Production of 2,3-Butanediol from Sucrose by *Klebsiella pneumoniae* NRRL B199 in Batch and Fed-Batch Reactors

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ABSTRACT

In batch mode, Klebsiella pneumoniae *growth and 2,3-butanediol/acetoin formation are increasingly inhibited by initial sucrose concentrations (S_0) over 60 g/L. At non inhibitory conditions, a maximum sucrose consumption rate of 1,5 g/L/h was measured. With S_0=204 g/L however, this rate decreased to 0.15 g/L/h.* K. pneumoniae *fermented 204 g/L sucrose to produce 84.3 g/L of a mixture 2,3-butanediol/acetoin with a yield of 0.41 g/g and a productivity of 1.06 g/L/h. Higher oxygen transfer rates improved the overall process rate but the product yield was reduced. Avoiding substrate inhibition, by performing the fermentation in fed-batch mode, a final 2,3-butanediol/acetoin concentration of 80.0 g/L was achieved. In this case, a productivity of 2.63 g/L/h and a product yield of 0.37 g/g were calculated.*

Key words: *Klebsiella pneumoniae*; 2,3-butanediol: batch process, fed-batch process

INTRODUCTION

2,3-Butanediol has potential applications in the manufacture of 1,3-butanediene, antifreeze, methyl ethyl ketone, polyurethane foams, and other chemical feedstockcs normally produced from substances derived from natural gas and crude oil. The biological production of 2,3-butanediol has been intensely studied throughout this century (MAGEE & KOSARIC, 1987). In view of its importance as raw material, the interest on this fermentation still remains.

The gram-negative bacterium *Klebsiella pneumoniae* produces 2,3-butanediol from different carbohydrates in good yields and productivities (MAGEE & KOSARIC, 1987). To obtain a high product concentration in batch mode, an important aspect is to start the process with a high sugar concentration. This, however, leads to the inhibition of both the cell growth and product formation. To avoid this problem, some authors have proposed to carry out the process in fed-batch mode (OLSON & JOHNSON, 1948; YU & SADDLER, 1983).

The use of glucose, xylose, or lactose as carbon source for this fermentation has been extensively studied. Nevertheless, except the work of PIRT & CALLOW (1958), little is reported on the kinetics of 2,3-butanediol production from sucrose, a cheap and abundant substrate.

This paper describes some results on the effect of sucrose concentration on the fermentative production of 2,3-butanediol, in batch and fed-batch systems.

MATERIALS AND METHODS

Microorganism: *Klebsiella pneumoniae* NRRL B199 was maintained on nutrient agar at 4°C. Inocula preparation was done in 500 mL flasks, containing 100 mL of medium at 37°C on a reciprocal shaker (120 rpm) for 17 hours. The

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medium was inoculated with 10% (v/v) of this culture.

Medium: PIRT & CALLOW (1958) medium was used in tests and inocula preparation. Salts and concentrated sucrose solutions were autoclaved separately at 110°C for 20 minutes. After mixing, the following composition in salts was obtained (g/L): $(NH_4)_2SO_4$, 7.2; $(NH_4)_2HPO_4$, 6.0; KOH, 0.45; EDTA, 0.51; $MgSO_4.7H_2O$, 0.30; $CaCl_2.6H_2O$, 0.09; $FeSO_4.7H_2O$, 0.0225; $ZnSO_4.7H_2O$, 0.0075; $MnSO_4.7H_2O$, 0.0038. The pH was corrected with H_3PO_4 to 5.5 for experiments in bioreactors or to 6.5 for inocula preparation.

Fermentation conditions: All tests were done in a 2L Microferm fermentor (New Brunswick Scientific Co.). The temperature was kept at 37°C and the pH was automatically controlled with 5N KOH at 5.5.

In batch experiments, the initial sucrose concentration varied from 20 to 204 g/L. The runs at low oxygen transfer rate (OTR $\cong$ 4 mmol O_2/L/h) were performed with an aeration rate of 0.8 L/min and an impeller speed of 230 rpm. In high OTR batch runs ($\cong$ 16 mmol O_2/L/h), the same aeration rate was used but the impeller speed was increased to 580rpm.

Fed-batch run started as a batch with 1,6 L of medium, containing 110 g sucrose/L. During the fermentation, 400 mL of medium, with 700 g sucrose/L, were intermittently added to the bioreactor. The time of feeding was indicated by the pH or the dissolved oxygen concentration whose values normally raise in the absence of the carbon source. The aeration/agitation conditions were 0.8 L/min and 580 rpm.

Oxygen transfer rate: OTR was determined before inoculation as described by PIRT (1975), with dissolved oxygen concentrations in medium calculated according to SCHUMPE & QUICKER (1982).

Analytical methods. Cell growth was measured by reading optical density of cell suspensions at 540nm. These turbidimetric measurements were converted to concentration (g dry mass / litre) by a correlation curve. Sucrose was assayed by the glucose oxidase method (Merck), after hydrolys with 2N HCl, using hydrolysed sucrose solutions as standards. 2,3-butanediol and acetoin were analysed by gas chromatography (FID) (Instrumentos Científicos CG Ltda), using a 1/8" x 1.0 m Porapak P column. Due to the equilibrium observed between 2,3-butanediol and acetoin, the analysis of results was done considering both substances together, as previously proposed by JANSEN *et al* (1984).

RESULTS AND DISCUSSION

The effect of the initial sugar concentration (S_0) on the process was evaluated in five batch runs carried out at low oxygen supply rate (4 mmol O_2/L/h). The results are shown in Table 1.

As shown in Table 1, cell yield ($Y_{X/S}$) decreased with increasing initial sugar concentration. With respect to the product yield ($Y_{P/S}$) however, an opposite trend was observed, with $Y_{P/S}$ varying from 0.33 g/g to 0.49 g/g (63 and 93% of the maximum theoretical yield), for initial sucrose concentrations up to 95 g/L.

In a previous paper (SILVEIRA *et al*, 1993), we have shown that sucrose consumption is predominantly directed to biomass formation and the maximum value of the specific growth rate (μ_x) is reached when measurable dissolved oxygen concentrations were present in the medium. Under oxygen limitation, the growth rate diminished and butanediol synthesis was strongly intensified to provide an additional reoxidation of NADH to be used by the cells in the Embdem-Meyerhoff pathway. Such behaviour was also reported by JANSEN *et al* (1984) for xylose. Thus, in runs with the same OTR, $Y_{X/S}$ and $Y_{P/S}$ were dependent on the amount of sucrose available for the culture under oxygen limitation conditions. With S_0 = 204 g/L, the lower product yield was robably influenced by the high initial sugar concentration. In spite of this, highest 2,3-butanediol/acetoin concentration (84.4 g/L) was achieved in this experiment.

In any process, the effect of the substrate concentration on cell growth is normally evaluated by observing the maximum specific growth rate in each condition. Due to the low OTR employed in this group of runs, however, dissolved oxygen concentration remained above zero for less than one hour and an accurate measurement of the maximum μ_x became impossible.

Table 1. Results of batch runs with different sucrose concentrations, at low OTR (4 mmol/L/h).

S_0 (g/L)	Time (h)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	R (%)	p (g/L/h)	dS/dt_{5h} (g/L/h)
20	11.4	0.084	0.33	62.7	0.60	1.16
41	16.3	0.065	0.38	72.2	0.96	1.40
61	20.0	0.055	0.39	74.1	1.20	1.50
95	34.5	0.045	0.49	93.2	1.34	0.74
204	80.0	0.029	0.41	77.9	1.05	0.15

S_0 - initial sucrose concentration
$Y_{X/S}$ - cell yield
$Y_{P/S}$ - 2,3-butanediol yield
R - 2,3-butanediol/acetoin yield in relation to the theoretical maximum (0.526 g/g sucrose)
p - volumetric productivity
dS/dt_{5h} - sucrose consumption rate in the first 5 h

Thus, the effect of S_0 on the process was evaluated by comparing the sucrose consumption rates in the first five hours of each run (dS/dt_{5h}). With S_0 up to 61 g/L, increasing dS/dt values were calculated. From this point, dS/dt_{5h} was markedly affected by sugar concentration. With S_0=204 g/L, for example, dS/dt_{5h} was tenfold less than with S_0=61 g/L.

Due to its influence on the final product concentration, and also its inhibitory effect on the cell growth at higher levels, S_0 intensely affected the volumetric productivity (p). As seen from Table 1, with S_0 between 20 and 95 g/L, increasing values of productivity were measured, whereas for S_0=204 g/L an expected fall of p was observed.

In experiments with OTR ≅ 16 mmol O_2/L/h (S_0 = 42 and 213 g/L), higher cell yields were obtained in comparison with the runs with similar S_0 and lower air supply. That biomass concentrations led to smaller fermentation times and, consequently, increasing productivities were found. On the other hand, the product yield was clearly reduced due to the high oxygen supply (Table 2). Such high OTR provided the occurrence of dissolved oxygen concentrations above zero for longer periods and, therefore,

allowed the measurement of the maximum specific growth rate in each experiment. The maximum values found for μ_x (0.83 h^{-1} for S_0=42 g/L and 0.37 h^{-1} for S_0=213 g/L) confirm the inhibitory effect of higher substrate concentrations.

As discussed, to reach high butanediol/acetoin concentrations in a batch operation, inhibitory sugar concentrations were needed. This problem could be avoided by operating in fed-batch mode, since the substrate required to achieve high levels of both cell and product could be kept at suitable levels.
By comparing the results of fed-batch run (Table 3) with those obtained in batch with the same amount of substrate and the same operational conditions (S_0=213 g/L and OTR ≅ 16 mmol O_2/L/h) (Table 2), one could conclude that fed-batch mode was a more efficient way to perform the 2,3-butanediol fermentation. To explain these results, the process could be divided in two parts: the initial batch and the fed-batch phase. Before adding fresh medium to the bioreactor (initial

batch), the high OTR provided the presence of dissolved oxygen concentrations above zero for approximately 5 hours. As such, the period under oxygen limitation in this phase was relatively short and a cell concentration of 20 g/L was reached after 11.4 hours (Fig. 1). The initial sucrose concentration of 110 g/L had no detrimental effect on *K. pneumoniae* growth, as demonstrated by the maximum specific growth rate of close to 0.8 h^{-1} (Fig. 2), similar to that measured with S_0 = 40 g/L and larger OTR. As such, high $Y_{X/S}$ and low $Y_{P/S}$ were observed at the end of the initial batch.

Table 2. Results of batch runs with different initial sucrose concentrations, at high OTR (16 mmol/L/h).

S_0 (g/L)	Time (h)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	R (%)	p (g/L/h)
42	7.6	0.21	0.24	45.6	1.33
213	40.5	0.11	0.29	55.1	1.53

S_0 - initial sucrose concentration
$Y_{X/S}$ - cell yield
$Y_{P/S}$ - 2,3-butanediol/acetoin yield
R - 2,3-butanediol/acetoin yield in relation to the theoretical maximum (0.526 g/g sucrose)
p - volumetric productivity

In the fed-batch phase, after the total depletion of the initial added sugar, sucrose concentration always remained under 40 g/L (Fig. 1).

Therefore, cell growth had as possible inhibitory factors the limitation of oxygen and the concentration of products. The interruption of growth after 13-14 hours of run could not be related to the concentration of product, smaller than 40 g/L at this time of process, since in batch run with S_0 of 204 g/L, when μ_x reached the value zero, a concentration of products of almost 60 g/L was present in the medium. Thus, that fact was probably due to an extreme oxygen limitation, a situation in which the oxygen supply would be enough just for maintenance of cells.

With respect to the decreasing specific product formation rate (μ_p) depicted in Figure 2, data from all experiments of this work showed that the maximum μ_p of each run had been obtained with values of μ_x smaller than 0.15 h^{-1} but greater than zero. This means that product formation was, in some extension, dependent on respiration, a condition in which the cells have a higher energy level.

As seen from Figure 1, between the time when cell growth was interrupted (13 to 14 hours) and the end of the run, most of the sugar contained in the fresh medium was led to the formation of product and a final 2,3-butanediol/acetoin concentration of 80 g/L was obtained (Fig. 1). Within that period, a $Y_{P/S}$ of 0.47 g/g (89.7% of the theoretical maximum) was obtained. The cell concentration during that phase remained close to 20 g/L, quite high for a bacterial culture, and allowed the occurrence of a relatively short fermentation time and, consequently, elevated productivity.

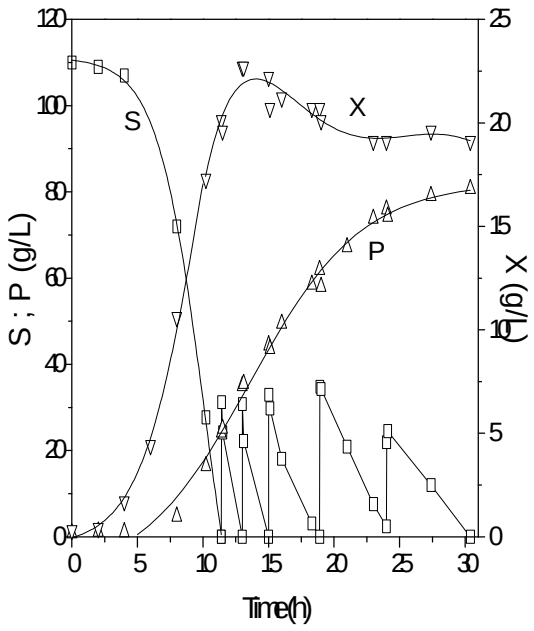

Fig. 1 - Variation of sucrose (S), 2,3-butanediol/acetoin (P), and cell (X) concentrations with time in the fed-batch run.

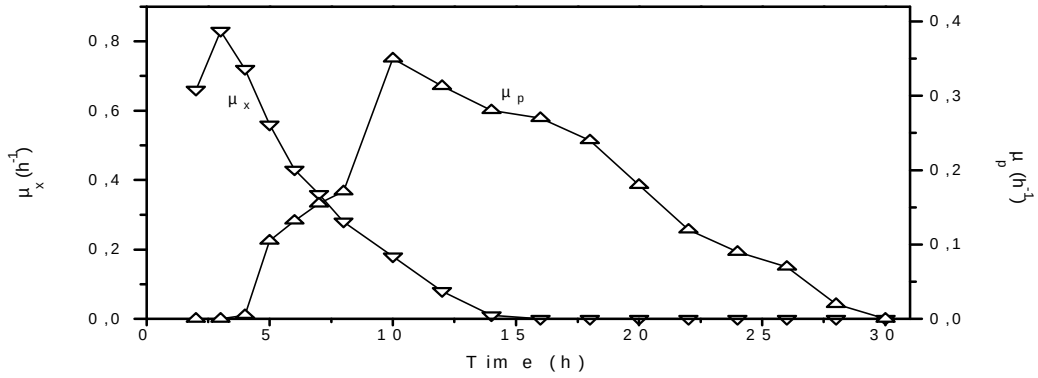

Fig. 2 - Variation of the specific growth rate (μ_x) and the specific product formation rate (μ_p) with time in the fed-batch run.

Table 3. Results of the fed-batch run.

	S_0 (g/L)	Time (h)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	R (%)	p (g/L/h)
Initial batch phase	110	11.4	0.18	0.22	41.8	2.05
Fed-batch phase	(131)[1]	19.0	0.024	0.47	90.2	3.27
Total	(219)[1]	30.4	0.084	0.37	71.0	2.63

[1] total sucrose added / final volume

S_0 - initial sucrose concentration or equivalent in fed-batch mode
$Y_{X/S}$ - cell yield
$Y_{P/S}$ - 2,3-butanediol/acetoin yield
R - 2,3-butanediol/acetoin yield in relation to the theoretical maximum (0.526 g/g sucrose)
p - volumetric productivity

Although fed-batch process requires further optimisation studies, the results of the present work show that this is an appropriate way to perform the fermentative production of 2,3-butanediol/acetoin, since it allows to obtain high product concentration with also high yield and productivity

RESUMO

Em regime descontínuo, o crescimento celular e a formação de 2,3-butanodiol/acetoína por *Klebsiella pneumoniae* sofrem inibição por concentrações iniciais de sacarose (S_0) acima de 60 g/L. Sob condições não inibidoras, uma velocidade máxima de consumo de sacarose de 1,5 g/L/h foi observada. Entretanto, com S_0 = 204 g/L, esta velocidade decresceu para 0,15 g/L/h. A fermentação de 204 g/L de sacarose por *K. pneumoniae* levou à formação de 84,3 g/L de mistura 2,3-butanodiol/acetoína, com uma conversão de 0,41 g/g e uma produtividade de 1,06 g/L/h. Um maior suprimento de oxigênio aumentou a velocidade global do processo mas reduziu a conversão em produto. Em regime descontínuo alimentado, a inibição foi evitada, tendo sido atingida uma concentração final de produtos de 80,0 g/L, com uma produtividade de 2,63 g/L/h e uma conversão de 0,37 g/g.

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