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Effect of High Oxygen Concentration on the Growth of a Ethanol Assimilating Yeast, *Candida* sp.

Masatoshi Matsumura and Jiroh Kobayashi

Institute of Applied Biochemistry, University of Tsukuba, Sakura-mura, Niihari-gun, Ibaraki 305

The effect of high oxygen concentration on cell growth and activities of some key enzymes associated with ethanol assimilation was studied by using a *Candida* sp. Measurements were made at the exponential phase of batch cultures with dissolved oxygen controlled at the levels ranging from 5% to 60% of the saturation concentration with pure oxygen of 1 atm. The results showed the existence of an upper critical value of dissolved oxygen for normal cell growth, above which the metabolism of ethanol assimilation was disturbed and both growth rate and cell yield were reduced. Kinetic examination of the relationship between cell growth and enzyme activities revealed that the reduction of growth rate and cell yield was caused by the depression of the enzyme activities of acetyl-CoA synthetase and isocitrate lyase.

There has been considerable interest in the use of oxygen-enriched aeration as a method for improving the oxygen transfer capability of fermentors. To increase oxygen transfer rates of a normal stirred fermentor either agitation speeds or gas flow rates must be increased. However, this approach leads to large power input for agitation and difficulties with foaming. The use of oxygen-enriched aeration enables one to increase oxygen transfer rates without increasing agitation speeds and gas flow rates, and to avoid difficulties with foaming. Oxygen-enriched aeration is therefore frequently employed in the cultivation of microorganisms with high oxygen demand.[1-3]

We previously developed a new fermentor in which pure oxygen could be utilized efficiently for biomass production from ethanol.[4] It is, however, essential to know the extent to which oxygen enrichment can be applied to the microorganism, because high oxygen concentration sometimes inhibits cell growth.[5] In the present work, batch cultures with dissolved oxygen controlled at various levels were conducted to clarify this extent for an ethanol-assimilating yeast. The effect of oxygen concentration on the activities of some enzymes associated with ethanol assimilation was also measured, and the relationship between the cell growth and these enzyme activities was investigated quantitatively.

Materials and Methods

Microorganism and culture medium The microorganism used was *Candida* EY-12, which was kindly given by the Institute of Physical and Chemical Research.

The medium used had the following composition per liter of tap water: ethanol, 15 g; $NH_4H_2PO_4$, 8.5 g; KH_2PO_4, 1.0 g; $Na_2HPO_4 \cdot 12H_2O$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_2 \cdot 6H_2O$, 0.01 g; yeast extract, 0.1 g. Ethanol and the other medium components were mixed after being sterilized seperately by membrane filtration and autoclaving, respectively.

Equipment and cultivation method Cultivation equipment, shown schematically in Fig. 1, consistent of a fermentor, a control unit for dissolved oxygen, and a gas circulation unit.

Dissolved oxygen concentration (DO) was controlled by manipulation of the oxygen partial pressure in the gas phase. When the oxygen concentration dissolved in the culture broth falls below a set value, the solenoid valve opens to allow pure oxygen flow to the head space of the fermentor. This supply of pure oxygen increases the driving force for oxygen transfer, and DO returns to the set value. In this manner DO is continuously and automatically controlled during cultivation without altering agitation speeds and gas flow rates.

Effluent gas was circulated in order to minimize substrate loss, which in the cultivation of cells on the

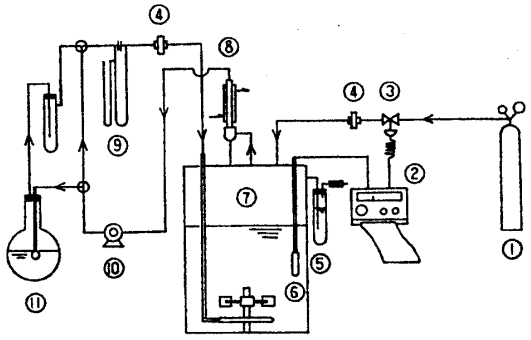

Fig. 1. Schematic flow sheet of the apparatus employed in batch cultivations.
1. Oxygen gas cylinder, 2. Controller of dissolved oxygen, 3. Solenoid valve, 4. Air filter, 5. Pressure controller, 6. Oxygen probe, 7. Jar fermentor, 8. Cooler, 9. Manometer, 10. Air circulation pump, 11. CO_2 absorber.

volatile lower alcohols can be considerable.[6] In our system the major portion of the sparged gas was recycled, and gas was exhausted only when pure oxygen was supplied, raising the total pressure. Therefore, the total amount of exhaust gas in our system was far smaller than in the usual aeration systems.

Preliminary experiments on the effects of CO_2 on the growth of *Candida* EY-12 showed that the absence of CO_2 in gas phase, as well as high CO_2 content, reduced the growth rate. Carbon dioxide was therefore added to the head space of the fermentor at an initial partial pressure of about 0.03 atm. When CO_2 accumulated the excess was removed by sparging part of the recycling gas through a 20% (w/v) KOH solution, and the partial pressure of CO_2 was controlled manually within the safety range of 0.1—0.2 atm.

Cells growing exponentially in a shake flask were used as the seed, and batch growth experiments at various DOs were carried out at 30°C and pH 5.0, adjusted automatically with an aqueous solution of 4N NaOH. The bench-scale fermentor (Marubishi MD-500) used was aerated at 1.0 vvm and agitation at 600 rpm.

Analysis The optical density of the culture broth was measured at 610 nm and converted into the concentration of dried cell mass using a calibration curve. The ethanol, acetaldehyde and acetate concentrations in the culture broth were determined by gas chromatography with F.I.D detector (Yanagimoto G-180). A glass colum (1.5 m×5 mm) was packed with 60—80 mesh chromosorb 105. Nitrogen gas flow rate was 40 ml/min. The injection port temperature was 110°C, the detector 200°C, and the colum oven was operated isothermally at 180°C.

The partial pressure of CO_2 in the gas phase was determined by gas chromatography with T.C.D detector (Yanagimoto G-1800). A stainless steel colum (6 m×4 mm) was packed with 60—80 mesh X-28, which was developed specially for the analysis of lower hydrocarbon gases by Yanagimoto Co., Ltd.

Elemental analysis of *Candida* EY-12 was done at the Chemical Analysis Center, Tsukuba University. Carbon, hydrogen and nitrogen contents were determined by C.H.N auto-analyzer and ash content gravimetrically by ashing at 800°C for 1 hr. Oxygen content was determined from the difference between total content and carbon, hydrogen, nitrogen and ash contents.

Enzyme assays Cell-free crude extract for enzyme assays was prepared as follows. Cells in the mid-exponential growth phase were harvested by centrifuging, washed with 50 mM Tris buffer, pH 7.7, and resuspended in the same buffer containing 10 mM $MgCl_2$. Cells were then distrupted in a Vibrogen Cell Mill (Edmund Bühler West German) with 0.3 mm glass beads for 20 min at 4°C. The supernatant obtained after centrifuging at $10,000 \times g$ for 30 min at 0°C was used as the crude extract.

Alcohol dehydrogenase (EC 1.1.1.1) activity was assayed by the method of Racker,[7] isocitrate dehydrogenase (EC 1.1.1.42) by the method of Cleland *et al.*,[8] isocitrate lyase (EC 4.1.3.1) by the method of Dixon and Kornberk,[9] and acetyl-CoA synthetase (EC 6.2.1.1) by the method of Jones and Lipman.[10] All spectrophotomic assays were performed on a Hitachi 100—50 recording spectrophotometer at 30°C. Protein content of crude extracts was determined by the Lowry method[11] using bovine serum albumin as standard.

Results and Discussion

Effect of DO on cell growth In this work DO was expressed as C_w/C_w^*, that is, relative to the dissolved oxygen concentration C_w^* in equilibrium with pure oxygen at 1 atm. The effect of oxygen concentration on cell growth was examined at various DOs in the range of $C_w/C_w^*=0.05$—0.6. Three examples of results for $C_w/C_w^*=0.05$, 0.4 and 0.6 are shown in Figs. 2—4.

By plotting the growth curves on semi-log paper it may be seen that the exponential growth is maintained until ethanol is almost totally consumed. However, specific growth rates and maximum cell concentrations decreased with increasing DO, and the growth ceased at $C_w/C_w^*=0.6$. The accumulation of acetate also became notable with the increase of DO, and at $C_w/C_w^*=0.6$ acetate was accumulated at a rate almost equal to the consumption rate of ethanol.

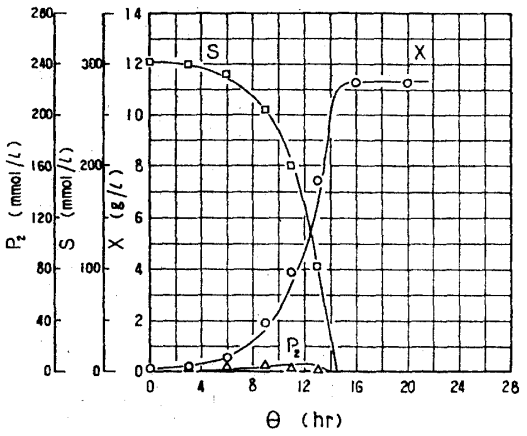

Fig. 2. Time course of batch cultivation at $C_w/C_w^* = 0.05$.
The solid lines are calculated from Eqs. (6)—(9). X cell concentration, S ethanol concentration, P_2 acetate concentration, C_w/C_w^* DO ratio to the saturation value with pure oxygen at 1 atm.

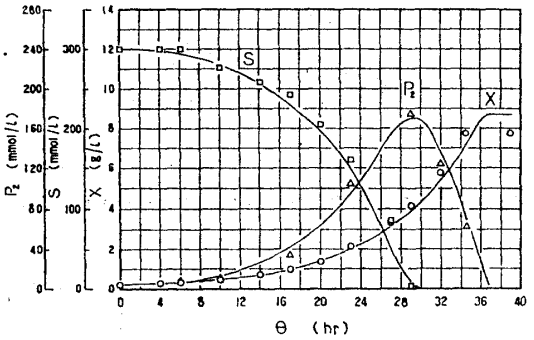

Fig. 3. Time course of batch cultivation at $C_w/C_w^* = 0.4$.
The solid lines are calculated from Eqs. (6)—(9).

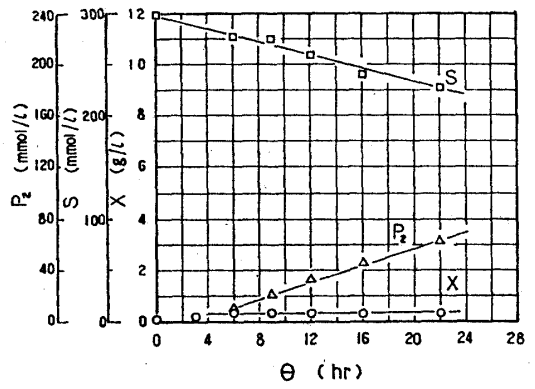

Fig. 4. Time course of batch cultivation at $C_w/C_w^* = 0.6$.

The remarkable accumulation of acetate at high DO was also observed by Páca and Grégr,[3] who studied the effect of increasing oxygen partial pressure in the aeration gas on the growth of *C. utilis* in a multistage tower fermentor. Considering the accumulation of this intermediate of ethanol oxidation, they estimated that the bottleneck in the metabolic pathway of ethanol should occur in the acetate activation to acetyl-CoA, or in one or more reactions of the tricarboxylic acid (TCA) or glyoxylic acid (GA) cycles.

The GA cycle is essential to the microorganism growing on twocarbon compounds such as ethanol and acetate. According to Kornberg and Elsden,[12] the net function of TCA cycle is a catabolic one for energy production, whereas the GA cycle plays an anabolic role in forming C_4-dicarboxylic acids which become the precursors of most cell constituents. That is, part of the acetyl-CoA formed from ethanol is further oxidized to CO_2 through the TCA cycle, while the remainder proceeds through the GA cycle into the anabolic pathways. Thus, as stated by Mor and Fiecher,[13] respiration quotient and cell yield are dependent on the distribution of acetyl-CoA into these catabolic and anaplerotic pathways.

In our experiments both growth rate and maximum cell concentration were reduced at higher oxygen concentration. From the results it is supposed that the distribution of acetyl-CoA is disturbed by high oxygen concentration.

Effect of DO on enzyme activity

The effect of DO on the activities of some enzymes associated with the metabolism of ethanol was investigated. In view of the results for the cell growth mentioned previously, the specific activities of alcohol dehydrogenase, acetyl-CoA synthetase, isocitrate lyase and isocitrate dehydrogenase were measured. The cell-free extract used in these enzyme assays was prepared from the cells in exponential growth phase at various DOs. As shown in Fig. 5, alcohol and isocitrate dehydrogenases were almost independent of oxygen concentration. Acetyl-CoA synthetase and isocitrate lyase, however, showed a remarkable depression of the activity at higher DO.

Based upon the Jacob-Monod model for the regulation of enzyme synthesis, Yagil[15] and van Dedem[16] studied quantitatively the

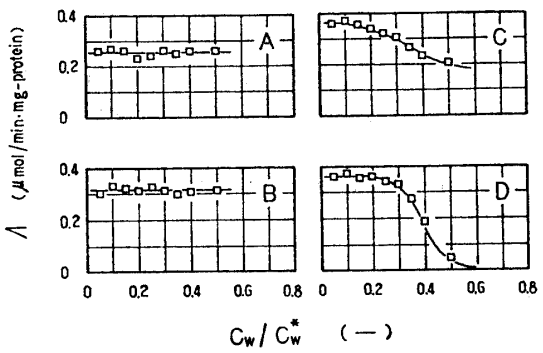

Fig. 5. Effect of dissolved oxygen concentration on specific enzyme activities.
A Alcohol dehydrogenase, B Isocitrate dehydrogenase, C Isocitrate lyase, D Acetyl-CoA synthetase.
The solid lines in Figs. 5-C and 5-D are calculated from Eq. (1) with the constants shown in Fig. 6.

interactions among repressor, operator and effector, and formulated specific enzyme concentration e as a function of effector concentration E. In systems where enzyme synthesis is repressed by an effector, the following equation was proposed.

$$\frac{e}{e_m} = \frac{1+K_1(E)^n}{1+(1+K_2(R_t))K_1(E)^n} \qquad (1)$$

where K_1 and K_2 are equilibrium constants, R_t is the total concentration of repressor, and e_m is the maximum enzyme concentration at the absence of effector.

Assuming that specific enzyme activity corresponds to specific enzyme concentration, and employing the specific enzyme activity at $C_w/C_w^*=0.05$ as e_m and DO as effector concentration, we obtained Yagil's plot as shown in Fig. 6. The applicability of Eq. (1) to these two enzymes was confirmed by comparing the calculated values with the experimental results as shown in Fig. 5.

Kinetics of cell growth A kinetic study of cell growth on ethanol at high DO was made based on the experimental results for the activity of key enzymes associated with the metabolism of ethanol.

Considering the functions of TCA and GA cycles explained by Kornberg[12] and Mor,[13] we assumed that ethanol was metabolized by the branched and irreversible enzymatic reactions shown in Fig. 7. Ethanol is first oxidized to acetaldehyde, then to acetate. The acetate produced is activated into

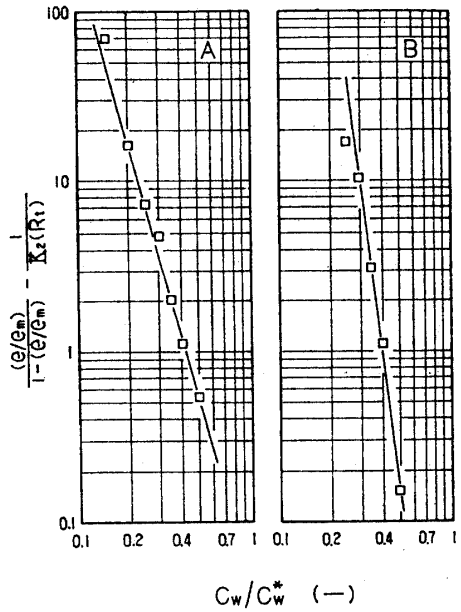

Fig. 6. Determination of constants in Eq. (1).
A Isocitrate lyase:
$n=4$, $K_1=30.7$, $K_2(R_t)=39.0$
B Acetyl-CoA synthetase:
$n=8.5$, $K_1=5.57$, $K_2(R_t)=2.78\times10^3$.

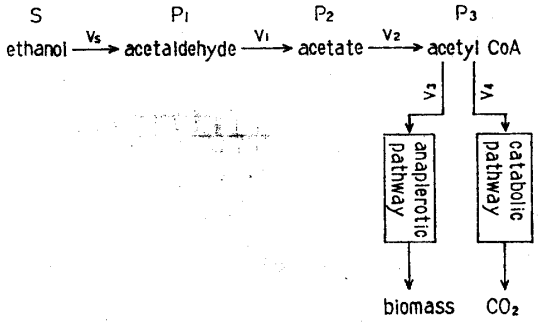

Fig. 7. Simplified scheme for metabolism of ethanol.

acetyl-CoA, and part of the acetyl-CoA flows through the GA cycle into the anaplerotic pathway with the specific velocity of v_3. The remainder of acetyl-CoA flows into the TCA cycle with the specific velocity of v_4 and is oxidized to CO_2. The distribution of acetyl-CoA into these catabolic and anaplerotic pathways is dependent on the activity of the enzymes which constitute the branching point. Since the TCA and GA cycles branch at isocitrate, the activities of isocitrate lyase and isocitrate dehydrogenase will affect the distribution of acetyl-CoA. We expressed the distribution coefficient η of acetyl-CoA into the anaplerotic pathway as $v_3/(v_3+v_4)$, and assumed that v_3 and v_4 were dominated by

the cleavage reaction rate of isocitrate with isocitrate lyase and the oxidation rate with isocitrate dehydrogenase. We also assumed that the specific growth rate μ was proportional to the rate v_3, and that the toxic influence of oxygen on growth rate and cell yield were caused by the depression of the activities of acetyl-CoA synthetase and isocitrate lyase, resulting in the change of v_2 and v_3.

To investigate the validity of these assumptions, the relationships between the concentration of dissolved oxygen and the specific growth rate in the exponential phase, where balanced growth occurred, was first obtained. According to the assumption,

$$\mu \propto v_3 = v_2 \eta \qquad (2)$$

The maximum values of specific growth rate μ_m and specific reaction rate of intermediates v_m was obtained at $C_w/C_w^*=0.05$, where the toxic effects of oxygen were not observed. By using these maximum values, Eq. (2) can be written as follows.

$$\frac{\mu}{\mu_m} = \left(\frac{v_2}{v_{2m}}\right)\left(\frac{v_3}{v_{3m}}\right)\left(\frac{v_{3m}+v_{4m}}{v_3+v_4}\right) \qquad (3)$$

The rate of enzyme reaction is generally expressed by the Michaelis-Menten equation. However, when excess acetate accumulates at high oxygen concentrations, the conversion rate v_2 of acetate to acetyl-CoA becomes zero order with respect to acetate. Under such high oxygen concentrations, the supply of acetyl-CoA becomes limiting and the concentrations of intermediates following acetyl-CoA are small. Thus the rates of the cleavage and oxidation reactions of isocitrate become first order with respect to acetyl-CoA provided that the reactions between acetyl-CoA and isocitrate are fast and in equilibrium.[17] Using the assumption that v_3 and v_4 are proportional to the rates of the cleavage reaction and the oxidation reaction, respectively, and considering that the specific concentration of isocitrate dehydrogenase e_4 is independent on oxygen concentration, we obtained the following equation from Eq. (3).

$$\frac{\mu}{\mu_m} = \left(\frac{e_2}{e_{2m}}\right)\left(\frac{e_3}{e_{3m}}\right)\Big/\left(1-\left(1-\frac{e_3}{e_{3m}}\right)\eta_m\right) \qquad (4)$$

where e_2 and e_3 are specific concentration of acetyl-CoA synthetase and isocitrate lyase, respectively. If the maximum distribution coefficient η_m of acetyl-CoA into the anaplerotic pathway is determined, specific growth rates at various oxygen concentrations can be obtained from Eqs. (1) and (4).

When ethanol is oxidized steadily without accumulation of intermediates, the relation $v_s=v_1=v_2=(v_3+v_4)$ holds and the distribution coefficient η of acetyl-CoA can be expressed as follows.

$$\eta \equiv \frac{v_3}{v_3+v_4} = \frac{v_3}{v_2} = \frac{v_3}{v_s} \qquad (5)$$

Equation (5) shows that the distribution coefficient of acetyl-CoA can be determined from the molar fraction of ethanol converted into cell component. In our experiments the steady oxidation of ethanol without accumulation of intermediates occurred at the exponential growth phase at $C_w/C_w^*=0.05$, and the elemental balance for cell synthesis, the maximum distribution coefficient η_m of acetyl-CoA, was determined as follows.

The elemental composition of *Candida* EY-12 was C:47, H:6.5, N:7.5, O:31.5 and ash: 7.5 weight %, and the molecular formula per 100 g of cell was expressed by $C_{3.9}H_{6.5}N_{0.64}O_{2.0}$. From this formula the following balance equations were obtained.

Cell synthesis

$$1.95\ C_2H_5OH + 2.08\ O_2 + NH_3 = C_{3.9}H_{6.5}N_{0.64}O_{2.0} + 4.1\ H_2O$$

Ethanol combustion

$$C_2H_5OH + 3\ O_2 = 2\ CO_2 + 3\ H_2O$$

The increment of cell mass ΔX was plotted against the amount of ethanol consumed ΔS at various times of the batch culture at $C_w/C_w^*=0.05$, and after the confirmation of the linear relationship between ΔX and ΔS the maximum cell yield was determined to be 0.79 g-cell/g-ethanol. This yield value shows that 2.75 moles of ethanol is required for the production of 100 g of cells, and from the above elemental balance equation it is clear that 1.95 moles of ethanol is converted into biomass and 0.8 moles of ethanol is consumed for energy production. From these values the maximum distribution coefficient η_m of

acetyl-CoA was determined to be 0.71.

Using these μ_m and η_m values obtained at $C_w/C_w^*=0.05$, we calculated specific growth rates at various oxygen concentrations from Eqs. (1) and (4) and compared these with the experimental values. As shown in Fig. 8, fairly good agreement was obtained between observed and calculated values, and it was proved that the toxic effect of high oxygen concentration on specific growth rate was caused by the depression of the specific enzyme activities of acetyl-CoA synthetase and isocitrate lyase.

We observed that high oxygen concentration diminished not only growth rate but also cell yield, and supposed previously that cell yield was also dependent on the distribution of acetyl-CoA into the anaplerotic pathway. In the batch culture with a considerable accumulation of intermediates, the constancy of cell yield on ethanol was not held. Therefore, to confirm this assumption for cell yield, we tried to apply Eq. (1), which held rigorously in the exponential growth phase, to the entire duration of batch culture, and calculated time courses of cell growth, ethanol consumption and acetate accumulation at high oxygen concentrations.

There is little information available on the relation between the intracellular concentration and extracellular concentrations of low molecular C_2-compounds such as ethanol, acetaldehyde and acetate. Based on the assumption employed by Prokop *et al.*,[19] that these low molecular C_2-compounds diffuse freely in and out of the yeast cell, we assumed that intracellular concentrations of these compounds were same as that in culture broth, and used the concentration per unit broth volume in the following equations. The oxidation rate of ethanol to acetaldehyde is described by the following Michaelis-Menten equation.

$$-\frac{1}{X}\frac{dS}{d\theta}=\frac{k_s e_s S}{K_s+S} \qquad (6)$$

where S and e_s are ethanol concentration and specific enzyme concentration of alcohol dehydrogenase, respectively. The saturation constant K_s was determined to be 10 mM by referring to the results of the preliminary experiment for the effect of ethanol concentration on the respiration rates of *Candida* EY-12. Since the specific activity of alcohol dehydrogenase was independent of oxygen concentration, the maximum oxidation rate of ethanol $k_s e_s$ was determined to be 10.4 mmol/g-cell·hr from the constant ethanol consumption rate at $C_w/C_w^*=0.6$, at which cell growth had almost ceased as shown in Fig. 4. The literature[18,19] reports the common accumulation of acetaldehyde in cultures of cells assimilating ethanol, and the toxic effect of this intermediate on cell growth has been discussed. However, in our experiments acetaldehyde was hardly detected at any oxygen concentration. This suggests that the oxidation rate of acetaldehyde is rapid and limited by the oxidation rate of ethanol. Under such conditions, the concentration of acetaldehyde becomes almost zero and the specific reaction rate v_1 in Fig. 7 is equal to v_s. Thus we obtained the following equation for the mass balance of acetate P_2 based on the assumption that the conversion of acetate into acetyl-CoA is a zero-order reaction with respect to acetate.

$$\frac{1}{X}\frac{dP_2}{d\theta}=\frac{k_s e_s S}{K_s+S}-k_{2e_{2m}}\left(\frac{e_2}{e_{2m}}\right) \qquad (7)$$

where $k_{2e_{2m}}$ is the maximum conversion rate of acetate into acetyl-CoA. From the experimentally observed negligible accumulation of acetate at the oxygen concentration of $C_w/C_w^*=0.05$, the maximum conversion rate $k_{2e_{2m}}$ was estimated to be nearly equal to $k_s e_s$. And the $k_{2e_{2m}}$ value of 10.2 mmol/g-cell·hr was confirmed later to give the best agreement between observed and calculated

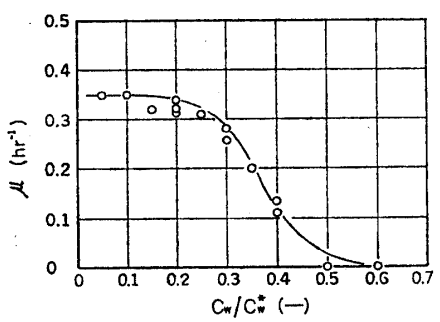

Fig. 8. Comparison of the observed specific growth rates with the rates calculated from Eq. (4).

values.

Using the assumptions mentioned previously for the distribution of acetyl-CoA into the catabolic and anaplerotic pathway, we obtained the following equations for acetyl-CoA, P_3 and cell mass, X.

$$\frac{1}{X}\frac{dP_3}{d\theta} = k_2 e_{2m}\left(\frac{e_2}{e_{2m}}\right) - \alpha k_4 e_{4m}\left(1-\frac{\eta_m}{1-\eta_m}\left(\frac{e_2}{e_{2m}}\right)\right)P_3 \quad (8)$$

$$\frac{1}{X}\frac{dX}{d\theta} = k_4 e_{4m}(Y_{X/P_3})\left(\frac{\eta_m}{1-\eta_m}\right)\left(\frac{e_2}{e_{2m}}\right)P_3 \quad (9)$$

In the above equations the unknown constants are Y_{X/P_3} and $\alpha k_4 e_{4m}$. Y_{X/P_3} represents the biomass yield on acetyl-CoA distributed into the anaplerotic pathway. This yield can be determined to be 0.0513 g-cell/mmol-acetyl-CoA from the elemental balance equation for cell synthesis. For $\alpha k_4 e_{4m}$, no rational determination method was found, so that $\alpha k_4 e_{4m}$ was determined to be 3.0 mmol/g-cell·hr by trial and error.

The relative enzyme activities of (e_2/e_{2m}) and (e_3/e_{3m}) at a given oxygen concentration were first obtained from Eq. (1). Then, by substituting these values into Eqs. (6)–(9), the changes of cell mass, ethanol and acetate in batch culture were calculated numerically by the Runge-Kutta-Gill method. As shown by the solid lines in Figs. 2 and 3, these calculated values agreed fairly well with the observed values. From these results it may be concluded that the growth of *Candida* sp. employed in this work is inhibited by the dissolved oxygen above $C_w/C_w^*=0.2$, and that the reduction of specific growth rate and cell yield are caused by the depression of enzyme activities of acetyl-CoA synthetase and isocitrate lyase. Therefore, when oxygen-enriched aeration is employed for the cultivation of *Candida* EY-12, the concentration of dissolved oxygen must be controlled so as not to exceed this upper critical value.

Nomenclature

C_w : dissolved oxygen concentration, mol/l

C_w^* : dissolved oxygen concentration in equilibrium with pure oxygen at 1 atm, mol/l

e : specific enzyme concentration, enzyme units/g-cell

e_m : specific enzyme concentration in the absence of effector, enzyme units/g-cell

E : effector concentration in Eq. (1)

k_3, k_2, k_4 : rate constants of ethanol oxidation, acetate activation and isocitrate oxidation, respectively, hr^{-1}

K_1, K_2 : equilibrium constant in Eq. (1)

K_3 : saturation constant for ethanol, mmol/l

P_1, P_2, P_3 : concentration of acetaldehyde, acetate and acetyl-CoA, respectively, m mol/l

R_t : total concentration of repressor in Eq. (1)

S : ethanol concentration, mmol/l

v_3, v_1, v_2 : specific reaction rates of ethanol oxidation, acetaldehyde oxidation and acetate activation, respectively, mmol/g-cell·hr

v_3, v_4 : specific mole flow rates of acetyl-CoA into anaplerotic and catabolic pathway, respectively, mmol/g-cell·hr

X : cell concentration, g/l

Y_{X/P_3} : biomass yield on acetyl-CoA distributed into the anaplerotic pathway, g-cell/mmol-acetyl-CoA

$Y_{X/S}$: biomass yield on ethanol, g-cell/g-ethanol

Greek

α : proportionality constant between v_4 and oxidation rate of isocitrate with isocitrate dehydrogenase, —

η, η_m : distribution coefficient of acetyl-CoA into the anaplerotic pathway and its maximum value, respectively, —

θ : cultivation time, hr

A : specific enzyme activity, mol/mg-protein·min

μ, μ_m : specific growth rate and its maximum value, respectively, hr^{-1}

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