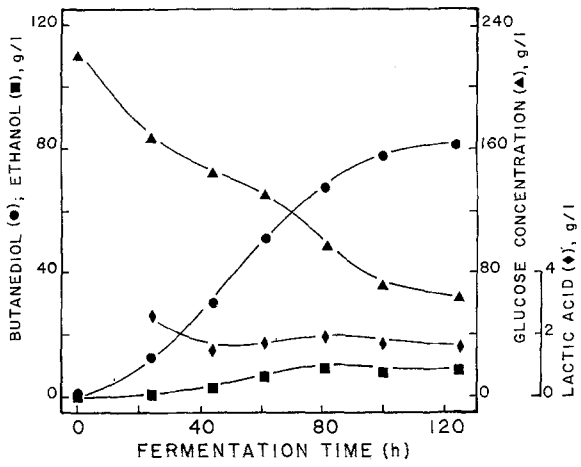

Fig. 4. Fermentation of high glucose concentration (219 g/l) by *Klebsiella oxytoca*. Lactic acid was added after 24 h of cell growth. ▲ glucose; ● butanediol; ■ ethanol; ◆ lactic acid.

the same conditions. These results are shown in Fig. 4.

The fermentation was faster than earlier experiments at high glucose levels without added lactate [5]. At the end of the fermentation (125 h), the butanediol concentration was 81 g/l with a productivity of 0.65 g/l·h. Maximum productivity was 0.84 g/l·h at 81 h, corresponding to a butanediol concentration of 68 g/l. Overall butanediol yield was 0.52 with 71% glucose utilization.

DISCUSSION

Sodium lactate inhibits growth of *Klebsiella oxytoca* under both aerobic and anaerobic conditions [4]. Under anaerobic conditions it also inhibits production of 2,3-butanediol. Cell growth decreases while specific production rate of butanediol and ethanol increases with low levels (<8 g/l) of added lactate. Higher levels of lactate, however, inhibited both growth and butanediol production. The mechanism of inhibition by lactate is not clear; its effect on growth and solvent production appears to be similar to that shown for *Saccharomyces cerevisiae* during ethanol production [3]. It is possible that the presence of lactate stimulates the enzyme(s) involved in the production of 2,3-butanediol. The induction of various enzymes, and hence the production of a number of chemicals, has been reported in *Alcaligenes europhus* when its metabolism is switched over from respiratory to limited respiratory [6].

It is interesting to note that lactate was apparently utilized by the culture in later stages of fermentation. It could be that conversion of pyruvate to lactate in the mixed butanediol pathway [2] is reversible. Butanediol yield (0.52) was higher than the theoretical value of 0.50 expected from glucose. This, together with the data in Fig. 3, suggest that the culture may be using lactate as a carbon source for cell maintenance or for product formation. The yield data is based upon total glucose utilized; available carbon from yeast extract and tryptone could also have been utilized.

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