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EFFECT OF SUCCINIC ACID ON 2,3-BUTANEDIOL PRODUCTION BY *KLEBSIELLA OXYTOCA*

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SUMMARY

The effect of succinic acid on the growth of *Klebsiella oxytoca* and its production of 2,3-butanediol was studied. Increasing succinic acid from 0 g/L to 30 g/L increased the final butanediol concentration. The maximum butanediol productivity occurred at an initial succinic acid concentration of approximately 10 g/L.

INTRODUCTION

There has been interest in industrial-scale production of butanediol from agricultural, logging, pulp and paper, and food industry wastes (Magee and Kosaric, 1987). In order to accomplish this overall conversion, cellulosic materials must first be hydrolyzed by microbial or enzymatic processes into simpler organic substrates which can then be further converted into butanediol in a second stage. 2,3-butanediol may readily be produced as this second stage from hexoses and pentoses by numerous microorganisms, including *Klebsiella oxytoca* (Leidingham and Neish, 1954; Sablayrolles and Goma, 1984; Janssen, et al. 1984). Since butanediol is produced via a mixed acid pathway, other end products such as ethanol, acetate, lactate, formate and succinate are commonly formed. Stormer (1977) noted that acetate, in its ionized form, induces acetolactate synthase formation, and thereby enhances the catalysis of pyruvate to butanediol. By analogy, a mixture of pentoses and organic acids resulting from a first stage of cellulosic conversion may be particularly attractive as substrates for butanediol production.

Ruminal anaerobic bacteria such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, can produce succinic acid very effectively as the principle end-product of cellulose degradation (Lo, et al. 1991; Weimer, 1992), but other ruminal organisms can

also form acetate, propionate and butyrate (Chiruvolu and Engler, 1992 and Blassig, et al. 1992). Although *F. succinogenes* has hemicellulolytic enzymes and thereby produces pentoses (Matte and Forsberg, 1992), this organism is unable to catabolize these pentoses (Matte, et al., 1992). The end-products of a fermentation of lignocellulose by *F. succinogenes* therefore are principally succinic acid and pentoses. The removal of the pentoses in some second stage process may reduce the inhibition of cellulolytic and hemicellulolytic enzymes, and thereby enhance the ruminal anaerobes' rate of cellulose degradation. In order to utilize this mixed product of the first stage of lignocellulose degradation for the further production of butanediol, the effect of succinic acid on the production of butanediol by *K. oxytoca* was studied.

EXPERIMENTAL METHODS

Growth Conditions

Klebsiella oxytoca (ATCC 8724) was maintained in nutrient broth and agar. The media used for inocula and for fermentations contained: $K_2HPO_4 \cdot 3H_2O$, 10.0 g/L; KH_2PO_4, 2.4 g/L; $(NH_4)_2SO_4$, 7.0 g/L; $(NH_4)_2HPO_4$, 4.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.01 g/L; $MnSO_4 \cdot H_2O$, 0.01 g/L; $FeSO_4 \cdot 7H_2O$, 0.01 g/L; yeast extract, 5.0 g/L. Inocula initially contained 125 mL of media with 20 g/L D-xylose, while 2.0 L fermentation experiments initially contained 30 g/L D-xylose with 0 - 30 g/L succinic acid. Organisms were transferred from slants directly into inoculum media in shake flasks incubated at 37°C for 20 hours. An inoculum was then transferred to a batch fermentor (Bioflow III, New Brunswick Scientific Co., Edison, NJ, USA). The pH was controlled at 5.3 - 5.4 by addition of 2M H_2SO_4 or 8M NaOH. Sterile air was sparged continuously at 1.0 vvm (2.0 L/min). Agitation was maintained at 350 rpm and temperature at 37°C.

Analytical Methods

Samples (each about 10 mL) were withdrawn from the fermentor and analyzed for growth and substrate and product concentrations. Growth was estimated by measuring optical density at 620 nm of a 1:4 dilution (DU650 spectrophotometer, Beckman Instruments, Inc., Fullerton, CA, USA). Dry cell weight was also found with some samples by drying a 10 mL volume of media at 55°C for 24 hours. Dry cell concentration (DC, g/L) was found to correlate with the 1:4 dilution optical density (20% OD) by the following equation: DC = 3.31 × (20% OD). D-xylose concentration was determined by the colorimetric orcinol assay (Herbert *et al.*, 1971). Concentrations of organic acids (i.e., succinic acid, citric acid and acetic acid) were measured by high pressure liquid chromatography (Model HIC-6A, Shimadzu Corp., Kyoto, Japan) with a PRP-X300 ion exclusion column (Hamilton, Reno, NV, USA). Concentrations of acetoin and 2,3-butanediol were determined by gas chromatography (Model HP5890II, Hewlett-Packard, Avondale, PA,

USA) using a flame ionization detector and a 6' x 2mm ID glass column packed with 60/80 Carbopack FTA (Supelco, Inc., Bellefonte, PA, USA).

RESULTS

Figure 1 shows typical growth results of *Klebsiella oxytoca* on 30 g/L xylose with different initial concentrations of succinic acid. For each of the fermentations, the final biomass concentration attained was about 4.6 g/L. However, when the initial concentration of succinic acid was 20 g/L (and 30 g/L, data not shown), the growth rate of the organism was reduced. Since the addition of succinic acid into the fermentation did not affect the final biomass concentration at the xylose concentration and air supply rate used, the cell mass yield from succinate was zero.

Figure 2 shows the degradation of the two substrates during the fermentations. A lag of 3 - 5 hours consistently occurred for the initiation of xylose degradation. Succinic acid degradation in contrast commenced immediately without a significant lag period. No diauxic growth was observed, and there was no evidence of catabolite repression. Since succinic acid is present in the tricarboxylic acid cycle, the organism probably already had sufficient succinic acid catabolizing enzymes to cause its immediate degradation. Succinic acid degradation was found to

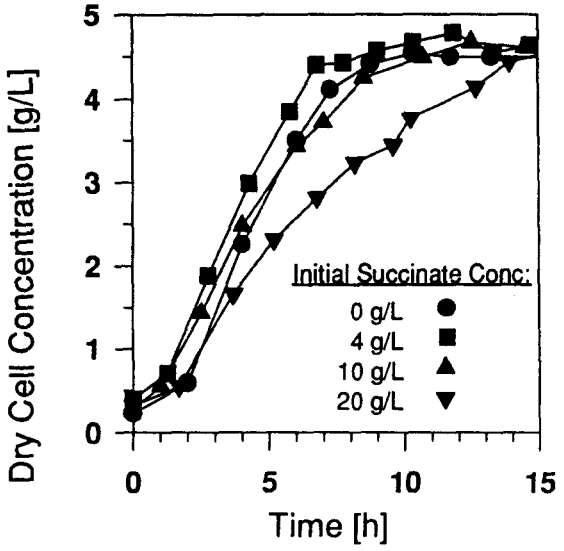

Figure 1: Growth of *K. oxytoca* with different initial concentrations of succinic acid.

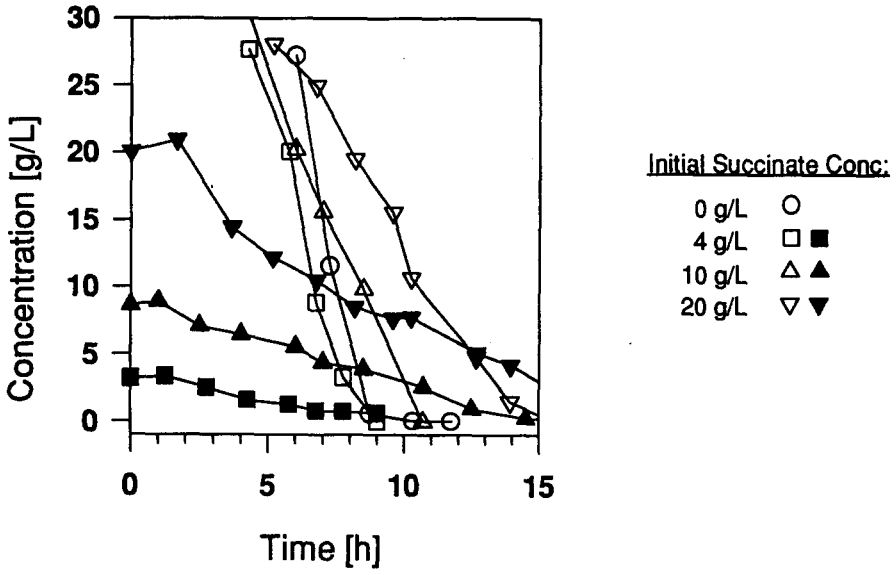

Figure 2: Utilization of xylose (hollow symbols) and succinic acid (filled symbols) by *K. oxytoca* with different initial concentrations of succinic acid.

Initial Succinate (g/L)	First Order Rate Constant Succinate Degradation (h^{-1})	Maximum Butanediol Conc. (g/L)	Butanediol Productivity (g/L·h)	Butanediol Yield from Succinate (g/g Suc.)
0	-	6.78	0.81	-
4	0.121	8.60	0.96	0.46
10	0.187	10.50	0.98	0.37
20	0.112	11.28	0.72	0.23
30	0.048	12.07	0.44	0.18

Table I: Summary of fermentations of xylose and succinic acid by *Klebsiella oxytoca*.

approximate a first order rate equation, and Table I displays the results of these calculations. These results indicate that the maximum rate of succinate degradation occurred in the fermentation having an initial succinic acid concentration of 10 g/L. Beyond this concentration, the reduction in succinic acid utilization may simply be due to the reduced biomass concentration at higher initial succinic acid concentrations.

Figure 3 shows the production of 2,3-butanediol and acetoin for the xylose/succinic acid fermentations. As shown in Figure 3

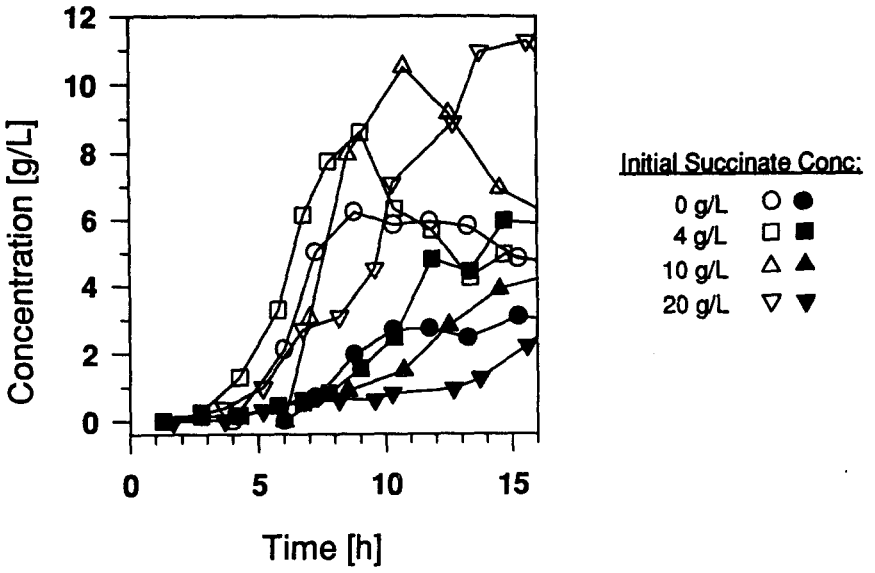

Figure 3: Production of 2,3-butanediol (hollow symbols) and acetoin (filled symbols) by *K. oxytoca* with different initial concentrations of succinic acid.

and Table I, the greater the initial concentration of succinic acid, the greater the maximum butanediol concentration. However, the greater the initial concentration of succinic acid, the lower the increase in butanediol production when more succinic acid was introduced. For example, 4 g/L of succinic acid resulted in an increase in the final butanediol concentration of 1.82 g/L compared with a fermentation without succinic acid. In contrast, an increase of initial succinic acid concentration from 10 g/L to 20 g/L resulted only in an increase in the final butanediol concentration of 0.79 g/L. The "yield" of butanediol produced as a result of additional succinic acid decreased with increasing succinic acid concentration. For each fermentation, the maximum concentration of 2,3-butanediol occurred at the time when the xylose concentration reached zero even though succinic acid did not disappear from the fermentation media for 2 - 5 hours after the disappearance of xylose.

The presence of succinic acid did not slow butanediol production up to an initial succinic acid concentration of 10 g/L. Although the maximum butanediol concentration achieved during a fermentation increased with initial succinic acid concentration, the butanediol productivity was the greatest at a 10 g/L initial succinic acid concentration.

CONCLUSIONS

Succinic acid does not inhibit the growth of *Klebsiella oxytoca* below a concentration of 10 g/L. These bacteria utilize all of the added succinic acid and produce additional butanediol, but less additional butanediol is produced the greater the initial succinic acid concentration. Irrespective of the initial concentration, succinic acid is degraded immediately by the cells. For all the fermentations, each of which initially contained 30 g/L xylose, succinic acid remained after the xylose was exhausted. The results indicate that the pentose-mixed acid mixture produced as a result of ruminal anaerobe cellulose degradation may increase the butanediol productivity by *K. oxytoca*, provided inhibitory concentrations are not exceeded.

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