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Effect of High Oxygen Partial Pressure on the Conversion of Sorbitol to Sorbose by *Acetobacter suboxydans**

SHIGEKI YAMADA, MITSURU WADA, and ICHIRO CHIBATA

Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., 16-89, Kashima-3-chome, Yodogawa-ku, Osaka 532

The conversion of sorbitol to sorbose by *Acetobacter suboxydans* was performed under oxygen-enriched atmosphere in shaken flasks and the effect of oxygen partial pressure upon the cell growth and the oxidizing activity was investigated.

By use of oxygen-enriched air the conversion was greatly accelerated, mainly because of extension of the oxygen transfer capability, up to an atmospheric concentration of oxygen of 40%. At higher concentrations of oxygen, growth inhibition due to oxygen was observed and the fermentation was reduced. However, when cultures grown in 40% oxygen-enriched atmosphere were used as inoculum, growth inhibition was not observed even in 80% oxygen-enriched atmosphere and the fermentation was markedly accelerated. This is because the cells grown in the enriched atmosphere adapted to the conditions, acquiring higher resistance to oxygen damage and enhanced oxidizing activity. Thus, the advantages of enriching the gas phase of shaken flasks with oxygen was demonstrated in the oxidation of sorbitol to sorbose by *Acetobacter suboxydans*.

In a typical aerobic fermentation oxygen is usually recognized as a substrate. To increase the productivity of aerobic fermentations oxygen must be supplied at a rate that meets the needs of the microorganism. The importance of oxygen supply in aerobic fermentations has been well documented in many reviews.[1~4]

Our previous report[5] showed that the oxygen transfer capability of shaken flasks is proportional to aeration coefficient K, *i.e.* the overall volumetric oxygen transfer coefficient involving both diffusibility through the cotton plug and across the gas-liquid interface $(1/K_1+1/K_2)^{-1}$ divided by the medium volume V_l, when the driving force (p_a-p_l) is constant.

$$n=K(p_a-p_l)$$

$$K=(1/K_1+1/K_2)^{-1}\cdot 1/V_l$$

where n is the total oxygen transfer rate per unit volume of culture fluid (mole O_2/ml·hr), K is the aeration coefficient (mole O_2/ml·hr), p_a and p_l are the oxygen partial pressures in the atmosphere and culture fluid (atm), V_l is the volume of culture fluid (ml), K_1 is the volumetric oxygen transfer coefficient through the cotton plug (mole O_2/atm·hr), and K_2 is the volumetric oxygen transfer coefficient across the gas-liquid interface (mole O_2/atm·hr).

Aeration efficiency in shaken flask cultures was compared with different types of equipment under various operating conditions. In the conversion of sorbitol to sorbose by *Acetobacter suboxydans* ATCC 621, the values of K under usual conditions were less than $2.0\sim2.5\times10^{-4}$ mole O_2/ml·atm·hr, except under extremely aerobic conditions. Thus, the theoretical maximum oxygen transfer rate in air was less than $4.0\sim5.0\times10^{-5}$ mole O_2/ml·hr, since oxygen partial pressure in air is about 0.2 atm. On the other hand, the oxidizing activity of the culture broth was approximately 7×10^{-5} mole O_2/ml·hr. Evidently the oxygen demand exceeded the oxygen supply and

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the fermentation exhibited oxygen deficiency. To obtain the maximum productivity oxygen transfer rates should be increased. As shown in our previous report, improvement of equipment performance is a particularly good means of achieving this. However, the above equation indicates that use of a large driving force, *i.e.* enriching the gas phase with oxygen, will also be an effective means of improving oxygen transfer. From this consideration, the conversion of sorbitol to sorbose was carried out in oxygen enriched atmosphere.

Wells *et al.*[6~7] showed that the oxidation of sorbitol to sorbose by *Acetobacter suboxydance* was markedly accelerated when the air pressure in a rotary drum fermentor was raised to 2 atm. Damodaran and Subramanian[8] also reported the use of oxygen instead of air for accelerating the above conversion, but detailed data were not included. Recently, Flickinger and Perlman[9] reported that the conversion of glycerol to dihydroxyacetone by *Gluconobacter melanogenus* increased when the dissolved oxygen partial pressure was controlled at 0.05 atm by use of oxygen-enriched aeration. In contrast, the oxidation of sorbitol to sorbose by *Acetobacter melanogenum* was found to be reduced in environments in which the atmospheric concentration of oxygen is 50% or above.[10,11] Numerous studies have also revealed that high oxygen partial pressure inhibits or delays the growth of many aerobic bacteria.[12,13] Consequently, the effect of high oxygen partial pressure on *Acetobacter* has not yet been adequately explored.

The present paper shows the effect of high oxygen partial pressure on the growth and the oxidizing activity of *Acetobacter suboxydans* ATCC 621 and the advantages of enriching the gas phase of shaken flasks with oxygen in the conversion of sorbitol to sorbose by this organism.

Materials and Methods

Microorganism and cultivation method *Acetobacter suboxydans* ATCC 621 was grown in a 5% sorbitol solution containing 2% corn steep liquor and 0.3% $CaCO_3$, and was transferred to the fermentation medium containing 20% sorbitol, 2% corn steep liquor and 0.3% $CaCO_3$. After several transfers in the fermentation medium, which increased the oxidizing capacity of this strain, the culture broth was used as inoculum. Usually the inoculum was prepared over several months by the above consecutive transfers. Unless otherwise noted, 100-ml portions of the medium in cotton-plugged 500-ml Sakaguchi flasks were inoculated with 10 ml of inoculum and incubated at 30°C for 48 hr under air on a reciprocating shaker at 140 strokes/min with an 8.5-cm stroke. These aerobic conditions were equivalent to $K=1.32\times10^{-4}$ mole O_2/ml·atm·hr. The fermentations under oxygen-enriched aeration were carried out with the apparatus shown in Fig. 1.

Apparatus for fermentation in oxygen-enriched atmosphere The apparatus for fermentation at different oxygen partial pressures is shown diagrammatically in Fig. 1. Sakaguchi flasks with two side holes for oxygen electrodes were employed. The top was closed with a rubber stopper fitted with gas inlet and outlet tubes. The oxygen-enriched gas was prepared in large reservoir and circulated by a diaphragm pump. During the fermentation the total pressure was held at 1 atm and the oxygen partial pressure in the circulating gas was kept constant. The circulating gas was passed through water in flask (A) shaking under the same conditions as the main fermentation flask (B). The moist gas then passed into the free space of the fermentation flask (B). Outlet gas from the fermentation flask was returned to the reservoir through KOH solution to remove CO_2. The reservoir was large enough that the oxygen partial pressure in it was not decreased appreciably by oxygen consumption during fermentation. The fermentations were carried out at 30°C in 50 ml of medium in flask B on a reciprocating shaker operating at 140 strokes/min.

Analytical methods The oxygen partial pressures in the cultures and in the gas phase were continuously measured with a membrane-type oxygen electrode (Beckman 777 oxygen analyzer). Aeration coefficient

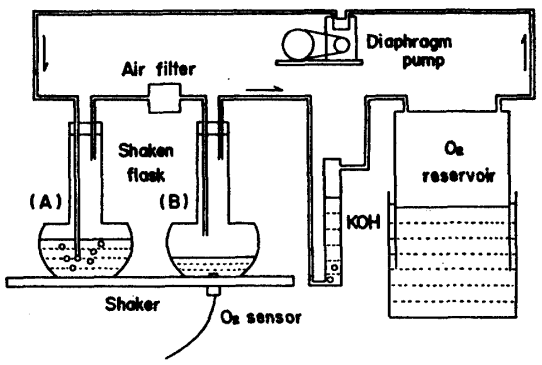

Fig. 1. Apparatus for shaken flask cultures in oxygen-enriched atmosphere.

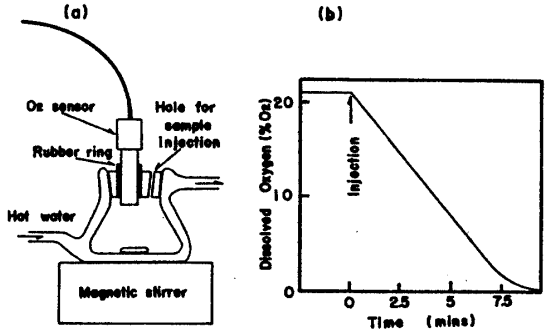

Fig. 2. (a) Apparatus for measuring oxidizing activity; (b) Plot of oxygen consumption.

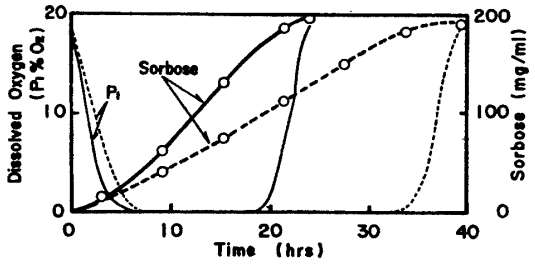

Fig. 3. Sorbose fermentation under air. ····· $K=1.32\times10^{-4}$ mole O_2/ml·atm·hr (cotton plug, 5 g; liquid volume, 100 ml; shaking speed, 140 strokes/min), —— $K=2.80\times10^{-4}$ mole O_2/ml·atm·hr (Toyoflon closure; liquid volume, 50 ml; shaking speed, 140 strokes/min).

and oxygen transfer rate were determined by the "steady state gas analytical method" described in our previous report.[5] Sorbose was determined in a Technicon Autoanalyzer according to the method of Hoffman.[14] The sorbitol oxidizing activity of cells, *i.e.*, the oxygen consumption rate of cells, was estimated form the decrease in the dissolved oxygen level in 20% sorbitol solution in a completely closed vessel, measured continuously with a membrane oxygen electrode (Fig. 2-a). The electrode was fitted tightly into the reaction vessel through a rubber ring mounted in the stopper of the vessel, so that leakage of air did not occur. The reaction vessel was almost completely filled with the air-saturated substrate solution, which was stirred magnetically with a teflon-coated stirring bar. The meter of the oxygen analyzer was set at full-scale deflection against 100% air saturation. A bacterial cell suspension was injected into the reaction vessel with a syringe through a very small hole in the stopper, filling the reaction vessel. Oxygen was consumed at a constant rate until the dissolved oxygen fell to an apparently critical level, nearly zero, and then more slowly (Fig. 2-b). Continuous recording of the oxygen consumption showed a straight line, and the oxygen consumption rate was readily determined from the slope of this straight line and the experimental relationship between full-scale deflection of the recorder and the oxygen concentration by Winkler's method. To avoid damaging cells by centrifuging and washing, the fermentation broth was used as the bacterial cell suspension.

Results and Discussion

Conversion of sorbitol to sorbose under air The fermentations in air were strongly affected by the aeration coefficient. Figure 3 shows typical fermentations carried out under conditions of high ($K=2.80\times10^{-4}$ mole O_2/ml·atm·hr) and low ($K=1.32\times10^{-4}$ mole O_2/ml·atm·hr) aeration coefficient. Shortly after inoculation, the dissolved oxygen level in the fermentation medium dropped rapidly, but remained appreciable because the cell concentrations were low and the oxygen demand was not so high. In this period sufficient oxygen was available, and the oxygen consumption and oxygen demand of the cultures were equal. As the cells increased exponentially, the oxygen demand increased rapidly, the dissolved oxygen level fell to a critical level, almost zero, and oxygen was supplied at the theoretical maximum oxygen transfer rate corresponding to the aeration coefficient. Subsequently oxygen demand exceeded the supply rate, and oxygen supply became limiting. The actual oxygen consumption rate was then equal to the theoretical maximum oxygen transfer rate corresponding to the aeration coefficient for the given operating conditions. Sorbose was also produced linearly at this phase, until quantitative conversion was attained. The data shown in Table 1 indicate that sorbose production rate in the steady state was determined strictly by the aeration coefficient. The specific oxidizing activity of the cells and the total oxidizing activity of the cultures are also shown in the same table. Neither activity was dependent on the aeration coefficient. The total oxidizing activity, namely oxygen demand, was greater than the actual oxygen transfer rate or sorbose production rate. In the oxidation of sorbitol to sorbose, one mole of oxygen corresponds to 2 moles of sorbose stoichiometrically.

Conversion of sorbitol to sorbose in atmospheres enriched with up to 40%

Table 1. Conversion of sorbitol to sorbose in air.[a]

K[b]	p_l in Steady state[c]	OTR[d]	SPR[e]	Cell[f]	Oxidizing activity	
					specific[g]	total[h]
1.32[i]	0	2.77	5.56	1.64	4.22	6.92
2.80[i]	0	5.85	9.68	1.65	4.27	7.05
3.19[j]	0	6.70	11.14	1.69	4.25	7.18

[a] The data show the values in the steady state during the period of maximum conversion.
[b] Aeration coefficient, 10^{-4} mole O_2/ml·atm·hr.
[c] Dissolved oxygen partial pressure, atm.
[d] Oxygen transfer rate, 10^{-5} mole O_2/ml·hr.
[e] Sorbose production rate, 10^{-5} mole sorbose/ml·hr.
[f] Dry cell weight, mg/ml.
[g] 10^{-5} mole O_2/mg cell·hr.
[h] 10^{-5} mole O_2/ml·hr.
[i] See Fig. 3.
[j] Without plug; liquid volume, 50 ml; shaking speed, 140 strokes/min.

oxygen The above results in an air environment suggested that the conversion would be accelerated by use of an oxygen-enriched atmosphere instead of air. Therefore, the fermentation was carried out under different partial pressures of oxygen by using the apparatus shown in Fig. 1. The course of the fermentation at an aeration coefficient of 3.19×10^{-4} mole O_2/ml·atm·hr under a 40% oxygen atmosphere (0.4 atm oxygen partial pressure) is shown in Fig. 4, and that of the ordinary fermentation in an air environment is shown by dotted lines for comparison. Under a 40% oxygen atmosphere dissolved oxygen levels did not fall to zero even at the time of maximum production. The cells grew well in sufficient oxygen, and the fermentation proceeded more rapidly. The oxidation was completed in 20 hr. The cell yield, the oxidizing activity of the cells and sorbose production rate are listed in Table 2. The enhancement by use of oxygen-enriched air was comparable to that obtained by improvement of equipment performance as described in our previous report.[5]

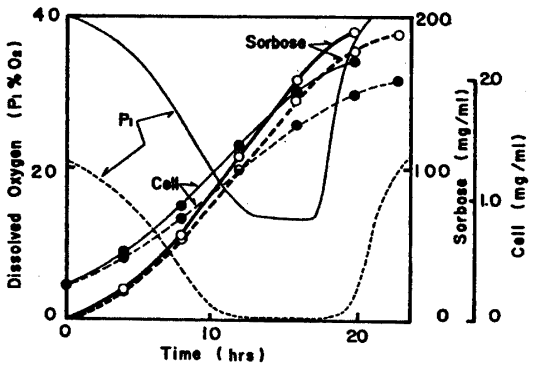

Fig. 4. Sorbose fermentation in a 40% oxygen atmosphere. —— 40% oxygen, ····· air; ○, sorbose concentration, mg/ml; ●, cell concentration, mg/ml.

Conversion of sorbitol to sorbose in atmospheres enriched with more than 60% oxygen The oxygen content of oxygen-enriched air was increased further. First, ordinary cultures growing in the cotton-plugged shaken flasks in an air environment were used as an inoculum. The results differ markedly from those in a 40% oxygen-enriched atmosphere. As a typical example, the fermentation in an 80% oxygen-enriched atmosphere is shown by dotted lines in Fig. 5. The cell growth was considerably inhibited and sorbose production rate was reduced even in the presence of abundant oxygen.

In contrast, when cultures grown at a high level of dissolved oxygen were used as inoculum, the fermentation was remarkably accelerated even in atmosphere enriched with more than 60% oxygen. *A. suboxydans* grown under a high oxygen level is considered to

Table 2. Conversion of sorbitol to sorbose in oxygen-enriched atmosphere.[a]

Expt.	Inoculum	Atmosphere	p_l in steady state[b]	OTR[c]	SPR[d]	Cell[e]	Oxidizing activity	
							specific[f]	total[g]
1	air-grown cells	air	0	6.69	11.14	1.69	4.25	7.18
2		40% O_2	0.12	8.93	16.39	1.92	5.20	9.99
3		60% O_2	0.51	2.87	6.30	0.85	5.05	4.29
4		80% O_2	0.73	2.23	4.20	0.51	4.78	2.44
5	40% O_2-grown cells[h]	60% O_2	0.29	9.89	17.00	2.02	5.45	12.30
6		80% O_2	0.36	13.40	18.33	2.11	5.75	12.13
7	80% O_2-grown cells[i]	80% O_2	0.43	11.80	16.74	2.15	5.96	12.82

[a] The data show the values in the steady state during the period of maximum conversion.
[b] Dissolved oxygen partial pressure, atm.
[c] Oxygen transfer rate, 10^{-5} mole O_2/ml·hr.
[d] Sorbose production rate, 10^{-5} mole sorbose/ml·hr.
[e] Dry cell weight, mg/ml.
[f] 10^{-5} mole O_2/mg cell·hr.
[g] 10^{-5} mole O_2/ml broth·hr.
[h] Cells grown in expt. 2 were used as inoculum.
[i] Cells grown in expt. 6 were used as inoculum.

acquire a higher resistance to oxygen inhibition. As an example, Fig. 5 shows the fermentation in which medium was inoculated with a culture grown in a 40% oxygen atmosphere and incubated in an 80% oxygen atmosphere. Both cell growth and sorbose production rates were increased remarkably and the fermentation was complete within 13 hr, in contrast to 22hr required when air was used. Similar desired results were obtained when a culture incubated in an 80% oxygen atmosphere was used as inoculum for the fermentation run in an 80% oxygen atmosphere. In these cases the dissolved oxygen level during the fermentation was always extremely high, and the dissolved oxygen level even at the point of maximum conversion were about twice the air saturation under normal atmospheric conditions. The oxidizing activities, the cell yield and the sorbose production rate are summarized in Table 2. The data indicate that the oxidizing activity is adaptively enhanced with an increase in dissolved oxygen level. The fermentation was thus accelerated by increasing both the oxidizing activity and the oxygen supply rate. In all cases, the quantitative conversion of sorbitol could be attained apparently without further metabolism of the accumulated sorbose, and the metabolic pathway of sorbitol in *A. suboxydans* seemed not to be greatly affected by such extremely high dissolved oxygen partial pressure. However, strictly speaking, good correlations between the oxidizing activity, the sorbose production rate and the oxygen transfer rate were not obtained when the oxygen concentration in the enriched gas was increased beyond 60%. The reason is not yet clear.

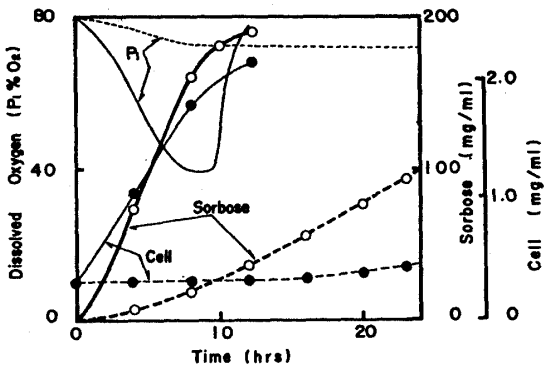

Fig. 5. Sorbose fermentation in an 80% oxygen atmosphere. —— Cultures grown in 40% oxygen atmosphere were used as inoculum; ····· cultures grown in air were used as inoculum; ○, sorbose concentration mg/ml; ●, cell concentration, mg/ml.

Conclusion

Under normal atmosphere, the productivity of the sorbose fermentation by *Acetobacter suboxydans* was directly proportional to oxygen supply rate. Dissolved oxygen partial pressure fell to zero even at high aeration coefficients. When oxygen transfer in the shaken flasks was increased by enriching the gas phase with oxygen, the fermentation was greatly accelerated through increases in both the oxygen supply rate and the cell growth. This phenomenon was observed at atmospheric concentrations of oxygen of up to 0.4 atm. Above this value, cell growth was inhibited, and although the specific oxidizing activity of the cells was not affected, sorbose production was reduced. The inhibitory level of dissolved oxygen could not be determined precisely since the dissolved oxygen was continually changing. Further, the inhibitory level was not absolute because it was dependent on the environment in which the inoculum was prepared. When a culture well fermented at a relatively high oxygen partial pressure, for instance, in a 40% oxygen-enriched atmosphere, was employed as inoculum, growth inhibition was not observed even in 80% oxygen atmosphere. In this case the dissolved oxygen level exceeded 0.4 atm, about twice the air saturation, during the fermentation. The cells grown at high oxygen level seemed to adapt to the environment and acquire high resistance to the oxygen inhibition. It was also interesting that the oxidizing activity of the cells adapted to high oxygen level was higher than that of cells grown in air. In conclusion, the acceleration of the conversion of sorbitol to sorbose by *Acetobacter suboxydans* in oxygen-enriched atmosphere can be accounted for by the following three factors: the oxygen deficiency is relieved by increasing oxygen transfer in the shaken flasks; oxygen-induced growth inhibition is overcome by adaptation of the cells; and oxygen induces an enhancement of the oxidizing enzyme activity. These results are not only of physiological interest but also suggest practical applications.

References

1) Lockhart, W. R., Squires, R. W.: *Advances Appl. Microbiol.*, **5**, 157 (1963).
2) Finn, R. K.: *Biochemical and Biological Engineering Science* (Blakebrough, N.) Vol. 1, 69, Academic Press, London and New York (1967).
3) Calderbank, P. H.: *Biochemical and Biological Engineering Science* (Blakebrough, N.) Vol. 1, 101, Academic Press, London and New York (1967).
4) Wimpenny, J. W. T.: *Process Biochem.*, **4**, 19 (1969).
5) Yamada, S., Wada, M., Chibata, I.: *J. Ferment. Technol.*, **56**, 20 (1978).
6) Wells, P. A., Stubb, J. J., Lockwood, L. B., Roe, E. T.: *Ind. Eng. Chem.*, **29**, 1385 (1937).
7) Wells, P. A., Lockwood, L. B., Stubb, J. J., Roe, E. T.: *Ind. Eng. Chem.*, **31**, 1518 (1939).
8) Damodaran, M., Subramanian, S. S.: *J. Sci. Ind. Research*, **10B**, 7 (1951).
9) Flickinger, M. C., Perlman, D.: *Appl. Environ. Microbiol.*, **33**, 706 (1977).
10) Mikhlin, E., Rozenberg, I.: *Biokhimiya*, **15**, 444 (1950).
11) Mikhlin, E., Rozenberg, I.: *Chem. Abst.*, **45**, 1650 (1951).
12) Haugaard, N.: *Physiological Rev.*, **48**, No. 2, 311 (1968).
13) Gottlieb, S. F.: *Annu. Rev. Microbiol.*, **25**, 111 (1971).
14) Hoffman, W. S.: *J. Biol. Chem.*, **120**, 51 (1937).

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