Computer coupled substrate feeding strategies for efficient conversion of D-sorbitol to L-sorbose in fed-batch culture

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Abstract

L-sorbose fermentation is of industrial importance since it is an intermediate step in the industrial production of vitamin C. It involves the biological oxidation of D-sorbitol to L-sorbose using *Acetobacter suboxydans*. Batch sorbose fermentation features substrate inhibition and hence it is impossible to obtain high sorbose productivity in batch bioreactors. Fed-batch fermentations can eliminate substrate inhibition and improve sorbose productivity. Fed-batch sorbose fermentations were conducted using two different nutrient feeding strategies. Fed-batch fermentation conducted by feeding nutrients containing 600 g l^{-1} of sorbitol at a constant feed rate of 0.6 l h^{-1} yielded a sorbose productivity of 12.64 g l^{-1} h^{-1}. While fed-batch fermentation conducted by feeding nutrients containing 600 g l^{-1} of sorbitol at a linearly decreasing feed rate (0.5–0.04 l h^{-1}) demonstrated a productivity of 17.01 g l^{-1} h^{-1}. The productivity obtained by feeding nutrients at a linearly decreasing feed rate was higher than that obtained by the constant fed-batch fermentation and, therefore, is a better substrate feeding strategy for improving sorbose productivity.

Keywords: *Acetobacter suboxydans*; Fed-batch fermentation; Vitamin C; Sorbitol; Sorbose; Feeding strategy

1. Introduction

L-sorbose has been used as an intermediate in the synthesis of vitamin C. L-sorbose is produced by the microbial oxidation of D-sorbitol using *Acetobacter suboxydans* [1,2]. Chemical oxidation of D-sorbitol leads to the formation of both D and L isomers of sorbose. The microbial process is preferred since it yields only the L-form of sorbose which is used in vitamin C synthesis.

D-sorbitol oxidation by *A. suboxydans* is carried out in conventional batch bioreactors. Bacterial growth is severely inhibited by sorbitol in a batch reactor [3–5]. Complete oxidation is possible up to an initial sorbitol concentration of 200 g l^{-1} within a reasonable period of time. However, the rate of sorbitol oxidation is drastically reduced when higher initial sorbitol concentrations are used [3] in batch processing. Since it is not possible to process high concentrations of sorbitol due to its inhibition, it is impossible to produce high sorbose concentration and productivity in batch fermentation. Moreover, batch processing also features very large unproductive downtime leading to low productivity. It is, therefore, desirable to use high productivity techniques for the cost effective production of sorbose. Fed-batch fermentations are ideally suited for processes in which the cell growth and product formation are sensitive to the limiting substrate concentration.

In fed-batch fermentations, nutrient medium containing high concentrations of sorbitol can be fed to the reactor which already contains partially fermented medium with a low sorbitol concentration. In this way, the ultimate concentration of sorbitol remains well below the inhibitory level and its negative effect on the growth is reduced. Consequently, the rate of oxidation increases and culture accumulates very high concentrations of sorbose in the fermentation medium. Further, a high concentration of sorbose in the fermentation medium also leads to the simultaneous crystallization of sorbose [6], which makes the sorbose recovery opera-

tions easy by sedimentation and filtration process. This eventually eliminates concentration of fermented broth and crystallization step for the sorbose recovery process.

It has been reported that the rate of sorbitol oxidation is accelerated by an increase in sorbose concentration, i.e. the process is autocatalytic [7,8]. However, the oxidation process is inhibited by very high concentrations of sorbose [7]. Eventhough continuous fermentation can overcome inhibition by sorbose by the continuous withdrawal of the inhibitory product from the fermenter, problems like culture instability and contamination are often encountered. Literature reports [6] indicate that up to a sorbose concentration of 770 g l^{-1} can be accumulated without significant inhibition by sorbose. Since sorbitol to sorbose bioxidation is not inhibited by sorbose as indicated above, fed-batch processes are the better options to achieve higher sorbose productivities. Some feeding strategies like exponential feeding [9] intermittent addition [7] and gradient feeding [4] had been reported for fed-batch sorbose fermentation. Srivastava et al. [9] initiated the fed-batch fermentation with an inital sorbitol concentration of 200 g l^{-1} and at 10 h of growth it was converted to fed-batch by feeding nutrients containing 700 g l^{-1} sorbitol at an exponentially increasing rate. Mori et al. [7] used an initial sorbitol concentration of 80 g l^{-1} and sorbitol powder was supplied to the fermenter intermittently for fed-batch cultivation in a fermenter using a DO-stat. They also used a pure oxygen supply to the fermenter to maintain 2–3 ppm level of oxygen. In the gradient fed-batch process adopted by Bosjnak et al. [4], batch fermentation with an initial sorbitol concentration of 120 g l^{-1} was converted to fed-batch at 10 h of growth. Nutrients containing increasing sorbitol concentration (0–400 g l^{-1}) was pumped into the reactor at constant feed rate. Lee [10] has extensively reviewed the use of various feeding strategies that couple the physical parameters for the fed-batch cultivation of *Escherichia coli*. The use of the dissolved oxygen signal as an indicator for substrate feeding in other fermentation systems has been very well demonstrated by Yano [11] for the fed-batch cultivation of *Candida brassicae* and *Protaminobacter ruber*.

In the present work, fed-batch fermentations were conducted using two different nutrient feeding strategies. In one strategy, nutrients containing sorbitol at high concentration (600 g l^{-1}) were fed at a constant rate of 0.6 l h^{-1} with the hope that exponentially growing culture will be able to convert the incoming sorbitol and the feeding process can be continued till the reactor is full. In the the other strategy, the highly concentrated sorbitol (600 g l^{-1}) was fed at a linearly decreasing feed rate. Feeding was initiated during the log phase of culture at a reasonably high feed rate (0.5 l h^{-1}) and it continued at a linearly decreasing rate. The advantage in this type of feeding was that with the deterioration of the culture, the sorbitol input to the reactor was also reduced. This ensured synchronization of the termination of substrate feeding and the end of fermentation.

2. Material and methods

2.1. Chemicals

A 70% (w/w) sorbitol solution was supplied by M/s Anil starch products, Ahmedabad (India). Yeast extract powder, ammonium dihydrogen phosphate and magnesium sulfate were obtained from M/s Qualigens fine chemicals, Mumbai (India).

2.2. Microorganism and inoculum development

NRRL B-72 strain of *A. suboxydans* was used for the present investigation. A 48-h old growth from agar slant was transferred into 10 ml medium in test tubes having the composition (g l^{-1}), sorbitol, 50; yeast extract, 5; ammonium dihydrogen phosphate, 3; magnesium sulfate, 1; pH, 6.0. The test tubes (static culture) were incubated at 30° C for 72 h. The growth was characterized by the appearance of thick pellicle on the surface and uniform turbidity. It was then transferred to 1 l shake flasks containing 100 ml medium having the composition (g l^{-1}), Sorbitol, 50; yeast extract, 5; ammonium dihydrogen phosphate, 3; magnesium sulfate, 1 and pH. 6.0. The flasks were incubated in a rotary shaker at 30°C and 250 rpm. Subsequent transfer into the fermenter was done when the biomass was about 2.5–2.8 g l^{-1}.

2.3. Inhibition studies

2.3.1. Substrate (sorbitol) inhibition

The effect of varying initial sorbitol concentration (S_0) on the growth of *A. suboxydans* was investigated in shake flask fermentations using liquid medium containing (g l^{-1}), yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0 and magnesium sulfate, 1.0. The initial sorbitol concentrations were (g l^{-1}), 100, 150, 200, 250, 350, 400 and 510. The initial pH was adjusted to 6.0 using 3N NaOH solution. The flasks were incubated in a rotary shaker (Adolf Kuhner, Germany) at 30°C and 250 rpm. The biomass concentration (X) was estimated by measuring the optical density (OD) of the cell suspension at intervals of 1 h during the initial phases of culture growth (upto 8 h). Specific growth rate was calculated from (In X) versus (t) plot. A graphical correlation between μ and S_0 was obtained.

2.3.2. Product (sorbose) inhibition

The effect of increasing sorbose (P) concentration on the growth of *A. suboxydans* was also examined in shake flask fermentations. The basal medium consisted of (g l^{-1}), D-sorbitol, 200; yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0 and magnesium sulfate, 1.0. Sorbose solutions of following concentrations (g l^{-1}), 50, 100, 150, 250 and 400 were autoclaved separately and added to the flasks. The initial pH was adjusted to 6.0 using 3N NaOH solution. The flasks were incubated in a rotary shaker (Adolf Kuhner, Germany) at 30°C and 250 rpm. The biomass measurement and calculation of μ was done in similar manner as described in Section 2.3.1. A graphical correlation between μ and P was obtained.

2.4. Fermentation process

2.4.1. Conditions of fermentation

The fed-batch fermentations were carried out in a 7.0 l capacity fermenter (Bioengineering A.G., Switzerland) equipped with two sets of flat blade turbine impellers. The aeration rate and agitation speed were 2.2 vvm and 700 rpm, respectively. The temperature was maintained at 30°C. The pH was controlled at 6.0 by using a pH controller and by the automatic addition of 3N NaOH/HCl.

2.4.2. Fed-batch fermentation at constant feed rate of 0.6 l h^{-1}, 600 g l^{-1} sorbitol

The fermentation was initiated as a batch with an initial sorbitol concentration of 100 g l^{-1} and a working volume of 2.5 l. The concentration (g l^{-1}) of other nutrients were, yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0 and magnesium sulfate, 1.0. At 8 h, when the exponential phase of growth and a biomass concentration of about 4.2 g l^{-1} were established, 2.0 l of nutrient medium containing 600 g l^{-1} was fed at a constant rate of 0.6 l h^{-1}. The concentrations of other nutrients in the medium were also increased proportionately so that they are non-limiting. After completion of feed addition, the fermentation was continued as a batch until all the sorbitol in the reactor was exhausted (as indicated by an abrupt increase in the dissolved oxygen concentration in the bioreactor).

2.4.3. Fed-batch fermentation with linearly decreasing feed rate

The fed-batch fermentation with linearly decreasing feed rate

$F = 500.0\text{-}62.64 \times \text{Time}$

(where F is in ml h^{-1} and time t is in hour), was initiated as a batch with an initial sorbitol concentration of 100 g l^{-1} and a working volume of 2.5 l. The concentrations of other nutrients were as given in section Section 2.4.2. At 6 h, when the biomass in the reactor reached a concentration of 3.8 g l^{-1}, 2.0 l of nutrient medium containing 600 g l^{-1} (with increased proportion of other nutrients) was fed at a linearly decreasing rate (0.5–0.04 l h^{-1}) using a computer coupled peristaltic pump (ALITEA U1-M, Sweden). After the completion of feed addition, the residual sorbitol in the reactor was allowed to ferment batchwise.

2.5. Analytical techniques

2.5.1. Biomass concentration

OD of the cell suspension at 600 nm was measured in a UV1KON 930 spectrophotometer (Kontron Instruments, USA). Biomass was estimated from a OD versus concentration (g l^{-1}) correlation which was established a priori as follows.

$$\text{Biomass (g } l^{-1}) = 0.73 \times 0D_{600}$$

2.5.2. Sorbitol and sorbose concentrations

Sorbitol and sorbose concentrations were estimated by HPLC (Waters Associates, USA) using a Supelcosil LC-NH_2 (Supelco, USA) column (25 cm × 4.6 mm ID) equipped with RI detector and using acetonitrile–water (75:25) as eluent with a flow rate of 1 ml min^{-1} at ambient temperature.

2.6. Productivity calculations in fed-batch fermentation

2.6.1. Calculation of the dynamic sorbose productivity

The productivity of the two fed-batch fermentation were calculated based on the mass balance around the bioreactor as follows.

$$\text{Observed fed-batch productivity} \frac{dp}{dr} = R_p - DP$$

where R_p is the actual rate of product formation, D (= Feed rate/Reactor Volume) is the dilution rate (h^{-1}) and P is the product concentration (in g l^{-1}) Actual fed-batch productivity R was calculated from the above formula for the fed-batch fermentation and plotted in Fig. 4.

2.6.2. Calculation of overall sorbose productivity

The overall sorbose productivity was calculated by the following expression

$$\frac{\text{Sorbose produced (gl}^{-1})}{\text{Total fermentation time (h)}}$$

after completion of fermentation.

3. Results and discussion

3.1. Inhibition studies

Fig. 1 represent effect of increasing initial sorbitol (substrate) concentration and sorbose (product) concentration on the culture specific growth rate (μ). From the figure it can be concluded that keeping sorbitol concentration in the range of 50–120 g l^{-1} the culture maintained a reasonably high specific growth rate (> 0.2 h^{-1}). The culture growth decreased with increasing initial sorbitol concentration and completely stopped at an initial sorbitol concentration (S_m) of 510 g l^{-1} (as obtained by extrapolation of the curve). These observation formed the basis for linearly decreasing feed rate fed-batch fermentation. The specific growth rate decreased with increasing sorbose concentration also. However even 400 g l^{-1} sorbose concentration could reduce the culture specific growth rate to the level of 0.1 h^{-1} only, which almost leveled off thereafter indicating relatively less inhibitory effect of sorbose on μ. The maximum sorbose concentration for total growth inhibition (μ = 0) was found to be 700 g l^{-1} by extrapolation of above curve. Literature reports also indicated that upto 628 g l^{-1} sorbose can be accumulated with out appreciable inhibition of sorbose [7], From above observations it can be concluded that the sorbose fermentation by *A. suboxydans* is not a severely product inhibited system.

3.2. Fed-batch fermentation at constant feed rate of 0.6 l h^{-1}, 600 gl^{-1} sorbitol

The effect of feeding of sorbitol (600 g l^{-1}) at a higher feed rate of 0.6 l h^{-1} on fermentation kinetics is shown in Fig. 2. The fermentation was completed in 25 h with a biomass and sorbose concentrations of 9.85 and 316.00 g l^{-1}, respectively. An overall sorbose

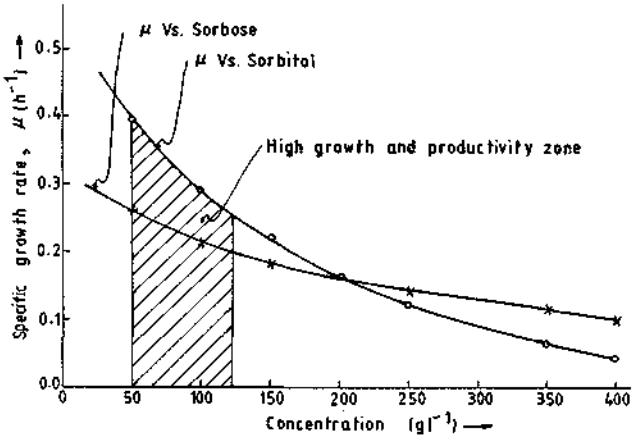

Fig. 1. Effect of increasing sorbitol (substrate) and sorbose (product) concentration on specific growth rate (μ).

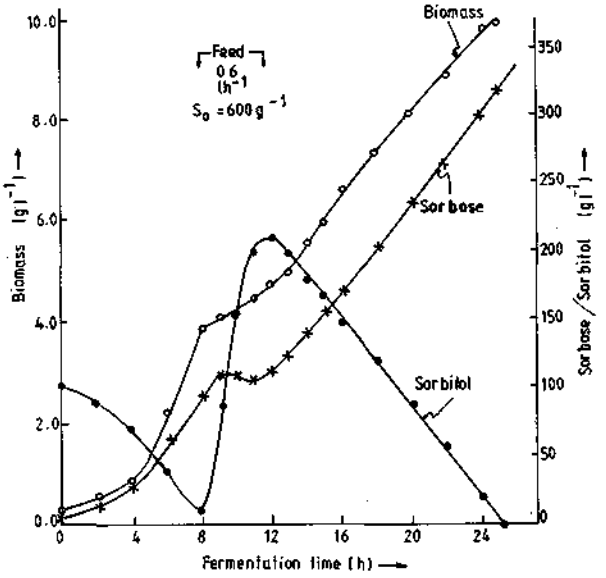

Fig. 2. Fed-batch sorbose fermentation by *A. suboxydans* at constant feed rate 0–8 h batch (S_0 = 100 g l^{-1}), 8–11.5 h fed-batch (inlet sorbitol = 600 g l^{-1}, feed rate = 0.6 l h^{-1}) 11.5–25 h batch fermentation.

productivity of 12.64 g l^{-1} h^{-1} was obtained at the end of fermentation. It can be also seen from Fig. 2 that the faster addition of fermentation medium led to a sudden accumulation of sorbitol during fed-batch cultivation from 10 to 210 g l^{-1} at 11.5 h in the reactor, which had severe adverse effect on μ as seen in Fig. 1 and it may be the reason of decrease in the sorbose productivity, thereafter, till the end of fermentation. This decrease was clearly indicated in the calculated values of sorbose productivities which featured relatively constant and and low sorbose productivity of 16.5 g l^{-1} h^{-1} after a feeding period from 11.5 h the end of fermentation (i.e., 25 h) (Fig. 4) These observations confirmed that the combined effect of increasing sorbose concentration and the sudden accumulation of sorbitol led to inhibition and osmotic stress at a stage when the culture was deteriorating. Therefore, in order to obtain high productivity along with high sorbose concentration, the feed rate must be such that the accumulation of sorbitol in the reactor should be below the inhibitory level, i.e. lower feed rates (constant) along with low inlet sorbitol concentrations are preferable.

3.3. Fed-batch fermentation with linearly decreasing feed rate

It can be seen from Fig. 2 for nutrient addition at constant rate, that the rapid addition of highly concentrated sorbitol during fed-batch fermentation led to the accumulation of sorbitol in the reactor from 10–210 g l^{-1} and higher sorbitol concentrations were inhibitory. In order to consume this sorbitol, the fermentation had

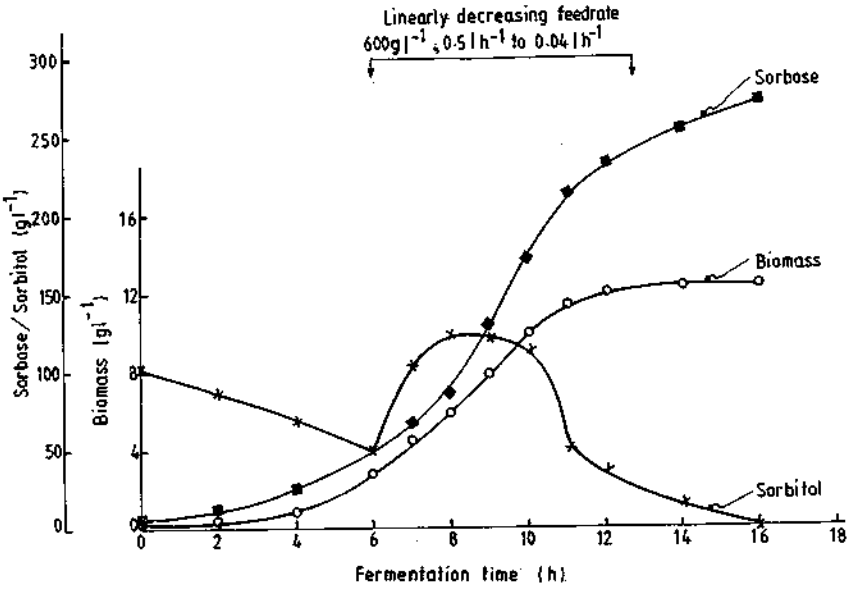

Fig. 3. Fed-batch sorbose fermentation by *A. suboxydans* with linearly decreasing feed rate 0–6 h batch ($S_0 = 100$ g l^{-1}), 6–13 h fed-batch (inlet sorbitol = 600 g l^{-1} at linearly decreasing feed rate) 13–16 h batch fermentation.

to be continued as a batch after completion of feed addition. To eliminate this 'secondary batch fermentation' and at the same time, to process highly concentrated sorbitol solution, it was decided to feed sorbitol (600 g l^{-1}) at a linearly decreasing rate, starting with high feed rate (0.5 l h^{-1}) when the culture was highly active metabolically and decreasing feed rate as the culture slowly deteriorates towards the end of the feeding. Ideally, the fermentation should be over when the fresh nutrient feed addition is completed, so that it eliminates the secondary batch processing which could reduce the overall processing time and enhanced sorbose productivity.

The time course of biomass, sorbose and sorbitol concentrations during the fed-batch cultivation with linearly decreasing feed rate is shown in Fig. 3. The fermentation was completed in 16 h with a biomass and sorbose concentrations of 12.41 and 272.37 g l^{-1}, respectively. The overall sorbose productivity obtained was 17.01 g l^{-1} h^{-1}. Using this feeding strategy the level of sorbitol accumulation in the bioreactor was also found to be less (in the range of 50–120 g l^{-1}) during the fed-batch cultivation period (6–13 h) as compared with 10–210 g l^{-1} during the feeding period (8–11.5 h) when constant feed rate of 0.6 h^{-1} (Fig. 1) was used. The reduced inhibitory effect of sorbitol on culture growth enhanced the biomass from 9.85 (Fig. 2) to 12.41 g l^{-1} (Fig. 3) at the end of fermentation. The secondary batch processing period to consume the residual sorbitol was also reduced from 13.5 h for fed-batch fermentation with constant feed rate of 0.6 l h^{-1} (Fig. 2) to 3 h for fed-batch fermentation with linearly decreasing feed rate (Fig. 3). The low sorbitol accumulation during the fresh feeding time ensured the higher rates of fermentation, as confirmed by high dynamic sorbose productivities in the range (20.106–58.0 g l^{-1} h^{-1}) during the feeding period of 7 h (6–13 h) (Fig. 4). The decrease in the overall process time (to 16 h) and the enhanced overall sorbose productivity (17.01 g l^{-1} h^{-1}) clearly indicated that the feeding of sorbitol at a linearly decreasing feed rate (particularly when the culture was deteriorating metabolically) was a better processing strategy for productivity improvement.

Several researchers have attempted to increase the sorbose productivity by implementing different feeding strategies in fed-batch fermentation [5,7,9]. Bosjnak et

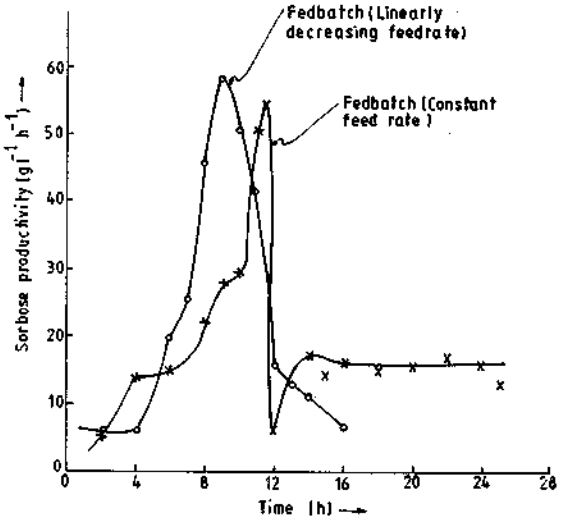

Fig. 4. Variation of sorbose productivity as a function of time in the fed-batch fermentations.

al. [5] attempted gradient fed-batch fermentation and obtained a sorbose productivity of 11.62 g l^{-1} h^{-1}. Mori et al. [7] used pure oxygen supply to the fermenter and enhanced the productivity to 44.85 g l^{-1} h^{-1}. However, it may be noted that use of pure oxygen may not be economically feasible for industrial production. Srivastava and Lasrado [9] cultivated *A. suboxydans* in fed-batch fermentation by feeding nutrients containing sorbitol exponentially and obtained a productivity of 12.6 g l^{-1} h^{-1}. The sorbose productivity of 17.01 g l^{-1} h^{-1} obtained by the linearly decreasing feed rate strategy of sorbitol addition, was highest among the literature reported values except Mori et al. [7]. The productivity of 12.64 g l^{-1} h^{-1} obtained by the constant fed-batch fermentation was almost comparable to that obtained by Srivastava and Lasrado [9]. Higher sorbose production was obtained in linearly decreasing feed mainly because fresh feed was added in healthy exponentially growing culture and feeding was maintained in such a way that the culture could grow under non limiting and non inhibitory growth conditions.

4. Conclusions

Fed-batch sorbose fermentation using sorbitol at a concentration of 600 g l^{-1} and a constant feed rate of 0.6 l h^{-1} demonstrated a sorbose accumulation and productivity of 316.00 g l^{-1} and 12.64 g $l^{-1}h^{-1}$, respectively in 25 h. Whereas, by feeding the same nutrient containing 600 g l^{-1} of sorbitol at a linearly decreasing feed rate (0.5–0.04 l h^{-1}), a sorbose concentration and productivity of 272.37 g l^{-1} and 17.01 g l^{-1} h^{-1} respectively were obtained in 16 h. Fast feeding of the sorbitol (and nutrients) elevated the fermenter sorbitol concentration quickly, which had to be fermented in the so called 'secondary batch fermentation' under inhibitory sorbitol concentrations. Linearly decreasing feed ensured high uninhibited and non limiting substrate supply when the culture was exponentially growing and slow down of substrate feeding rate when culture was detonating and the reactor volume was approaching full working capacity.

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