# Ammonium Phosphate as a Sole Nutritional Supplement for the Fermentative Production of 2,3-Butanediol from Sugar Cane Juice

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*Klebsiella pneumoniae*, 2,3-Butanediol, Production Medium

The production of 2,3-butanediol by *Klebsiella pneumoniae* from sugar cane juice supplemented with different salts was studied. This microorganism is able to degrade sucrose present in sugar cane juice containing ammonium phosphate as the sole nutritional supplement. With a sugar cane juice-based medium containing ~180 g sucrose / l and 8.0 g $(NH_4)_2HPO_4$, / l, over 70 g 2,3-butanediol plus acetoin / l were formed. This result is comparable to that achieved with a sugar cane juice-based medium containing several nutrients, although the kinetic profiles of these runs presented significant differences. With the ammonium phosphate-enriched medium, cell growth was initially favoured by both the strong oxygen supply and the higher water activity due to the lower concentration of nutrients. After 14 h, the limitation in some nutrients led to the interruption of cell growth, and decreasing rates for product formation and substrate consumption were observed. During the stationary phase of this run, sucrose was preferentially converted to product, and the substrate was completely depleted after 35 h of the process. With the complete medium, the substrate was totally consumed after 36 h of run. In this case, the higher initial concentration of nutrients reduced the overall process rate but sustained the cell growth for 27 h. Conversion yields of 0.40 g product / g sucrose and productivities close to 2.0 g/ lxh were obtained under both conditions.

## Introduction

For several decades 2,3-butanediol has been recognised as a potential substitute for petrol derivatives in the manufacture of products like 1,3-butanediene, antifreeze, methyl ethyl ketone, polyurethane foams, and other chemical feedstocks (Magee and Kosaric, 1987). Although the number of studies on the fermentative production of 2,3-butanediol decreased in the last years, due to the relative stability of the oil market, the wide range of possible applications for this substance has sustained the interest on this process as an alternative in case of oil shortage.

Mostly, research on the production of 2,3-butanediol has involved the facultative anaerobe bacterium *Klebsiella pneumoniae* which releases this substance as the main product of its carbohydrate metabolism (Magee and Kosaric, 1987). Besides pure carbohydrates as xylose (Jansen *et al.*, 1984), glucose (Sablayrolles and Goma, 1984), and sucrose (Pirt and Callow, 1958; Silveira *et al.*, 1998), raw materials such as cheese whey (Barret *et al.*, 1983) and sugar cane molasses (Afschar *et al.*, 1991) have been examined. However, no information is available about the use of sugar cane juice (SCJ), a low-cost substrate abundant in sugar-producing countries.

As described by Lima *et al.* (1982), SCJ contains many important nutrients for microorganisms but it is a poor source of nitrogen and phosphorous. In the industrial ethanol production by yeasts, for instance, these elements must be added to SCJ-based media. According to the same authors, in some cases sugar cane juice should also be enriched with magnesium and other metallic ions. In this context, the aim of the present work was to define a minimal nutrient supplementation for sugar cane juice to be used in fermentative production of 2,3-butanediol by *K. pneumoniae.* Furthermore, the kinetics of 2,3-butanediol fermentation using SCJ-based media was also examined.

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## Material and Methods

### *Microorganism*

Klebsiella pneumoniae NRRL B199, obtained from the Northern Regional Research Laboratory (USA), was used in all experiments. The strain was maintained on nutrient agar at 4 °C.

### *Media*

Sugar cane juice to be used in media preparation was previously treated to remove suspended solids. The pre-treatment involved the following steps: i) heating to 100 °C; ii) cooling to 25 °C and centrifugation; iii) correction of pH to 9.0 with $Ca(OH)_2$ and centrifugation; iv) correction of pH to 6.0 with 2 N HCl.

In the preparation of the complete medium (M1), pre-treated sugar cane juice was supplemented with the salt composition defined by Pirt and Callow (1958), as follows (g/l): $(NH_4)_2SO_4$, 7.2; $(NH_4)_2HPO_4$, 6.0; KOH, 0.45; EDTA, 0.51; $MgSO_4x7H_2O$, 0.30; $CaCl_2x6H_2O$, 0.09; $FeSO_4.7H_2O$, 0.0225; $ZnSO_4.7H_2O$, 0.0075; $MnSO_4x7H_2O$, 0.0038. Further media consisted of pre-treated SCJ supplemented with $(NH_4)_2HPO_4$ (1.0 to 10.0 g/l), $(NH_4)_2SO_4$ (7.2 g/l), and $MgSO_4x7H_2O$ (0.30 g/l), as mentioned within the text. Media used in shake-flasks experiments were buffered at pH 5.5 with 0.3 M 2-N-morpholinoethanesulfonic acid / potassium salt (MES / MES.K). Diluted sugar cane juice and salts solutions were sterilised separately at 121 °C for 20 min, and mixed prior to inoculation.

### *Inoculum*

For shake-flask experiments, inoculation was done by transferring nutrient-agar-grown cells (17 hours at 37 °C) directly to the media. For bioreactor runs, inocula were prepared in 500 ml flasks containing 100 ml of each medium, at 37 °C, on a New Brunswick Incubator Shaker 25D reciprocal shaker (120 rpm) for 17 h. The media were inoculated with the volume of these cultures necessary to give 0.2 g cells / l in the bioreactor.

### *Experimental conditions*

Shake-flask runs were carried out at the same conditions described for inocula preparation, with SCJ media diluted to ca. 150 g/l of sucrose. In these experiments, variation of volume due to evaporation was corrected by adding distilled water every 24 h. Bioreactor runs were performed in a 5-liter Braun Biotech Biostat MD equipment, containing 3.5 l of medium (~ 180 g/l sucrose), at an impeller speed of 500 rpm and aeration of 1.5 l/min. The temperature was kept at 37 °C and the pH was controlled at 5.5 with 5 M NaOH. The initial oxygen transfer coefficient ($k_La$), measured by the method reported by Moo-Young and Blanch (1987), was set at 95 $h^{-1}$.

### *Analytical methods*

Cell growth was measured by reading optical density of cell suspensions at 560 nm in a Shimadzu UV-160A instrument. These turbidimetric measurements were converted to concentration (g/l) by a correlation curve. Sucrose was assayed by using the glucose oxidase / peroxidase test-kit of CELM (Cia. Equipadora de Laboratórios Modernos, Brazil), after hydrolysis with 2 N HCl, using hydrolysed sucrose solutions as standards as described by Silveira *et al.* (1993). 2,3-butanediol and acetoin were analysed by liquid chromatography (Merck-Hitachi), with a refraction index detector at 50 °C, using a Hamilton PRP-X300 column and ultra-pure water as eluent. Due to the equilibrium observed between 2,3-butanediol and acetoin in fermentation runs, the analysis of results was done considering both substances together as previously proposed by Jansen *et al.* (1984).

## Results and Discussion

From the results of shake-flask experiment with medium M2 (Table I), it is clearly demonstrated that SCJ must be supplemented with nutrients for the production of 2,3-butanediol by *K. pneumoniae*. With pure pre-treated SCJ diluted to 140–150 g sucrose / l, only 8.5% of the initial substrate was consumed after 48 hours of cultivation to form 0.6 g/l of butanediol / acetoin. With media M1 and M3 to M5, sucrose was completely depleted and similar product yields were achieved. The higher cell yields found both with media M1 and M3 can be justified by the presence of ammonium sulfate as well as some other nutrients in M1. Comparison of results with media M4 and M5 shows that the supplementation of SCJ with $MgSO_4.7H_2O$ has no significant effect on the process.

Table I. Results of shake flasks cultivation of *Klebsiella pneumoniae* on different sugar cane juice-based media (initial sucrose concentration, 140 –150 g/l, cultivation time, 48 h).

| Medium | M1 | M2 | M3 | M4 | M5 |
|---|---|---|---|---|---|
| Sucrose consumed (%) | 100 | 8.5 | 100 | 100 | 100 |
| Cell yield (g/g) | 0.065 | 0.041 | 0.058 | 0.051 | 0.050 |
| Product yield (g/g) | 0.44 | 0.039 | 0.41 | 0.42 | 0.43 |

M1 – complete medium.
M2 – non-supplemented sugar cane juice (SCJ).
M3 – SCJ supplemented with $(NH_4)_2HPO_4$ (6.0 g/l), $(NH_4)_2SO_4$ (7.2 g/l), and $MgSO_4x7H_2O$ (0.3 g/l).
M4 – SCJ supplemented with $(NH_4)_2HPO_4$ (6.0 g/l) and $MgSO_4x7H_2O$ (0.3 g/l).
M5 – SCJ supplemented with $(NH_4)_2HPO_4$ (6.0 g/l).

A second set of shake-flask experiments was done in order to correlate the concentrations of sucrose and ammonium phosphate for SCJ enrichment. As shown in Table II, when SCJ (140–150 g sucrose / l) was supplemented with 1.0 to 4.0 g $(NH_4)_2HPO_4$ / l, significant residual sucrose concentrations were found in the media even after 48 hours of cultivation. On the other hand, media containing 5.0 to 10.0 g/l of this salt resulted in the total consumption of the substrate and the achievement of similar 2,3-butanediol / acetoin yields.

Table II. Results of shake flasks cultivation of *Klebsiella pneumoniae* on sugar cane juice supplemented with different concentrations of ammonium phosphate (initial sucrose concentration, 140–150 g/l, maximum cultivation time, 48 h).

| $(NH_4)_2HPO_4$ (g/l) | Sucrose consumed (%) | Cell yield (g/g) | Product yield (g/g) |
|---|---|---|---|
| 1.0 | 23.3 | 0.063 | 0.10 |
| 2.0 | 34.2 | 0.062 | 0.30 |
| 3.0 | 62.8 | 0.048 | 0.32 |
| 4.0 | 88.7 | 0.042 | 0.36 |
| 5.0 | 100.0 | 0.046 | 0.38 |
| 6.0 | 100.0 | 0.049 | 0.41 |
| 8.0 | 100.0 | 0.056 | 0.40 |
| 10.0 | 100.0 | 0.050 | 0.40 |

From the results presented in Table II, one can see that sucrose consumption is approximately proportional to the concentration of $(NH_4)_2HPO_4$ from 1.0 to 5.0 g/l. As such, it is possible to estimate the amount of the salt necessary to the complete degradation of a certain concentration of sucrose in SCJ.

The kinetics of this fermentation was studied in a bioreactor employing both the complete medium (M1; Experiment I) and also SCJ supplemented with 8.0 g $(NH_4)_2HPO_4$ / l (Experiment II). According to the data shown in Table II, such an ammonium phosphate concentration was in excess. However, it was chosen to assure the total consumption of the sucrose present in SCJ (~ 185 g/l). The final results of these experiments are resumed in Table III and depicted in Figures 1 and 2.

Table III. Results of cultivation of *Klebsiella pneumoniae* in bioreactor on media based on sugar cane juice (initial sucrose concentration, 180 g/l).

| | Medium M1 | $(NH_4)_2HPO_4$ (8.0 g/l) |
|---|---|---|
| Process time (h) | 36 | 35 |
| Cell yield (g/g) | 0.065 | 0.049 |
| Product yield (g/g) | 0.39 | 0.40 |
| Productivity (g/ lxh) | 1.97 | 2.03 |
| Maximal specific growth rate ($h^{-1}$) | 0.36 | 0.58 |
| Maximal specific product formation rate ($h^{-1}$) | 0.45 | 0.53 |

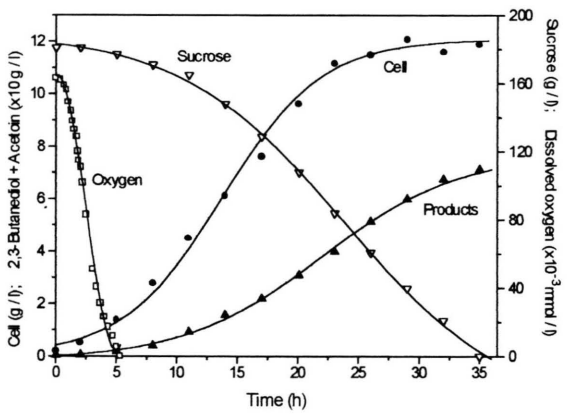


Fig. 1. Time course of cultivation of *Klebsiella pneumoniae* on complete medium.

As shown in Table III, with the complete medium (M1) a higher cell yield (0.065 g/g) was achieved due to the larger amount of nutrients, in particular nitrogen, present in this medium. With respect to the formation of 2,3-butanediol / acetoin, similar final concentrations (~ 71 g/l), yields, and productivities were obtained with both media in 35–36 hours of fermentation. However, the kinetic profiles of substrate consumption, cell growth, and product formation in each run was quite different, as depicted in Figs 1 and 2.

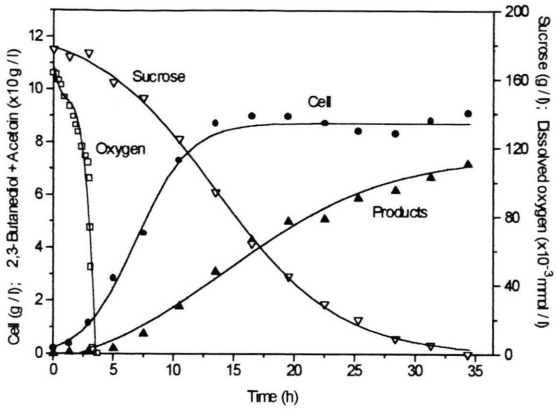


Fig. 2. Time course of cultivation of *Klebsiella pneumoniae* on pre-treated sugar cane juice supplemented with 8.0 g/l $(NH_4)_2HPO_4$.

As previously reported by different authors (Jansen and Tsao, 1983; Sablayrolles and Goma, 1984; Silveira *et al.*, 1993), 2,3-butanediol fermentation is strongly affected by the oxygen supply. In the presence of dissolved oxygen concentrations higher than 10% of saturation, the maximal specific growth rate ($\mu_{X,m}$) occurs, and negligible amounts of fermentative products are formed. Whereas in run with M1 medium this phase lasts 5 h and $\mu_{X,m}$ was 0.36 $h^{-1}$, in the experiment with ammonium phosphate-enriched SCJ, non-limiting dissolved oxygen concentrations remained in medium for only 3.5 h and $\mu_{X,m}$ was 0.58 $h^{-1}$. The lower $\mu_{X,m}$ in Experiment I was probably due to the composition of the complete medium, with high concentration of salts, that reduced the water activity and therefore affected the cell growth. The negative effect of low water activities on *K. pneumoniae* growth was previously reported by Esener *et al.* (1981).

Under oxygen limitation, the specific growth rates decreased, and butanediol / acetoin synthesis was enhanced to provide the re-oxidation of NADH to be used by the cells in the Embden-Meyerhof-Parnas pathway. In this condition, the maximal product formation rates were observed (0.45 and 0.53 $h^{-1}$, for Experiments I and II, respectively).

From Figures 1 and 2, one can see that cell growth was interrupted after 27 h of run in Experiment I and 14 h in Experiment II, approximately, although large amounts of substrate were still present in both media. Among the possible reasons for the interruption of cell growth, limitation of some nutrient is the most probable alternative, considering the relatively low product concentration and the intense oxygen supply. Following the interruption of cell growth, decreasing rates for 2,3-butanediol / acetoin formation and sucrose consumption in both runs were observed (Figure 3), and the productivities were affected, particularly in Experiment II. This finding is in agreement with the work of Silveira *et al.* (1998) who showed that a specific growth rate slightly greater than zero was preferred for maximum 2,3-butanediol / acetoin production.

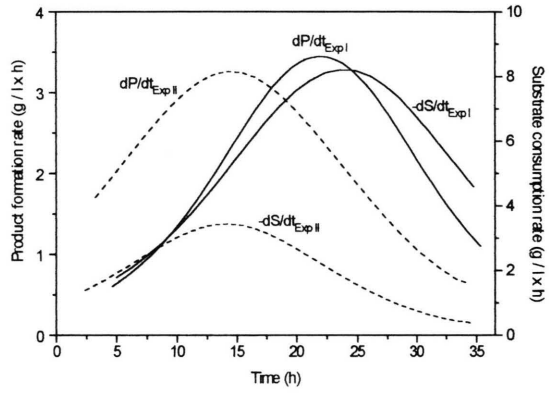


Fig. 3. Variation of product formation rate (dP/dt) and substrate consumption rate (-dS/dt) during the cultivation of *Klebsiella pneumoniae* on complete medium (Exp I) and on $(NH_4)_2HPO_4$ - enriched sugar cane juice medium (Exp II).

Whereas during the cell growth phase in Experiment II almost 50% of the initial substrate was consumed to produce approximately 30 g/l 2,3-butanediol / acetoin (0.34 g product / g substrate consumed) and almost 9 g/l cells (0.1 g cells / g substrate consumed), at the same process time (14 h) in Experiment I, less than 20% of the initial sucrose was utilised by the cells to form only 14 g product / l (0.40 g/g) and 6 g biomass / l (0.18 g/g). Despite the higher partial yields for both cells and product calculated for the experiment with the complete medium, the sucrose consumption rate in the first hours of the experiment with ammonium phosphate-enriched SCJ was significantly higher because of the lower solids content of this medium.

In Experiment I, such a product conversion yield (0.40 g/g) remained approximately constant

up to the beginning of the stationary phase, at 27 h of the process, when the conversion was reduced (~ 0.34 g/g). On the contrary, during the non-growth phase of Experiment II (14 to 35 h of run), sucrose was predominantly consumed toward the formation of 2,3-butanediol / acetoin and a enhanced conversion yield of 0.46 g product / g consumed substrate was calculated for this period (87.5% of theoretical maximum). The reason for these apparently contradictory results is not clear. As such, in Experiment II, the lower value for the conversion yield observed during the cell growth phase was compensated for by the better conversion occurring in the stationary phase.

Our results show that ammonium phosphate, at a concentration of 8 g/l, can be used as the sole nutrient for the batch production of 2,3-butanediol from SCJ by *K. pneumoniae*. This fact demonstrates that the 2,3-butanediol fermentation could be industrially performed with a simple and cost-effective medium. However, taking into account that the SCJ composition is variable, the optimal concentration of $(NH_4)_2HPO_4$ must be defined for each particular sample of SCJ. In addition, considering that better productivities are reached when that process is carried out in fed-batch mode (Silveira *et al.*, 1998), further optimisation studies are required for the use of ammonium phosphate-enriched SCJ in this type of fermentation system.

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