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2,3-Butanediol production by *Enterobacter aerogenes* in continuous culture: role of oxygen supply

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Summary. The influence of oxygen on growth and production of 2,3-butanediol and acetoin by *Enterobacter aerogenes* was studied in continuous culture. At all dilution rates (D) studied cell mass increased steadily with increasing oxygen uptake rate (OUR), hence the micro-aerobic cultivation was energy-limited. The biomass yield on oxygen increased with D which suggests that cells need more energy for maintenance functions at lower D. At each D an optimal OUR giving highest volumetric productivity for the sum of butanediol and acetoin was found. The optimal OUR increased with D. The occurrence of optimal OURs results from the various effects of O_2 on growth and specific productivity. The latter was found to be a linear function of the specific OUR irrespective of D. At optimal OUR the cells proved to have equal specific OURs and equal specific productivities representing a fixed relationship between fermentative and respiratory metabolism. The product yield based on glucose and corrected for biomass formation was 80%. A product concentration as high as 43 g/l was obtained at $D = 0.1\ h^{-1}$ while the volumetric productivity was the highest at $D = 0.28\ h^{-1}$ (5.6 g/l and hour). The findings further indicate that growth and product generation are obviously non-associated phenomena. Hence, high productivities may be achievable by cell recycling and cell immobilisation systems.

Introduction

In spite of the present low oil price the production of valuable chemicals from renewable resources such as carbohydrates could be still attractive (Young et al. 1989). In particular, the fermentative production of 2,3-butanediol (BD) appears to be a promising bioconversion process as the chemical synthesis of BD is unambiguously more costly than the microbial route (Rehm 1980). In addition, there is an increasing demand for "natural products" such as BD which can be used as a versatile solvent and as an intermediate compound for the chemical industry, for instance in the production of polymers and as a chiral component for asymmetric syntheses.

2,3-Butanediol is produced from carbohydrates by enteric bacteria of the genera *Enterobacter* and *Klebsiella* and by bacilli such as *Bacillus polymyxa*. The fermentative production of 2,3-butanediol by *E. aerogenes* or *K. pneumoniae* proceeds with satisfactory yields and end-product concentrations. Moreover, these organisms metabolize many of the sugars present in hemicellulose and cellulose hydrolysates (Yu and Saddler 1982). *E. aerogenes* and *K. pneumoniae* are facultative anaerobes that are capable of both oxidative and fermentative energy generation. The fermentation proceeds in the presence of oxyen and is even stimulated by aeration. The rate of oxygen supply determines which fractions of substrate are fermented to products, oxidized by respiration, or assimilated to cell material (Pirt and Callow 1958; Sablayrolles and Goma 1984; Jansen et al. 1984; Ramachandran and Goma 1987; de Mas et al. 1988).

Pirt and Callow (1958) as well as Ramachandran and Goma (1987) investigated the effect of oxygen on 2,3-butanediol production in continuous culture. In the study of Pirt and Callow (1958) the oxygen uptake rate (OUR) was calculated from measurements of oxygen and carbon dioxide in the effluent gas of the culture by means of an Orsat apparatus at only two dilution rates. Ramachandran and Goma (1987) have used the initial oxygen transfer rate $(k_La C_L^*)_0$ to study the effect of oxygen supply. However, as both the volumetric mass transfer coefficient (k_La) and saturation solubility of O_2 in the culture media (C_L^*) depend on the respective culture conditions the parameter $(k_La C_L^*)_0$ does not necessarily express the real amount of oxygen available in the culture, but is certainly a reasonable estimate.

Recently, a two-stage continous process for BD production with *K. pneumoniae* was proposed by Tran-Dinh et al. (1987). In the first stage, cells were grown at high oxygen supply (OUR = 65 mmol/l and hour) and fed to the second stage operated at a low OUR (= 10

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mmol/l and hour). Under such conditions 180 g/l glucose could be converted to 77 g/l BD (+4.4 g/l acetoin), the overall productivity being about 2.3 g/l and hour. The downstream processing of BD fermentation broths has been reviewed by Sridhar (1989).

In this work the influence of oxygen supply and dilution rate (D) on the continuous production of BD by *E. aerogenes* has been studied in detail. The particular objective was to provide preliminary data for the design of a process for continuous BD production by making use of a microorganism that is not subjected to special safety restrictions.

Materials and methods

Strain. Enterobacter aerogenes DSM 30053 obtained from the German Collection of Microorganisms (DSM) was used in this study.

Culture medium. The composition of the nutrient medium per litre was: 2.5 g KH_2PO_4; 12 g $(NH_4)_2SO_4$; 0.75 g $MgSO_4 \cdot 7H_2O$; 0.02 g NaCl; 0.02 g $Na_2MoO_4 \cdot H_2O$; 0.02 g $CaCl_2$; 0.02 g $MnSO_4 \cdot H_2O$; 0.10 g $FeSO_4 \cdot H_2O$; 0.14 mg $ZnCl_2$; 0.38 mg $CoCl_2 \cdot 6H_2O$; 0.034 mg $CuC_2 \cdot H_2O$; 0.048 mg $NiCl_2 \cdot 6H_2O$; 0.2 mg biotin; 4 mg *p*-aminobenzoic acid; 4 mg thiamin hydrochloride; 1.0 g yeast extract. Depending on D and OUR the glucose inlet concentration was adjusted to values giving a glucose concentration of about 10 g/l in the bioreactor. The medium for inoculum and preculture contained 20 g/l glucose and additional 10 g/l K_2HPO_4.

Culture conditions. Inocula for the bioreactor preculture were prepared in 250-ml shake flasks containing 50 ml medium. The flasks were incubated at 30° C for 16 h. The preculture was grown in a 500-ml shake flask containing 250 ml medium with 5% inoculum. It was transferred to the bioreactor after 12 h of incubation. The fermentation was carried out in a 4-l Setric (SGI, Toulouse, France) bioreactor with a working volume of 2 l. The volume was controlled by a balance connected with a Philips weight control unit PR 1592 (Philips, Eindhoven, NL). The bioreactor was equipped with pH, pO_2, temperature, antifoam and agitation speed controls. Temperature, pH value and aeration rate were maintained at 30° C, 5.5 and 0.35 vvm, respectively. The pH was controlled by addition of either 2 *N* KOH or 2 *N* HCl. The antifoam used was Desmophen (Bayer, Leverkusen, FRG) in a concentration of 0.1 ml/l. Whenever the D and/or stirring speed (for variation of the OUR) of the continuously operated system were changed, at least four residence times were allowed to pass before steady state parameters were assessed.

Analytical methods. Glucose concentration was measured by means of a sugar analyser (YSI Model 27, Yellow Springs Instruments, Ohio, USA) operating with a glucose oxidase enzyme electrode.

Cell concentration was measured as dry weight (DW). Cells of 10 ml culture were centrifuged in metal tubes at 15000 rpm and dried at 80° C for 48 h. Cell concentration was also measured as absorbance at 578 nm.

The determination of products was carried out with a gas chromatograph (Hewlett Packard 5800 A, Palo Alto, Calif, USA), a flame-ionisation detector, and a 2-m glass column with Chromosorb 101 (Chrompack, Frankfurt, FRG) operated with N_2 as carrier gas at 250° C evaporation temperature, 300° C detector temperature and 175° C column temperature) connected to a Spectraphysics integrator SP 4240. *n*-Butanol was used as the internal standard.

Oxygen uptake rate. The OUR was determined from measurement of the oxygen and carbon dioxide content in the effluent gas of the culture. A paramagnetic oxygen analyser (Oxygor, Maihak, Hamburg, FRG) and an infrared analyser (Unor 4N, Maihak, Hamburg, FRG) were used to measure O_2 and CO_2 mole fractions in the effluent gas. The subsequent equation was applied to calculate the OUR from off-gas analysis

$$\mathrm{OUR} = k_L a\, c_L^* = \frac{\dot{V}_G}{V_M V_L}\left(x^0_{O_2} - \frac{1-x^0_{O_2}}{1-x^1_{O_2}-x^1_{CO_2}}\, x^1_{O_2}\right) \qquad (1)$$

in which $\dot{V}_G$ is the gas flow rate, V_M the molar volume (l/mol), V_L the fermentation working volume, and x^0 and x^1 the mole fractions at gas inlet and outlet, respectively.

Results and discussion

Continuous cultivation of *E. aerogenes* was performed at $D = 0.1$, 0.16, 0.2 or 0.28 h^{-1}. At all Ds different OURs were obtained at constant aeration rate by variation of the impeller speed. In all cultivations the pO_2 in the media was zero. Therefore, OUR corresponded to $k_L a c_L^*$. However, as the effect of the products on O_2 solubility (c_L^*) is not known a reliable estimation of $k_L a$ is not possible. The OUR data as calculated from the off-gas analysis by Eq. 1 were plotted vs impeller speed (N) in Fig. 1.

As expected the OUR increased with increasing stirrer speed. However, there were significant differences in the slope of this increase which was about twice as high at $D = 0.1\ h^{-1}$ as at $D = 0.28\ h^{-1}$. Of course, it is understood that the apparent dependency of the OUR (or $k_L a c_L^*$) on D was not an effect of the D as such, but had to be attributed to the differences in product composition that are given in Table 1 for the runs carried out at $N = 400$ rpm.

Obviously, it was particularly the appearance of increasing amounts of products which caused enlarged mass transfer rates with decreasing D. One can assume that the main products (BD + acetoin) as well as volatile by-products (ethanol + acetate) increased both c_L^* and $k_L a$, thus having caused the observed increase in OUR with decreasing D. In batch fermentations with *K. pneumoniae* (*Aerobacter aerogenes*) Sablayrolles and Goma (1984) also found that $k_L a$ increased with increasing BD concentration.

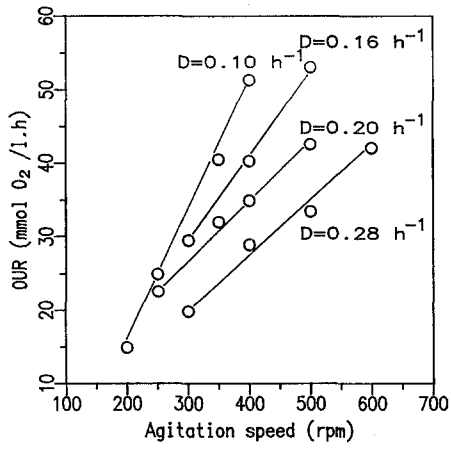

Fig. 1. Oxygen uptake rate (OUR) vs agitation speed N (in rpm) at different dilution rates (D)

Table 1. Results of cultivations at constant impeller speed ($N=400$ rpm)

D (h^{-1})	Biomass (g DW/l)	OUR (mmol/l h)	BD (mmol/l)	Acetoin (mmol/l)	Ethanol (mmol/l)	Acetate (mmol/l)
0.10	9.6	51.3	253	79.7	22.8	34.5
0.16	9.5	40.3	239	71.6	8.1	22.1
0.20	8.8	34.9	220	45.4	13.0	12.7
0.28	8.7	28.9	145	28.5	20.7	13.5

D = dilution rate; DW = dry weight; OUR = oxygen uptake rate; BD = 2,3-butanediol

The effect of the OUR on cell and product concentration (BD + acetoin) is shown in Fig. 2 for $D=0.1$ and $D=0.28\ h^{-1}$. For $D=0.16$ and $0.2\ h^{-1}$ intermediate figures were obtained. At all *Ds* the biomass concentration increased with the OUR indicating that cell growth was oxygen limited. However, in contrast to the study of Ramachandran and Goma (1987) with *K. pneumoniae* the cell concentration did not increase linearly with OUR. One suspects that the linear dependency of cell concentration on the OUR was actually an artifact since Ramachandran and Goma (1987) did not measure the OUR during cultivation but calculated it from k_La data determined in sterilized media.

For all *Ds* it was found that the product concentration (i.e. BD + acetoin) passed a maximum value as function of the OUR. The ethanol concentration decrased with increasing OUR, while acetate increased. In Fig. 3, product concentration, yield (based on glucose consumed), and productivity observed under optimal OUR are plotted vs *D*. The product concentration declined from 43.1 g/l at $D=0.1\ h^{-1}$ to 19.9 g/l at $D=0.28\ h^{-1}$. However, due to higher volume throughput, the productivity increased from 4.3 to 5.6 g/l per hour. These productivities were about twice as large as that reported by Tran-Dinh et al. (1987) for a two-stage continuous process with *K. pneumoniae*. However, one has to consider that the productivity obtained by Tran-Dinh et al. (1987) refers to a considerably higher product concentration, which facilitates product recovery. In additon, Tran-Dinh et al. (1987) achieved product yields in the range of 90% while in this study with *E. aerogenes* the product yield was only 77.5% at $D=0.1\ h^{-1}$ and decreased to 62% at $D=0.28\ h^{-1}$.

Both BD and acetoin formation by *E. aerogenes* is accompanied by respiration, cell growth, maintenance and by-product generation, as the intermediate pyruvate can be transformed to acetate and ethanol via aetyl coenzyme A (CoA). With regard to the extent of the various phenomena O_2 plays an important role as it influences ATP availability and the nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide ($NAD/NADH_2$) pool. In Fig. 4 the biomass/oxygen yield coefficient Y_{X/O_2} expressed as grams cell DW per mol O_2 is plotted vs OUR. The biomass yield decreased with OUR for each *D*. The explanation is that cells under O_2-limiting conditions also obtain energy from fermentative metabolism. When assuming that cell growth requires a given amount of ATP at a given specific growth rate more energy is obtained from fermentative metabolism at low OURs, and hence biomass yield on O_2 will increase.

Figure 4 also shows that the Y_{X/O_2} increased with *D*. This result can be explained as follows: anticipating that the efficiency of respiratory ATP production is the same at all *Ds* it has to be inferred that more energy is consumed for non-growth-associated functions at lower *D*. Such behaviour has been observed for other strains by other authors (Marr et al. 1963; Pirt 1975; Tempest and Neijssel 1984; Haughney et al. 1988). Of course, the reason is that more energy is required for maintenance at low *D* as, for instance, cells consume more energy to perform osmotic work for maintaining concen-

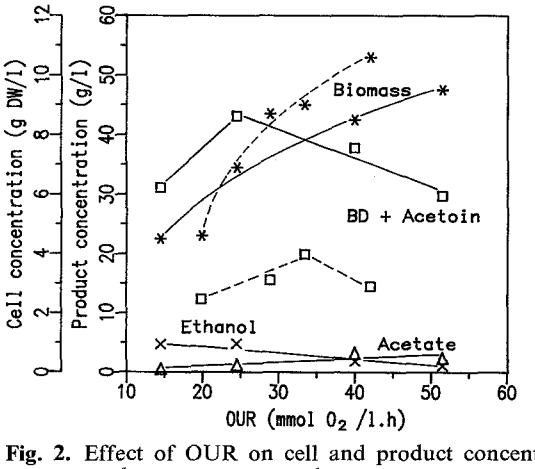

Fig. 2. Effect of OUR on cell and product concentrations: —, $D=0.10\ h^{-1}$; ---, $D=0.28\ h^{-1}$; BD = 2,3-butanediol

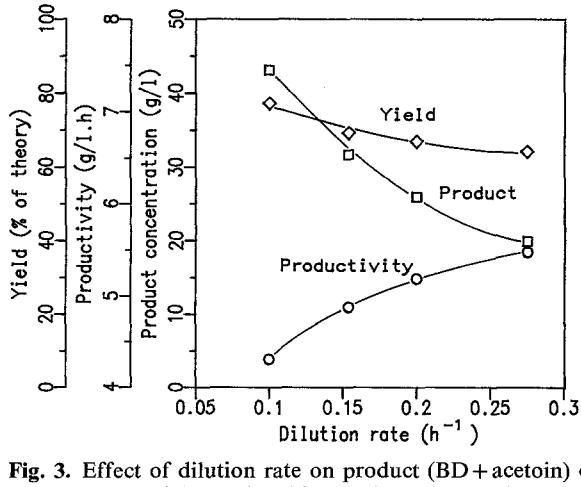

Fig. 3. Effect of dilution rate on product (BD + acetoin) concentration, productivity and yield at optimal oxygen supply

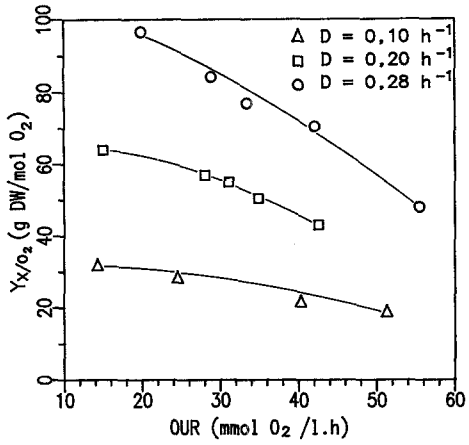

Fig. 4. Biomass yield per oxygen consumed (Y_{X/O_2}) as a function of OUR

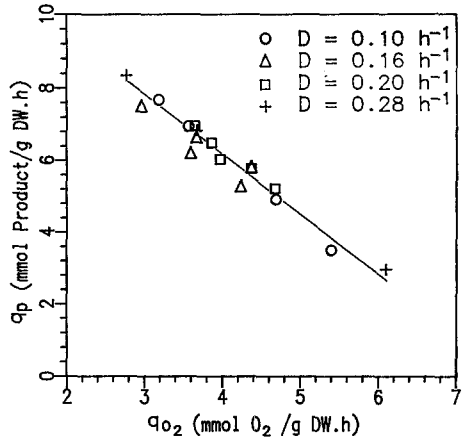

Fig. 5. Specific productivity (q_p; BD+acetoin) as a function of the specific OUR (q_{O_2})

tration gradients between the cell interior and its environment at low D due to higher product concentrations.

The specific productivity (q_p) is plotted as a function of the specific OUR (q_{O_2}) for all *Ds* studied in Fig. 5. It turned out that the specific productivity was only a function of the specific OUR irrespective of D. Particularly interesting is that the data points for optimal OUR (see Fig. 2) at various *Ds* fall together at $q_p \approx 6.8$ mmol products/g DW per hour and $q_{O_2} \approx 3.6$ mmol O_2/g DW per hour. The exact numerical values calculated from the cultivation at optimal OUR are given in Table 2. Also given are the specific glucose consumption rates which increase with increasing D. The reason is that with increasing D biomass concentration increases as well. Hence, more glucose is used for biomass formation. Assuming that 1.29 g glucose is needed to generate 1 g cell mass (Schlegel 1985) one can calculate a specific glucose consumption rate ($q^*_{G/P}$) which refers only to product formation. As shown in Table 2 $q^*_{G/P}$ turns out to be approximately constant, the mean value being about 1.52 g glucose/g DW and hour at optimal OUR. It is understood that the yield coefficient ($Y_{P/G}$; products referred to glucose consumption for formation of products and by-products)

$$Y_{P/G} = q_P / q^*_{G/P} \qquad (2)$$

is constant and amounts to 0.4 g products (BD+acetoin)/g glucose at optimal oxygen uptake. This corresponds to a yield of 80% of the theoretical value and implies that at optimal OUR a constant fraction (about 20%) of the intermediate pyruvate is converted to acetyl CoA, which is either transformed to acetate (by ATP generation) and/or reduced to ethanol (with $NADH_2$ consumption).

The figures in Table 2 also reveal that there is a fixed relationship between energy generation by fermentative and oxidative metabolisms provided that the oxygen supply is optimal for volumetric productivity. If the oxygen supply is suboptimal with regard to volumetric productivity, even higher specific productivities are observed at low OUR (cf. Fig. 5). Under such conditions respiration and oxidative phosphorylation is suppressed and energy for growth and maintenance is mainly generated by the fermentative route. It is therefore the low biomass concentration at low OUR which seemingly leads to higher specific productivities than the optimal value. On the other hand, at an OUR higher than the optimal value (i.e. about 3.6 mmol O_2/g dry cell mass and hour) respiration (and probably oxidative phosphorylation) becomes dominant as compared to fermentation. Thus high biomass concentrations are obtained and hence lower specific productivities.

The data shown in Fig. 5 do not indicate any dependency of product formation on D (hence growth

Table 2. Specific rates of oxygen (q_{O_2}) and glucose consumption (q_C) and specific productivity (sum of BD and acetoin) (q_p) at optimal OUR

D (h^{-1})	q_{O_2} (mmol O_2/ g DW h)	q_P (g product/ g DW h)	q_G g glucose/ g DW h)	$q^*_{G/P}$ g glucose g DW h)
0.10	3.56	0.62	1.62	1.50
0.16	3.66	0.61	1.73	1.53
0.20	3.63	0.61	1.81	1.55
0.28	3.67	0.60	1.87	1.51

$q^*_{G/P}$ = specific glucose consumption rate after subtraction of glucose consumption for biomass formation (1.29 glucose/g DW)

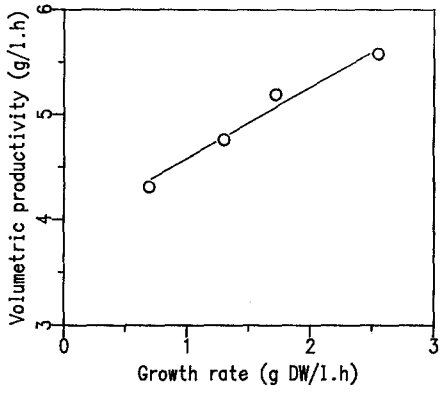

Fig. 6. Volumetric productivity (BD + acetoin) as a function of growth rate

rate). Therefore product formation is not associated with cell growth. This is particularly evident for the results obtained at optimal OUR as shown in Fig. 6. The product formation rate is a linear function of the growth rate, but the straight line does not pass through the origin. At very low or even zero growth rates remarkably high volumetric productivities can apparently be expected. For achieving high volumetric productivities it may therefore be advisable to make use of cell recycling or cell immobilization systems.

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