HIGH DENSITY PRODUCTION OF SORBOSE FROM SORBITOL BY FED-BATCH CULTURE WITH DO-STAT

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Gluconobacter suboxydans was cultivated in a fed-batch culture with a DO-stat under the condition that none of the components in the basal medium limited the growth of the microorganism and the production of sorbose. Final concentration of sorbose reached 460 g/_l_ after 16 hrs with CSL medium and 628 g/_l_ after 14 hrs with YE medium, respectively. Conversion ratio of sorbitol to sorbose was almost maintained at about 0.9 for CSL medium and about 1.0 for YE medium during the cultivation. The effect of sorbose concentration on growth was investigated in test tube culture. Decrease in specific growth rate and a lag phase were observed with increase in sorbose concentration in culture medium. The growth was inhibited considerably at 300 g/_l_ of sorbose concentration and was not observed at sorbose concentrations over 510 g/_l_. The effect of osmotic pressure and water activity on growth was examined. Growth rate and sorbose production rate decreased as osmotic pressure increased or water activity decreased due to the accumulation of sorbose.

Introduction

In fermentation industries, batch culture is mainly used for microbial production. As the initial concentration of carbon source is restricted in the batch culture, high concentration of products is not obtained. In fed-batch culture, carbon source and/or other nutrients are fed and higher productivity can be achieved. In the case of biomass production in the fed-batch culture, however, it was difficult to obtain high concentration of biomass because oxygen concentration is apt to become a growth-limiting factor. Thus, the DO-stat[27], a device to keep concentration of dissolved oxygen in the broth (abbreviation: DO) constant, has been developed. With the attachment of the DO-stat, pure oxygen or oxygen gas mixed with air could be supplied to the fermentor without toxicity due to high DO. In fact, high concentration of biomass (85 g/*l* for *P. ruber*, 125 g/*l* for *E. coli* and 138 g/*l* for *C. brassicae*) was obtained in the fed-batch culture with the DO-stat under the condition that none of the components in the basal medium limited the growth of the microorganisms[14,26]. On the other hand, few studies[9] on high concentration of microbial products other than biomass have been reported.

Many studies[2,23–25] on sorbose fermentation have been carried out since the finding by Bertrand[3] of a microorganism capable of oxidizing sorbitol. With the development of vitamin chemistry, L-sorbose became a useful intermediate for the synthesis of L-ascorbic acid (vitamin C) from glucose. Thus, sorbose fermentation, in which D-sorbitol is oxidized to L-sorbose, has been an important step in vitamin C production[16]. Some findings on the metabolic mechanism of oxidation of sorbitol from the viewpoint of enzymology have been reported[1,20]. In the field of fermentation technology, on the other hand, various kinds of culture conditions have been reported but were mostly based upon the batch culture. The maximum sorbose concentration reported[25] is 190 g/*l* as far as we know. In fed-batch culture, on the other hand, it is possible to keep concentrations of various nutrients within suitable ranges during cultivation.

In the present work, *Gluconobacter suboxydans* was cultivated in a fed-batch culture in which sorbitol was fed intermittently, with the DO-stat under the condition that none of the components in the basal medium limited the growth of the microorganism and the production of sorbose. The respiration activity of the microorganism obtained at various cultivation times in the fed-batch culture was measured in the fresh medium. The effect of sorbose concentration on growth was examined in test tube culture. Osmotic pressure and vapor pressure in the supernatant were

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Table 1 Medium compositions for *G. suboxydans*

Component	Concentration YE medium	Concentration CSL medium
KH_2PO_4	10 g/*l*	10 g/*l*
$(NH_4)_2SO_4$	3	3
$MgSO_4 \cdot 7H_2O$	1	1
$FeSO_4 \cdot 7H_2O$	20 mg/*l*	20 mg/*l*
$MnSO_4 \cdot nH_2O$	20	20
$CaCl_2 \cdot 2H_2O$	40	40
$CoCl_2 \cdot 6H_2O$	4	4
$AlCl_3 \cdot 6H_2O$	1	1
H_3BO_3	0.5	0.5
Yeast extract (Oriental)	20 g/*l*	—
Corn steep liquor (Ohji)	—	20 g/*l*
D-sorbitol	variable	variable
pH	5.0	4.0

measured and the effect of osmotic pressure and water activity on the growth of the microorganism and the production of sorbose was examined.

1. Experimental

1. 1 Microorganism and chemicals

The organism used in this study was *Gluconobacter suboxydans* ATCC 621. All the chemicals used were of reagent grade. Water was deionized after distillation.

1. 2 Cultivation

The compositions of basal medium are shown in **Table 1**.

For the cultivation of *G. suboxydans* with YE medium, pH and temperature were automatically controlled at 5.0 with 33% ammonia water and at 30°C, respectively. For the cultivation with CSL medium, pH and temperature were controlled at 4.0 and at 30°C, respectively. DO was automatically controlled at 2–3 ppm with the DO-stat. The seed culture was prepared as follows. A loop of the microorganism from a stock culture was inoculated into 5 m*l* of a medium consisting of 10% sorbitol, 2% corn steep liquor and 0.3% calcium carbonate (pH: 7.0) in a test tube (18×180 mm) and cultured for 48 hr at 30°C on a reciprocal shaker. This test tube culture (1 m*l*) was transferred into 100 m*l* of the basal medium (sorbitol concentration: 100 g/*l*) in a 500 m*l* Sakaguchi flask, and then cultured for 48 hr at 30°C on a reciprocal shaker. This seed culture (200 m*l*) was transferred into a jar-fermentor equipped with a four-bladed disc-turbine impeller ($D_i/D_T=0.5$) and three baffles (initial working volume: 1 *l*, Iwashiya Co., Type MB). The initial concentration of sorbitol in the culture medium was 80 g/*l* and sorbitol powder was supplied to the culture intermittently by the DO-stat and manual operation during the cultivation. No essential components other than sorbitol and oxygen were supplied to the fermentor during the cultivation because their levels at the end of the cultivation were not deficient in the previous experiments. Foam was suppressed, when necessary, by the addition of sterilized 20% Silicone KM-72 as an antifoamer. In the middle and late stages of the cultivation, pure oxygen gas was supplied to the fermentor to keep DO level constant.

For evaluation of the effect of sorbose on the growth of the microorganism, the following two kinds of test tube culture were carried out on a reciprocal shaker at 30°C.

Method (a): In various periods of the fed-batch cultivation with YE medium, culture broth was taken and cells separated from the broth by centrifugation were suspended in a sterilized NaCl (0.9%) solution. This suspension was further diluted with the same solution so that the concentration of biomass was between 3.0 g/*l* and 4.0 g/*l*, and then this dilute suspension was used as the inoculum. Ten drops (about 0.3 m*l*) of this inoculum were transferred into a test tube (18×180 mm) with the YE medium (6 m*l*, sorbitol concentration; 50 g/*l*, pH: 5.0), containing no sorbose or comparable amount of sorbose in the broth at each sampling time in the fed-batch culture.

Method (b): Culture broth at the late stage (at 22 hr after the inoculation) of logarithmic phase in the test tube culture with YE medium (sorbitol concentration; 100 g/*l*) was used as the inoculum. Ten drops of this culture broth were transferred into the test tube with the YE medium (6 m*l*, sorbitol concentration; 100 g/*l*, pH: 5.0) containing various concentrations of sorbose.

1. 3 Analytical method

Growth of the microorganism was evaluated by measuring the optical density at 570 nm of the culture broth with a Shimadzu Spectronic 20 spectrophotometer. The cell concentration was also determined by weighing after the centrifuged cells were dried. The ratio of optical density to dry cell weight [g/*l*] was 2.69.

Concentrations of sorbose and sorbitol were determined by gas chromatography as follows. The centrifuged supernatant (0.2 m*l*) diluted with water was mixed with 0.2 m*l* of a solution of inositol·$2H_2O$ (5 g/*l*) as internal standard, and then this mixture was freeze-dried. The dried material, after being dried in a dessicator overnight, was trimethylsilylated as described by Sweeley[19]. This silylated sample (1 μl) was injected into a gas chromatograph (Hitachi Seisakusho Co., model 163) equipped with a flame ionization detector and a 2-m stainless steel column packed with 1.5% Silicone OV-1, Shimalite W (80–100 mesh). Conditions of the gas chromatography were as follows; injection temperature: 200°C, initial column temperature: 180°C, increasing rate of column temperature: 2°C/min. The ratios of peak height on chart for 1 mg

Table 2 Lists of concentrations of biomass and sorbose at 12 hrs cultivation in fed-batch culture of *G. suboxydans* with CSL medium and YE medium at various pH values

Medium	pH	X [g/l]	P [g/l]
CSL	3	0.38	—
	4	2.09	365
	5	2.83	252
	6	3.75	138
	7	1.12	82
YE	4	3.12	362
	5	3.65	403
	6	3.25	341

—: not measured

of sorbose and 1 mg of sorbitol to that for 1 mg of inositol·$2H_2O$ were 1.18 and 1.24, respectively. During the cultivation, sorbitol was supplied intermittently to the culture broth. Residual concentrations of sorbitol and sorbose and volume of the culture broth were determined at various cultivation times, and conversion ratio of sorbitol to sorbose was evaluated.

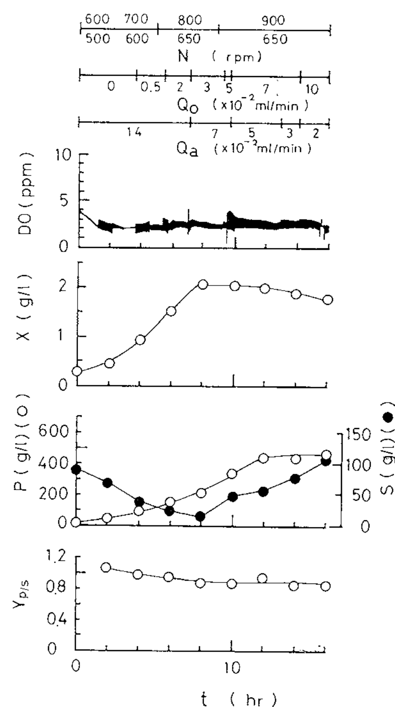
DO was measured with an oxygen sensor (Beckman, Fieldlab oxygen analyzer). The analysis of respiration activity of the microorganism was carried out as follows. The YE medium (3.1 ml, sorbitol concentration; 10 g/l) which was saturated with air was agitated by a stirrer in a glass vessel (total volume; 3.3 ml) equipped with an oxygen sensor at 30°C. Then, 0.2 ml of the culture broth was transferred into the vessel and DO decrease was monitored after the vessel was closed. The respiration activity was expressed as the oxygen consumption rate per unit biomass.

For measurement of the osmotic pressure of the centrifuged supernatant, the supernatant (1 ml) was mixed with water (5 ml). Osmotic pressure of this mixture was measured with an osmometer (Advanced Instruments, Advanced Osmometer Models 3L, 3W).

The analysis of vapor pressure of the supernatant was carried out as described by Taniguchi[21]. Water activity[4] was expressed as the ratio of the vapor pressure of the supernatant to that of water at 30°C.

2. Results

Fed-batch cultures of *G. suboxydans* with CSL medium were carried out for 12 hrs by maintaining the medium pH constant. As shown in **Table 2**, the maximum sorbose concentration was obtained at pH 4.0. Thus, the fed-batch culture for longer cultivation time was carried out at pH 4.0 (**Fig. 1**). High concentration of sorbose (460 g/l) was obtained after 16 hrs cultivation. For the first 4 hrs, the specific growth rate was constant (0.37 hr^{-1}). Linear growth was observed from 4 hrs to 8 hrs. Concentration of biomass was maximum (2.06 g/l) at 8 hrs and then decreased gradually. The DO level was controlled at 2–3 ppm



DO-level was controlled by switching upper and lower agitation speeds of impeller. Upper and lower agitation speeds and flow rates of air and pure oxygen gas were changed manually as shown in the upper part of the figure.

Fig. 1 Cultivation results of *G. suboxydans* in fed-batch culture with DO-stat (CSL medium)

with the DO-stat during the cultivation. Concentration of sorbose increased logarithmically until 10 hrs and production rate of sorbose decreased gradually. Conversion ratio of sorbitol to sorbose decreased gradually from 1.0 to 0.8. When corn steep liquor (20 g) was added to the fermentor at 8 hrs in another fed-batch culture with CSL medium at pH 4.0, concentration of biomass increased until 12 hrs (maximum biomass concentration: 2.47 g/l at 12 hrs), but the time-course of sorbose production corresponded to that with no addition of corn steep liquor at 8 hrs.

Fed-batch cultures with YE medium were carried out by maintaining the medium pH constant. As shown in Table 2, the maximum sorbose concentration was obtained at pH 5.0. Thus, the fed-batch culture for longer cultivation time was carried out at pH 5.0 (**Fig. 2**). High concentration of sorbose (628 g/l) was obtained at 14 hrs. For the first 8 hrs, the specific growth rate was constant (0.30 hr^{-1}). The growth rate of the microorganism decreased after 8 hrs and concentration of biomass was maximum (3.33 g/l) at 12 hrs and then decreased gradually. The DO level was controlled at 2–3 ppm with the DO-stat but after 12 hrs it fell to almost 0 ppm, although agitation was carried out at high speed (900 rpm) and pure oxygen

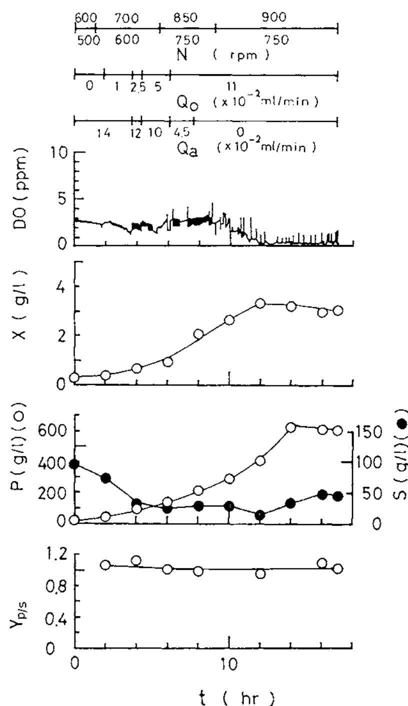
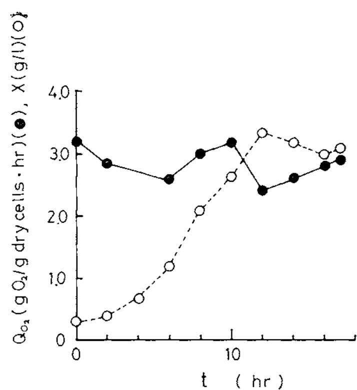


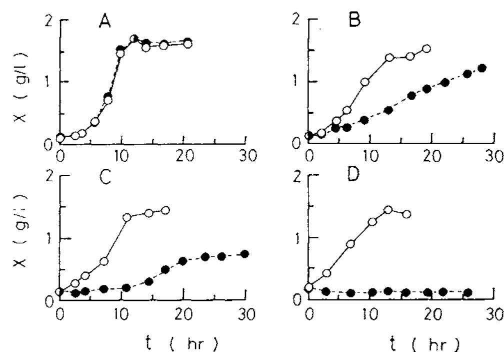
Fig. 2 Cultivation results of *G. suboxydans* in fed-batch culture with DO-stat (YE medium)



The culture broth was obtained at each cultivation time in the fed-batch culture shown in Fig. 2. Growth curve in this figure is the same as shown in Fig. 2.

Fig. 3 Time-courses of Q_{O_2} (●) and growth (○) in fed-batch culture

gas (about 0.9 vvm) was supplied to the fermentor. In the late stage of the cultivation shown in Fig. 2, the growth of the microorganism became oxygen-limited. Concentration of sorbose increased logarithmically for the first 14 hrs and then decreased a little. Conversion ratio of sorbitol to sorbose was kept at about 1.0 during the cultivation. When yeast extract (20 g) was added to the fermentor at 8 hrs in another fed-batch culture with YE medium at pH 5.0, concentration of biomass was maximum (4.35 g/*l*) at 12 hrs, but sorbose production rate was retarded. Maximum sorbose concentration (590 g/*l*) was obtained at 17 hrs.



Cultivation time in Fig. 2 and concentration [g/*l*] of sorbose in test tube were as follows. A: 0 hr, 20 (22) B: 8 hr, 168 (214) C: 10 hr, 300 (293) D: 14 hr, 506 (628). Figure in parentheses is concentration [g/*l*] of sorbose in the supernatant at each cultivation time in Fig. 2 obtained by the analysis. Solid lines show cultivation results when sorbose was not added to culture medium. Dotted lines show those when amounts of sorbose shown above were added to culture medium.

Fig. 4 Effects of cultivation time and sorbose on growth of *G. suboxydans*

In the other fed-batch experiments, inorganic phosphorus, nitrogen and metal ions as the essential components other than sorbitol and oxygen were found not to be growth-limiting factors during the cultivation.

Respiration activity in the fresh medium of the microorganism was measured at each cultivation time in the fed-batch culture shown in Fig. 2. As shown in **Fig. 3**, respiration activity was maintained between 2.5 and 3.0 g O_2/g dry cells·hr during not only the growth but also the declining phases.

To examine the effect of sorbose concentration on the growth of the microorganism, the test tube cultivation described in Method (a) was carried out by using the culture shown in Fig. 2 as the inoculum. As shown in **Fig. 4**, growth of the microorganism obtained at each cultivation time of the fed-batch culture showed almost the same pattern, when sorbose was not added to the culture medium. On the other hand, when sorbose was added to the culture medium, the decrease in specific growth rate and the lag phase were observed with increase in sorbose concentration. In the case of sorbose addition at low concentration (20 g/*l*), the growth coincided with that for no addition and linear growth was observed on addition of 170 g/*l* of sorbose. Growth was not observed at all on addition of sorbose at higher concentration (510 g/*l*). From these experiments, it was found that the retardation and stoppage of the growth rate shown in Fig. 2 were not caused by the accumulation of metabolic inhibitor(s) but were due to the accumulation of sorbose.

To make this clearer, another test tube cultivation described in Method (b) was carried out by using the culture broth taken at the late stage of logarithmic phase in the test tube culture as the inoculum. As shown in **Fig. 5**, the decrease in specific growth rate and the lag phase were observed as concentration of sorbose in culture medium increased and no growth at all was observed in the case of the addition of sorbose at higher concentration (620 g/*l*).

Figure 6 shows the effects of osmotic pressure and water activity on the growth of the microorganism and the production of sorbose. As concentration of sorbose increased, osmotic pressure increased gradually. Water activity decreased gradually till 10 hrs (A_w=0.847) and then decreased rapidly to 0.758, approaching this value after 12 hrs. The cultivation time when water activity decreased rapidly coincided with the time when concentration of biomass reached the maximum. The following relation is known[4,18] between osmotic pressure and water activity.

$$\pi = \frac{-RT \ln A_w}{V_w}$$

By replotting both osmotic pressure and logarithm of reciprocal of water activity ($-\ln A_w$) against cultivation time, time-courses similar of each other were obtained.

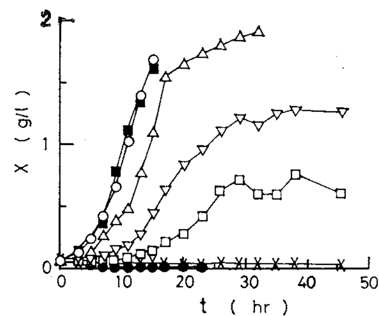
3. Discussion

In the present fed-batch cultures of *G. suboxydans* with CSL medium and YE medium, final concentrations of sorbose reached 460 g/*l* at 16 hrs and 628 g/*l* at 14 hrs, respectively. The final volume of the culture was 1.5 *l* with CSL medium and 1.2 *l* with YE medium. It may be said that high production rate of sorbose was obtained by the fed-batch culture with the DO-stat.

As shown in Figs. 1 and 2, differences in both maximum biomass concentration and oxygen uptake rate of the microorganism might be mainly due to the difference in the natural nutrients used. It was reported that *G. suboxydans* ATCC 621 required pantothenate, *p*-aminobenzoate, nicotinate and some amino acid(s) as growth factor[7,22]. In fact, yeast extract includes these growth factors abundantly but their concentrations in corn steep liquor are low.

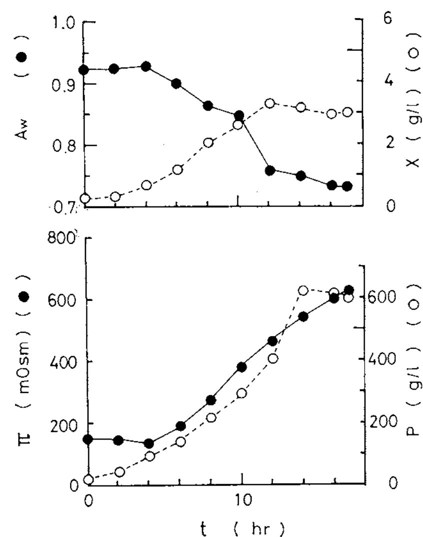
Kono and Asai[11] indicated that the sorbose fermentation by *G. suboxydans* studied by Fencl *et al.*[6] was an example of the fermentation process in which product formation is associated with growth. As shown in the present experiments, however, a pattern not associated with growth was recognized when sorbose concentration became high.

As described above, additions of yeast extract and corn steep liquor did not increase the production rate of sorbose. DO was maintained at 2–3 ppm. Sorbitol



Concentration [g/*l*] of sorbose in culture medium was as follows. ■: 0, ○: 22, △: 90, ▽: 210, □: 290, ×: 620, ●: 660.

Fig. 5 Effect of sorbose concentration on growth of *G. suboxydans*



Time-courses of growth and sorbose production shown in this figure are the same ones as shown in Fig. 2.

Fig. 6 Effects of osmotic pressure and water activity on growth of *G. suboxydans* and sorbose production

concentration was almost controlled below 5–10% not to be at such high level as to cause growth inhibition as pointed out by Kölblin *et al.*[10]. Asai *et al.*[2] indicated that iron inhibited sorbose fermentation. Thus, fed-batch cultivation with the YE medium containing no ferrous sulfate was carried out. Time-courses of growth and sorbose production coincided with those shown in Fig. 2. Furthermore, analysis of metabolic products in the supernatant separated from the final broth in Fig. 2 was carried out. Acetate and lactate were detected but their concentrations were not at such high level as to cause growth inhibition. It was also confirmed by the test tube culture experiments, as shown in Fig. 4, that the time-courses of growth in fresh medium were similar to each other. The evolution of carbon dioxide may be small as shown in the metabolic characteristics of the micro-

organism[5]. From these facts, it may be considered that the essential components in the medium were not deficient and not at such high level as to cause growth inhibition and that metabolic products other than sorbose did not cause growth inhibition.

As shown in Fig. 6, the cultivation time when water activity decreased rapidly coincided with the time when concentration of biomass reached the maximum. Concentration of sorbose reached the maximum when osmotic pressure reached a high level (538 mOsm) and water activity decreased to 0.748. The increase in osmotic pressure and the decrease in water activity may be mainly due to the accumulation of sorbose because concentrations of biomass and the essential components other than sorbitol were at such low levels and sorbitol concentration was controlled below 5–10% during the cultivation. Sayed *et al.*[17] reported that high osmotic pressure resulted in both a substantially lower growth rate and lower peak yield of *Ureplasma urealyticum*. Prior *et al.*[15] indicated that decrease in growth rate of *Pseudomonas fluorescens* and the catabolism effect of glucose were due to a decrease in water activity. Most bacteria are unable to grow[13] at water activity less than 0.9, and most yeasts and moulds are unable to grow[8,12] at water activity less than 0.85. Thus, it may be considered that retardations and stoppage of growth and sorbose production rates were due to the accumulation of sorbose to an inhibitory level, which caused both the increase in osmotic pressure and the decrease in water activity. From these facts, it may be difficult to obtain higher sorbose concentration than 628 g/*l*.

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Nomenclature

A_w	= water activity in supernatant	[—]
D_i	= diameter of impeller	[m]
D_T	= diameter of fermentor	[m]
DO	= dissolved oxygen concentration in culture broth	[ppm]
P	= concentration of sorbose	[g sorbose/*l*]
Q_a	= flow rate of air	[m*l*/min]
Q_O	= flow rate of pure oxygen gas	[m*l*/min]
Q_{O_2}	= respiration activity of microorganism in fresh medium	[g O_2/g dry cells·hr]
S	= concentration of sorbitol	[g sorbitol/*l*]
t	= cultivation time	[hr]
X	= concentration of biomass	[g dry cells/*l*]
$Y_{P/S}$	= conversion ratio of sorbitol to sorbose	[g sorbose produced/g sorbitol consumed]
π	= osmotic pressure in six-fold diluted supernatant	[mOsm]

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