Productivity Improvement in L-Sorbose Biosynthesis by Fedbatch Cultivation of *Gluconobacter oxydans*

RAGHAVAN NAIR GIRIDHAR1 AND ASHOK KUMAR SRIVASTAVA1*

Department of Biochemical Engineering & Biotechnology, Indian Institute of Technology Delhi, New Delhi-110 016, India

Received 31 January 2002/Accepted 16 April 2002

The effect of increased (100, 200 and 300 g\textsuperscript{l} \textsuperscript{-1}) initial sorbitol concentrations (\(S_0\)) was investigated in the sorbitol to sorbose bioconversion process. Batch cultivations with a \(S_0\) of 100, 200 and 300 g\textsuperscript{l} \textsuperscript{-1} were completed at 10, 14 and 24 h with a corresponding overall sorbose productivity of 10.1, 14.3 and 12.4 g\textsuperscript{l} \textsuperscript{-1} h\textsuperscript{-1} respectively. The decrease in sorbose productivity at \(S_0=300\) g\textsuperscript{l} \textsuperscript{-1} was attributed to the inhibition by sorbitol of culture growth and product formation. In order to eliminate substrate inhibition, two identical fed-batch cultivations were performed in which a highly concentrated (500 g\textsuperscript{l} \textsuperscript{-1}) sorbitol solution was added at a constant feed rate (0.2 l h\textsuperscript{-1}) in the exponential phase of growth. The Fed-batch culture initiated with \(S_0=225\) g\textsuperscript{l} \textsuperscript{-1} exhibited an enhanced accumulation of sorbose (336.2 g\textsuperscript{l} \textsuperscript{-1}) but with a processing time of 24 h, and a productivity of 14.0 g\textsuperscript{l} \textsuperscript{-1} h\textsuperscript{-1}. While, the fed-batch culture initiated with \(S_0=100\) g\textsuperscript{l} \textsuperscript{-1} accumulated 279.7 g\textsuperscript{l} \textsuperscript{-1} of sorbose with an increase in productivity of 17.6 g\textsuperscript{l} \textsuperscript{-1} h\textsuperscript{-1} in 16 h.

[Key words: vitamin C, D-sorbitol, L-sorbose, *Gluconobacter oxydans*, fed-batch cultivation]
MATERIALS AND METHODS

Chemicals A 70% (w/w) D-sorbitol solution was supplied by M/s Anil starch products, Ahmedabad, India. The yeast extract powder, ammonium dihydrogen phosphate and magnesium sulfate were obtained from M/s Qualigen fine chemicals, Mumbai, India. All chemicals were of Analytical Reagent (AR) grade.

Microorganism Strain NRRL B-72 of Gluconobacter oxydans was used in this study.

Culture media

Standard liquid medium The composition (in g l⁻¹) of the standard liquid medium used was as follows: D-sorbitol, 5.0; yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0; magnesium sulfate, 1.0; the pH of the medium was adjusted to 6.0 by the addition of 3N aq. NaOH/HCl.

Culture maintenance media The culture was maintained on agar slants prepared by adding 2% (w/v) agar to the standard liquid medium. A 48-h growth at 30°C was preserved at 4°C.

Inoculum development A loop of microorganism from the slant was transferred to 10 ml of liquid medium in test tubes. The test-tubes were incubated at 30°C for 72 h. The growth was characterized by the appearance of a thick pellicle on the surface and uniform turbidity. The sample was then transferred into 1.0-l capacity flasks containing 100 ml of liquid medium. The flasks were incubated in a rotary shaker (Adolf Kuhner, Germany) at 30°C and 250 rpm. Subsequent transfer into the bioreactor was done when the biomass concentration in the flasks was about 2.5 to 3.0 g l⁻¹.

Batch cultivation Batch cultivation was carried out in a 7.0-l capacity bioreactor (Bioengineering AG, Switzerland) equipped with 2 sets of flat blade-turbine impellers under the following conditions: working volume, 4.5 l; aeration rate, 2.2 vvm; agitation speed, 700 rpm; temperature, 30°C. pH was adjusted to 6.0 by the automatic addition of 3N aq. NaOH/HCl. The cultivation medium was the standard liquid medium described above except that the initial sorbitol concentration was either 100, 200 or 300 g l⁻¹.

Fed-batch cultivation Fed-batch cultivation was initiated as batch cultures with a working volume of 2.5 l. Initial sorbitol concentrations were 100 and 225 g l⁻¹. For fed-batch cultivation, a medium containing 500 g l⁻¹ sorbitol was fed at a constant feed-rate of 0.2 l h⁻¹ (at appropriate times during the fermentation) till the total volume reached 4.5 l (full working capacity of the fermentor). The concentrations of other nutrients were also increased proportionally in the feed, so as to be non-limiting. The fermentation was continued as a batch after the addition of sorbitol to consume the residual sorbitol till completion as indicated by the abrupt increase in dissolved oxygen in the reactor. The dissolved oxygen concentration was measured with a dissolved oxygen probe (Ingold, Lemexa, KS, USA).

Analytical techniques

Biomass estimation The optical density (OD) of a suitably diluted sample was measured at 600 nm in a UV-VIS 950 spectrophotometer (Kontron Instruments, Zurich, Switzerland). Biomass was estimated from an OD vs. concentration (g l⁻¹) correlation which was determined a priori as follows:

\[ \text{Biomass (g l}^{-1}) = 0.73 \times \text{OD}_{600} \]

Sorbitol and sorbose concentrations Sorbitol and sorbose concentrations were estimated by HPLC (Waters Associates, Milford, MA, USA) using a Supelcosil LC-NH₂ column (25 × 4.6 mm i.d.; Supelco, Bellefonte, PA, USA) equipped with RI detector and using acetonitrile-water (75:25) as eluent with a flow rate of 1 ml min⁻¹ at ambient temperature.

Productivity calculations in fed-batch fermentation

Calculation of the dynamic sorbose productivity The productivity of the two fed-batch fermentations was calculated based on the mass balance around the bioreactor as follows:

\[ \frac{dQ}{dt} = R_0 - DQ \]

where \( R_0 \) is the actual rate at which component Q (X and P) is formed, D (= feed rate/reactor volume) is the dilution rate (h⁻¹) and \( Q \) is the concentration of component (in g l⁻¹). The actual fed-batch productivity \( \frac{dQ}{dt} \) was calculated from the above formula for the fed-batch fermentation and plotted in Figs. 2 (for fed-batch culture started with \( S_0 = 100 \text{ g l}^{-1} \)) and 3 (for fed-batch culture started with \( S_0 = 225 \text{ g l}^{-1} \)).

Calculation of overall sorbose productivity The overall sorbose productivity was calculated as follows:

\[ \text{Sorbose produced (g l}^{-1}) = \frac{\text{Total Fermentation time (h)}}{\text{after the completion of fermentation.}} \]

RESULTS AND DISCUSSION

The batch kinetics of the biosynthesis of sorbose by Gluconobacter oxydans for initial sorbitol concentrations (\( S_0 \)) of 100, 200 and 300 g l⁻¹ are shown in Fig. 1. The batch cultivation with \( S_0 = 100 \text{ g l}^{-1} \) demonstrated 98.6% conversion with an overall sorbose productivity of 10.10 g l⁻¹ h⁻¹ in 10 h. An overall sorbose productivity of 14.3 g l⁻¹ h⁻¹ was obtained in 14 h with 98.7% conversion when \( S_0 = 200 \text{ g l}^{-1} \) was used. Whereas, the productivity decreased to 12.4 g l⁻¹ h⁻¹ at \( S_0 = 300 \text{ g l}^{-1} \). It was observed that 99.3% of the sorbitol fed into the bioreactor could be converted to sorbose, however the processing time increased to 24 h in the case of sorbitol, indicating a severe inhibition by sorbitol (substrate) of microbial growth.

The effect of increasing the initial sorbitol (substrate) concentration and sorbose (product) concentration on the culture-specific growth rate (\( \mu \)) had also been studied by Giridhar and Srivastava (11). It was observed that the cultures exhibited a high specific growth rate (0.2 h⁻¹) only at low sorbitol concentrations (up to 150 g l⁻¹). When the sorbitol concentration in the fermentation broth was greater than 150 g l⁻¹, it led to a decrease in \( \mu \) values indicating substrate (sorbitol) inhibition. By extrapolation (and also by independent experiments) it was concluded that the culture growth was totally inhibited (\( \mu = 0 \)) when the maximum initial sorbitol concentration (\( S_m \)) was 310 g l⁻¹. A decrease in the \( \mu \) values was also observed upon a gradual increase in the product (sorbose) concentration in the fermentation broth. However, the decrease was not as significant as that of sorbitol. For example, by increasing the sorbose concentration to 400 g l⁻¹, the specific growth rate decreased to 0.1 h⁻¹ only. The specific growth rate almost remained constant with any further increase in the sorbose concentration thereafter. This established a less inhibitory effect of the product (sorbose) on \( \mu \). By extrapolation, the maximum sorbose concentration for total growth inhibition (\( \mu = 0 \)) was found to be 700 g l⁻¹. Muri et al. (7) also indicated that up to 628 g l⁻¹ of sorbose can be accumulated without appreciable inhibition of sor-
36 GIRIDHAR AND SRIVASTAVA

FIG. 1. Kinetics of L-sorbose biosynthesis by *G. oxydans* in batch cultivation. Symbols: open circles, *S*<sub>b</sub>=100 g/l; filled circles, *S*<sub>b</sub>=200 g/l; open squares, *S*<sub>b</sub>=300 g/l.

From the above reports it can be concluded that the fermentation of sorbose by *Gluconobacter oxydans* is not a product-inhibited system.

To further confirm the relative magnitude of substrate (sorbitol) inhibition, the variation in the volumetric rates of biomass (*r*<sub>b</sub>) and sorbose (*r*<sub>s</sub>) production with the time course of batch cultivation was estimated and plotted in Figs. 2 and 3. A volumetric rate of biomass formation of 1.2 g/l h<sup>-1</sup> was obtained at 6 h when *S*<sub>b</sub>=100 g/l was used. Thereafter it decreased essentially due to lack of availability of sorbitol (as shown in Fig. 1). While, the maximum volumetric biomass formation rate was only 0.7 g/l h<sup>-1</sup> at 8 h for *S*<sub>b</sub>=200 g/l. The decrease in the maximum volumetric rate of biomass formation at 8 h (as shown in Fig. 3) when enough sorbitol was available (as reflected in Fig. 1) at *S*<sub>b</sub>=200 g/l indicated that the growth was severely substrate-inhibited.

The maximum volumetric rate of sorbose production was found to be 16.2 g/l h<sup>-1</sup> at 6 h (for *S*<sub>b</sub>=100 g/l) and 22.5 g/l h<sup>-1</sup> at 8 h (for *S*<sub>b</sub>=200 g/l) of the batch cultures. The decrease in the volumetric rates for both cases after the maximum value could be attributed to decreased substrate availability (for *S*<sub>b</sub>=100 g/l) in the medium or due to inhibited biomass formation rates (for *S*<sub>b</sub>=200 g/l), respectively.

FIG. 2. Variation in the volumetric rates of sorbose production (*r*<sub>s</sub>) and biomass production (*r*<sub>b</sub>) for batch (with *S*<sub>b</sub>=100 g/l) and fed-batch (started with batch *S*<sub>b</sub>=100 g/l) cultures.

However, in order to take advantage of the relatively high rates of biomass production at 6 h and 8 h for *S*<sub>b</sub>=100 g/l and 200 g/l respectively, and to eliminate the corresponding decline in maximum volumetric sorbose production rates due to the reduced substrate availability, addition of concentrated sorbitol (with nutrients) was added at 6 and 8 h for fed-batch cultivation initiated with *S*<sub>b</sub>=100 g/l and *S*<sub>b</sub>=200 g/l.

Fed-batch fermentation (no. 1) with an initial substrate concentration of *S*<sub>b</sub>=100 g/l was completed in 16 h (as shown in Fig. 4) and yielded an ultimate sorbose concentration of 279.7 g/l with an overall productivity of 17.6 g/l h<sup>-1</sup>. The fed-batch (no. 2) culture initiated with *S*<sub>b</sub>=225 g/l was completed in 24 h (as shown in Fig. 5) with an accumulated sorbose concentration of 336.2 g/l (as compared to an accumulation of 199.9 g/l when the batch with *S*<sub>b</sub>=200 g/l was processed). It was observed that the accumulation was greater in the latter case, but the overall volumetric productivity of sorbose decreased to 14.0 g/l h<sup>-1</sup>. It is important to note that the total sorbitol accumulation during the cultivation period (6–15 h) with *S*<sub>b</sub>=100 g/l was in the range of 40–60 g/l (Fig. 4) as compared to 125–175 g/l for the fed-batch culture (period 6–18 h) initiated with *S*<sub>b</sub>=225 g/l (Fig. 5). This excess accumulation of substrate (sorbitol) in the medium might have contributed significantly to culture inhibition and thereby resulted in the observed in-
increase in process time and decrease in productivity for fed-batch cultivation initiated with $S_o=225 \text{ g/l}$.  

A comparison of the variation in volumetric rates of biomass ($r_{\text{VX}}$) and sorbose ($r_{\text{JS}}$) production with the time course of fed-batch cultivation (initiated with $S_o=100 \text{ g/l}$ and $S_o=225 \text{ g/l}$) is also shown in Figs. 2 and 3. The trend toward a decrease in the batch volumetric rates of biomass and sorbose production for $S_o=100 \text{ g/l}$ (Fig. 2) and $S_o=225 \text{ g/l}$ (Fig. 3) were reversed in fed-batch cultivation when fresh feed was added at 6 h for $S_o=100 \text{ g/l}$ and 8 h for $S_o=225 \text{ g/l}$, respectively. The highest volumetric rates for biomass and sorbose production were 1.8 $\text{g/l h^{-1}}$ and 46.0 $\text{g/l h^{-1}}$, respectively, for fed batch no. 1 with $S_o=100 \text{ g/l}$ (Fig. 3). The corresponding rates for the fed-batch culture initiated with $S_o=225 \text{ g/l}$ were 1.3 $\text{g/l h^{-1}}$ and 43.50 $\text{g/l h^{-1}}$, respectively (Fig. 4). The higher volumetric rates of biomass and sorbose formation observed in fermentation no. 1 (initiated with a batch having $S_o=100 \text{ g/l}$) could have been maintained for a longer period of time if the fresh nutrient feeding had continued. But due to the limitation of the bioreactor volume, this was not possible.  

The overall sorbose productivity of 17.6 $\text{g/l h^{-1}}$ obtained in this investigation was higher than previously reported values. By gradient fed-batch cultivation, Bosnjak et al. (4), obtained a productivity of 11.6 $\text{g/l h^{-1}}$ while Srivastava and Lasrado (12) obtained a productivity of 12.6 $\text{g/l h^{-1}}$ using exponential fed-batch cultivation. Mori et al. (7) enhanced the productivity to 44.8 $\text{g/l h^{-1}}$ using sorbitol powder and a pure oxygen supply to the bioreactor. However, the use of sorbitol powder and a pure oxygen supply may not be economically feasible for the industrial production of sorbose and therefore, is not the best option. The results obtained in the present study were also compared with those for continuous culture sorbose fermentation with or without cell recycling. Continuous L-sorbose fermentation with (100% cell recycling) or without cell recycling using an inlet sorbitol concentration of 200 g/l, was investigated (13) at different dilution rates from $D=0.05 \text{h^{-1}}$ to $D=0.3 \text{h^{-1}}$. A maximum biomass concentration of 8.44 g/l and sorbose concentration of 177 g/l (with a sorbose productivity of 17.7 g/l h^{-1}) was obtained at a dilution rate of 0.1 h^{-1} without cell recycling. However, for $D=0.1 \text{h^{-1}}$ with cell recycling (using an inlet sorbitol concentration of 200 g/l), a biomass concentration of 19.5 g/l and sorbose concentration of 181.4 g/l h^{-1} was accumulated in the bioreactor which resulted in an increase in sorbose productivity of 18.1 g/l h^{-1}. It was observed that on increasing the rate of dilution from 0.1 h^{-1}...
to 0.3 h⁻¹, there was a significant effect on the extracellular fermentation products, e.g., biomass, sorbose and sorbitol. The present study indicates that feeding a highly concentrated sorbitol solution (500 g l⁻¹) at a constant rate (0.2 l h⁻¹) into a partially converted sorbitol medium (batch, S₀=100 g l⁻¹) yields a greater concentration of sorbose (279.7 g l⁻¹) in 16 h leading to high overall volumetric sorbose productivity (17.6 g l⁻¹ h⁻¹) which otherwise is not possible in batch processing.

Conclusions

The substrate inhibition at higher initial sorbitol concentrations in batch L-sorbose biosynthesis was prominently due to the decrease in sorbose productivity as the initial concentration of sorbitol in the nutrient medium was varied from 100 g l⁻¹ to 200 g l⁻¹. The decrease in the volumetric rates of biomass and sorbose production with the time course of batch fermentation further confirmed the inhibitory effect of sorbitol. The inherent substrate inhibition was eliminated by adopting a constant fed-batch fermentation strategy to process higher quantities of sorbitol and improve sorbose productivity.

The higher sorbose productivity of 17.6 g l⁻¹ h⁻¹ and sorbose accumulation of 280 g l⁻¹ obtained by initiating the fed-batch fermentation with a sorbitol concentration of 100 g l⁻¹ indicated the improved processing technique as compared to fed-batch fermentation initiated with a higher sorbitol concentration, of 225 g l⁻¹. It was possible to accumulate sorbose at 336.2 g l⁻¹ but with a decreased productivity of 14 g l⁻¹ h⁻¹ with a fed-batch fermentation initiated with a sorbitol concentration of 225 g l⁻¹. A high level of sorbose was also obtained by batch fermentation with an initial sorbitol concentration of 300 g l⁻¹ but with significantly a lower productivity, 12.4 g l⁻¹ h⁻¹. Therefore, it is worthwhile initiating fed-batch fermentation with 100 g l⁻¹ and maintaining the same non-inhibitory sorbitol concentration throughout for better yields and productivity.

REFERENCES