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Fed-Batch Sorbose Fermentation Using Pulse and Multiple Feeding Strategies for Productivity Improvement

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Abstract Microbial oxidation of D-sorbitol to L-sorbose by *Acetobacter suboxydans* is of commercial importance since it is the only biochemical process in vitamin C synthesis. The main bottleneck in the batch oxidation of sorbitol to sorbose is that the process is severely inhibited by sorbitol. Suitable fed-batch fermentation designs can eliminate the inherent substrate inhibition and improve sorbose productivity. Fed-batch sorbose fermentations were conducted by using two nutrient feeding strategies. For fed-batch fermentation with pulse feeding, highly concentrated sorbitol (600 g/L) along with other nutrients were fed intermittently in four pulses of 0.5 liter in response to the increased DO signal. The fed-batch fermentation was over in 24 h with a sorbose productivity of 13.40 g/L/h and a final sorbose concentration of 320.48 g/L. On the other hand, in fed-batch fermentation with multiple feeds, two pulse feeds of 0.5 liter nutrient medium containing 600 g/L sorbitol was followed by the addition of 1.5 liter nutrient medium containing 600 g/L sorbitol at a constant feed rate of 0.36 L/h till the full working capacity of the reactor. The fermentation was completed in 24 h with an enhanced sorbose productivity of 15.09 g/L/h and a sorbose concentration of 332.60 g/L. The sorbose concentration and productivity obtained by multiple feeding of nutrients was found to be higher than that obtained by pulse feeding and was therefore a better strategy for fed-batch sorbose fermentation.

Keywords: sorbitol, sorbose, fed-batch fermentation, pulse feed, multiple feed, dissolved oxygen, *Acetobacter suboxydans*

INTRODUCTION

L-Sorbose fermentation is an intermediate step in the manufacture of L-ascorbic acid (vitamin C) by Reichstein synthesis [1]. It is the only biochemical step in the entire production process. It involves the oxidation of D-sorbitol by *Acetobacter suboxydans* to L-sorbose. Chemical oxidation of D-sorbitol leads to the formation of D and L-sorbose, while microbial oxidation yields only L-sorbose. Therefore, microbial oxidation is industrially used for L-sorbose production.

Sorbose fermentation is a typical fermentation example which distinctly demonstrates substrate inhibition. In a batch reactor, the rate of oxidation decreases with increasing initial concentration of sorbitol [2-4]. Complete oxidation of sorbitol is possible only up to an initial sorbitol concentration of 200 g/L within a reasonable period of time. However, there will be a sharp decline in the sorbose yields when initial sorbitol concentrations above 200 g/L are used for batch fermentation [2-4]. Therefore, it is impossible to obtain high concentrations of sorbose (and hence high productivity) in batch fermentations.

In order to obtain high sorbose productivity, it is necessary to process large quantities of sorbitol under non-inhibitory levels with maximum utilization of reactor volume. In fed-batch fermentation, nutrient medium containing very high concentration of sorbitol can be added to the reactor, which already contains partially fermented medium with low sorbitol concentration. Thus the ultimate concentration of sorbitol in the reactor can be kept well below inhibitory levels. In the case of fermentations, which have inhibition due to substrate, fed-batch fermentation is one of the most available processes for obtaining maximum specific growth rate during cultivation, leading to faster oxidation of substrate into product.

An efficient method of feeding nutrients and appropriate timing of feed into the reactor are very essential parameters for achieving high productivity in fed-batch fermentation. The rate of product formation is also affected by changing the feeding strategy. For sorbose fermentation system, the over supply of sorbitol to the fermenter should be prevented to avoid inhibition of cellular growth while feeding of small amounts of sorbitol causes substrate limitation which eventually result in low product concentrations. The most suitable concentration and the time of addition of sorbitol decide its

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ultimate concentration in the reactor.

Some feeding strategies like exponential feeding, [4] intermittent addition [5] and gradient feeding [6] had been reported for fed-batch sorbose fermentation. Srivastava and Lasrado [4] initiated the fed- batch fermentation with an initial sorbitol concentration of 200 g/L and at 10 h of growth it was converted to fed-batch by feeding nutrients containing 700 g/L sorbitol at an exponentially increasing rate. Mori *et al.* [5] used an initial sorbitol concentration of 80 g/L and sorbitol powder was supplied to the fermenter intermittently for fed-batch cultivation in a fermenter using a DO-stat. They also used 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.* [6], batch fermentation with an initial sorbitol concentration of 120 g/L was converted to fed-batch at 10 h of growth. Nutrients containing increasing sorbitol concentration (0-400 g/L) was pumped into the reactor at constant feed rate. Feeding methods that couple addition of nutrients with measurement of various physical parameters like dissolved oxygen concentration, pH, microbial heat and carbon dioxide evolution rate appears to be more efficient than coupling the nutrient feeding with the time of cultivation. Lee [7] has extensively reviewed the use of various feeding strategies that couple the physical parameters for the fed-batch cultivation of *E. coli*. The use of DO-signal as an indicator for substrate feeding in other fermentation systems has been very well demonstrated by Yano *et al.* [8] for the fed-batch cultivation of *Candida brassicae* and *Protaminobacter ruber*. The present study, investigates the use of pulse feeding and multiple feeding strategies for feeding fresh sorbitol medium for the fed-batch cultivation of *Acetobacter suboxydans* to microbially oxidize D-sorbitol to L-sorbose.

MATERIALS AND METHODS

Chemicals

The sorbitol used in this study was of commercial grade (70% w/w) supplied by M/s Anil starch products, Ahmedabad, India. Yeast extract powder, ammonium dihydrogen phosphate and magnesium sulphate were purchased from M/s Qualigens fine chemicals, Mumbai, India. All chemicals were of analytical reagent (AR) grade.

Microorganism and Maintenance

The present investigation was carried out using NRRL B-72 strain of *Acetobacter suboxydans*. Cultures were maintained on agar slants having the composition (g/L): sorbitol, 5.0; yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0; magnesium sulphate, 1.0; agar 20. The initial pH was 6.0. Samples taken after 48 h growth at 30°C was preserved at 4°C.

Inoculum Development

A loop full of microorganism from the slant was transferred into 0.01 liter medium in test tubes having the composition (g/L): Sorbitol, 5.0; yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0; magnesium sulphate, 1.0; pH 6.0. The test tubes were incubated at 30°C for 72 h. The growth was characterised by the appearance of a thick pellicle on the surface of the medium and uniform turbidity. It was then transferred into 1.0 liter capacity flasks containing 100 mL medium of composition (g/L): sorbitol, 5.0; yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0; magnesium sulphate, 1.0; pH, 6.0. The flasks were incubated in a rotary shaker (Adolf Kuhner, Switzerland) at 30°C and 250 rpm. Subsequent transfer into the fermenter was done when the biomass concentration was about 2.8 to 3.0 g/L.

Fermentation Process

Conditions of Fermentation

The fed-batch fermentations were carried out in a 7.0 litre capacity fermenter (Bioengineering A.G., Switzerland) equipped with two sets of flat blade turbine impellers. The working volume was 4.5 litres. The aeration rate and agitation speed were 2.2 vvm and 700 rpm respectively. The temperature was maintained at 30°C and the pH at 6.0 by the automatic addition of 3 N NaOH and 3 N HCl.

Fed-batch Fermentation with Pulse Feed

Fed-batch fermentation with pulse feed was started as a batch with an initial sorbitol concentration of 100 g/L. The concentration (g/L) of other nutrients were; yeast extract powder, 5.0; ammonium dihydrogen phosphate, 3.0 and magnesium sulphate, 1.0. Four pulse feeds of 0.5 liter, 600 g/L sorbitol along with increased proportion of other nutrients so that they are not limiting, were fed when the dissolved oxygen concentration in the reactor increased abruptly (at hours 7, 12, 15 and 20) indicating the depletion of carbon source. The fermentation was stopped when all the sorbitol in the medium was exhausted as indicated by the increased DO signal.

Fed-batch Fermentation with Multiple Feeds

The fed-batch fermentation with multiple feeds was started as a batch with an initial sorbitol concentration of 100 g/L. The concentration of other nutrients in the medium were as given above in the fed-batch fermentation with pulse feed. Two pulse feeds of 0.5 litres nutrient medium containing 600 g/L sorbitol (with increased proportion of other nutrients) were followed by the addition of 1.5 liters of nutrient medium containing 600 g/L sorbitol at a constant feed rate of 0.36 L/h. First nutrient pulse addition was done when the culture was in the exponential phase (biomass = 2 g/L) Further nutrient additions were done when the dissolved oxygen

concentration in the reactor started increasing indicating the substrate depletion. After two pulse additions a constant feed rate of 0.36 L/h (Sorbitol concentration 600 g/L) was maintained till the reactor was full. The fermentation was terminated when no residual sorbitol in the reactor was available as indicated by the increased DO signal.

Dissolved Oxygen (DO) Concentration

An *in situ* Ingold dissolved oxygen probe was used for the measurement of DO concentration in the fermenter. The DO concentration is indicated as % pO_2.

Analytical Techniques

Biomass Concentration

Optical density (OD) of the suitably diluted samples was measured at 600 nm in a UVIKON 930 spectrophotometer (Kontron Instruments, USA). Biomass was estimated from an OD vs concentration (g/L) correlation, which was determined *a priori* as follows:

Biomass concentration of a known volume of the samples were determined gravimetrically after measuring the OD at 600 nm. A standard curve was plotted between OD of the samples and their respective biomass concentrations to arrive at the following correlation.

$$\text{Biomass (g/L)} = 0.73 \times OD_{600}$$

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 at ambient temperature.

RESULTS AND DISCUSSION

Fed-batch Fermentation with Pulse Feed

Fig. 1 shows the kinetics of fed-batch fermentation with four pulse feeds of 0.5 litre containing S_0 = 600 g/L sorbitol. The fermentation was completed in 24 hours with a final biomass and sorbose concentrations of 12.24 and 320.48 g/L respectively. The fermentation also demonstrated a sorbose productivity of 13.35 g $L^{-1}h^{-1}$. It can be seen from Fig. 1 that the maximum sorbitol concentration in the reactor was about 105 g/L at 7.5 hours of fermentation after the addition of first feed pulse consisting of 0.5 litre of 600 g/L sorbitol medium. Second sorbitol feed shot was quickly consumed. Presumably due to high (and active) biomass concentration in the reactor. However the last two sorbitol shots not only diluted the existing sorbose concentration but it took longer time (4-5 h) for total substrate consumption. This may be due to the dilution effect resulting from additional sorbitol feed for not so young (deteriorating) culture. However, by pulse feeding, the ultimate concentration of sorbitol in the reactor could be maintained well below 200 g/L (high inhibitory concentration [4]) throughout the fermentation process.

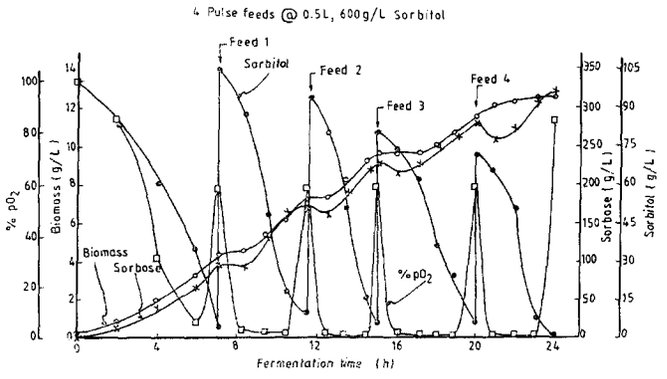

Fig. 1. Fed-batch sorbose fermentation by *Acetobacter suboxydans* with pulse feed: 0-7 h batch (S_0 = 100 g/L), 7-24 h fed-batch (4 pulse feeds of 0.5 L, 600 g/L sorbitol, at 7,12,15 and 20 h) fermentation.

Fed-batch Fermentation with Multiple Feeds

In fed-batch fermentation with pulse feed (as described above) the fresh nutrient additions were done when DO increased, this invariably led to increase in substrate, concentration but at the expense of dilution of existing product concentration in the bioreactor. This may be because towards the end of the batch growth (when substrate concentration is close to zero) the culture activity eventually deteriorates, and at that time growth and production rate during the feeding period of pulse substrate does not match the dilution rate. Therefore it would be beneficial if the fresh nutrient feed shots are added a little bit before the end of the fermentation preferably in the exponential phase of growth. Similarly continuous linear feed as opposed to one time feed shot (pulse) may continuously supply the depleting nutrients and not unnecessarily dilute the existing fermentation broth with respect to biomass and product. In order to eliminate excess accumulation of high sorbitol concentrations in the fermentation broth, it was decided to feed the nutrients at a relatively lower but constant feed rate of 0.36 L/h with S_0 = 600 g/L after the second pulse feed as had been used in Fig. 2. It was anticipated that during continuous feeding the incoming sorbitol will be quickly consumed by the vigorously growing culture and not result in dilution due to fresh sorbitol feed as had happened in the earlier pulse feed case.

Kinetics of fed-batch fermentation with multiple feeds (2 pulse feeds of 0.5 L, 600 g/L followed by fresh medium (with 600 g/L sorbitol) addition at a feed rate of 0.36 L/h) is shown in Fig. 2. The fermentation was completed in 24 h with a sorbose productivity of 15.09 g $L^{-1}h^{-1}$. The final biomass and sorbose concentrations were 12.64 and 332.60 g/L, respectively. The sorbose

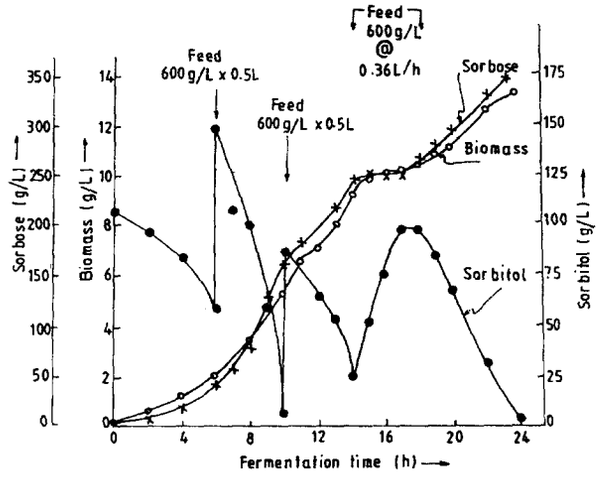

Fig. 2. Fed-batch sorbose fermentation by *Acetobacter suboxydans* with multiple feeds: 0-6 h batch (S_0 = 100 g/L), 6–18 h fed-batch (two pulse feeds of 0.5 L, 600 g/L sorbitol followed by 600 g/L sorbitol at constant feed rate of 0.36 L/h), and 18-22 h batch fermentation.

concentration and productivity obtained by multiple feeding was found to be higher than that obtained by pulse feeding of the nutrients. The improvement in sorbose accumulation and productivity could be attributed due to the following reasons.

- The first pulse feed was added during the exponential growth phase of the culture therefore the dilution due to fresh medium addition was not reflected at all in the observed values of biomass and substrate as was the case with pulse feeding presumably because the incoming substrate was used up quickly during the feeding time and thereafter very little dilution effect was observed during the addition of second pulse feed and later on during the constant feed. The culture growth clearly demonstrated pseudo steady state with respect to substrate and biomass during the constant feeding period indicating the fresh nutrient feeding exactly balanced the growth and product formation rate of the culture.
- Also the sorbitol feeding during constant feed basically maintained high turn over production rates and vigourously growing culture (particularly with out any dilution), which eventually resulted in highly productive fermentation. The high sorbose concentration (332.60 g/L) and productivity (15.09 g $L^{-1} h^{-1}$) obtained by the multiple feeding of sorbitol made it a better processing strategy compared to pulse feeding alone in response to DO increase.

It was rather interesting to compare the productivities of this fermentation with the literature reported fermentations. Srivastava and Lasrado [4] obtained a productivity of 12.6 g $L^{-1} h^{-1}$ by exponential feeding. Mori *et al.* [5] using pure oxygen supply and feeding sorbitol powder to the fermenter enhanced the productivity to 44.85 g $L^{-1} h^{-1}$. Bosjnak *et al.* [6] could get a productivity of 11.62 g $L^{-1} h^{-1}$ by gradient fed-batch fermentation. The present work demonstrated a productivity of 13.35 g $L^{-1} h^{-1}$ for pulse feeding and 15.09 g $L^{-1} h^{-1}$ for multiple feeding of nutrients for fed-batch fermentation, which are higher compared to the above investigators except Mori *et al.* [5]. However, the use of sorbitol powder and pure oxygen supply to the fermenter used in their investigation [5] may not be economically feasible for large scale industrial fermentations.

CONCLUSIONS

The substrate limitation in sorbitol to sorbose fermentation was prominently indicated by abrupt increase in dissolved oxygen signal. The nutrient (substrate) limitation of sorbitol to sorbose fermentation by *Acetobacter suboxydans* was eliminated by adopting "Add substrate when low" technique(s). In one fed-batch fermentation pulse feed design was implemented. 0.5 Liter sorbitol medium (containing 600 g/L sorbitol) was added in four, one-time shots (pulses) in response to abrupt increase of DO signal in the fermentation. This resulted in an overall sorbose productivity of 13.35 g $L^{-1} h^{-1}$ and final sorbose concentration of 320.48 g/L. In another fed-batch fermentation design two shots (pulses) of fresh nutrient (0.5 Liter, sorbitol concentration 600 g/L) were added and thereafter a constant fresh nutrient supply (sorbitol concentration 600 g/L) at a rate of 0.36 L/h was maintained in the reactor till it was full. The above fed-batch resulted in sorbose accumulation of 332.60 g/L with an overall productivity of 15.09 g $L^{-1} h^{-1}$. The pulse feed during the exponentially growing culture and slow continuous substrate feeding were much beneficial in improving the productivity of fermentation as it did not excessively dilute the existing biomass and product concentrations in the reactor. The high sorbose concentration and productivity obtained by multiple feeding strategies made it a better processing technique compared to the pulse feeding alone.

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