On-Line Monitoring of Mould's Activity in Submerged Citric Acid Production*

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Based on experiments carried out on both synthetic media with saccharose and natural one with beet molasses, the correlations between gases exchange and the activity of *Aspergillus niger* strain during citric acid fermentation were estimated and used in on-line process control. For a continuous monitoring of biomass growth, the carbon dioxide evolution rate was chosen as a parameter that properly reflects all processes connected with biomass development and cells maintenance. For acidogenic activity control, the estimated volumetric product formation rate was used. The parameters were calculated in real time based on the difference between oxygen uptake and carbon dioxide evolution rates.

Citric acid (CA) is produced mainly by submerged fermentation with *Aspergillus niger* strains. High efficiency of CA production requires to measure many direct process parameters followed by their optimization analysis in real time. It is then necessary to apply the on-line computer control with simultaneous elimination of human in making a direct decision [1—3]. Most parameters employed to process control are based on environmental conditions such as temperature, pH, redox potential, oxygen concentration, *etc.* These parameters are used in traditional methods of citric acid biosynthesis control and do not directly represent the metabolic state of cells. A new trend in optimization techniques is to use methods that reflect the actual activity of microorganisms [4].

In the present study, the oxygen uptake rate, carbon dioxide evolution rate and its correlation with activity of biomass to citric acid excretion was studied and adapted to on-line computer control of citric acid biosynthesis process.

EXPERIMENTAL

Aspergillus niger W78B (strain collection of the Food Biotechnology Department, Wroclaw University of Economics, Wroclaw, Poland) used in an industrial production of CA on synthetic and natural media was used throughout this study. The synthetic medium with saccharose and natural one with beet molasses were used in these experiments. The composition of the synthetic medium was the following: 126 g saccharose, 2 g NH_4NO_3, 0.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, tap water to 1 dm^3, and HCl for adjustment of pH to 2.7. The natural medium contained: beet molasses in amount that was needed to obtain 126 g of sugars, 1.0 g $K_4Fe(CN)_6 \cdot 3H_2O$, 0.05 g $ZnSO_4 \cdot 7H_2O$, 0.15 g KH_2PO_4, tap water to 1 dm^3, and HCl for pH adjustment to 6.0.

Fermentations were performed in stirred tank reactor (STR) MicroFerm Fermenter MF114 (New Brunswick Scientific Co., New Brunswick, New Jersey, USA) and Biomer (Food Biotechnology Department, Wroclaw University of Economics, Wroclaw, Poland), with 14 dm^3 and 7 dm^3 capacity, respectively. Citric acid concentration was determined with pyridine and acetic anhydride and by isotachophoresis [5]. For dry cell mass determination, samples were vacuum-filtered. Biomass was washed and dried to constant mass.

Oxygen concentration in fermentation gases was determined using paramagnetic oxygen analyzer Servomex 1100A (Servomex International Ltd., Crowborough, Great Britain). The carbon dioxide was measured by infrared Guardian II Carbon Dioxide Monitor (Edinburgh Sensors Ltd, Edinburgh, Scotland). Air flow-rate at the inlet was measured by electronic rotameter ERG 2000 (Beta Erg, Warsaw, Poland). On the basis of continuously measured gas parameters, the derivative parameters were calculated in real time applying research computer system SysLab Bio [3].

Oxygen uptake (Q_{O_2}) and carbon dioxide evolution (Q_{CO_2}) rates were calculated according to the equa-

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tions

$$Q_{O_2} = \frac{\dot{V}_P}{V_r V_m}\left[\varphi_{O_2P} - \varphi_{O_2}\cdot\left(\frac{1-\varphi_{O_2P}-\varphi_{CO_2P}}{1-\varphi_{O_2}-\varphi_{CO_2}}\right)\right] \quad (1)$$

$$Q_{CO_2} = \frac{\dot{V}_P}{V_r V_m}\left[\varphi_{CO_2}\cdot\left(\frac{1-\varphi_{O_2P}-\varphi_{CO_2P}}{1-\varphi_{O_2}-\varphi_{CO_2}}\right) - \varphi_{CO_2P}\right] \quad (2)$$

where V_m is the air molar volume, Q_{CO_2} carbon dioxide evolution rate, Q_{O_2} oxygen uptake rate, $\dot{V}_P$ air flow-rate, V_r medium volume, φ_{CO_2} carbon dioxide volume fraction in fermentation gases, φ_{CO_2P} carbon dioxide volume fraction in air, φ_{O_2} oxygen volume fraction in fermentation gases, and φ_{O_2P} oxygen volume fraction in air.

RESULTS AND DISCUSSION

Parameters directly connected with metabolic state of cells that reflect the actual activity of microorganisms in CA biosynthesis can be chosen based on the mass balance.

The mass flow during citric acid fermentation from saccharose, limited to five basic elements (carbon, hydrogen, oxygen, nitrogen, and phosphorus) can be presented in the following way [6]

$$x\mathrm{CH_{11/6}O_{11/12}}\ \text{(saccharose)} + y\mathrm{O_2} + z\mathrm{NH_4^+} + w\mathrm{PO_4^{3-}} \rightarrow \mathrm{CH_aO_bN_cP_d}\ \text{(biomass)} + u\mathrm{CO_2} + v\mathrm{H_2O} + s\mathrm{CH_{4/3}O_{7/6}}\ \text{(citric acid)} \quad (A)$$

This equation takes into account two simultaneous fundamental processes running in citric acid production, mainly synthesis of biomass and citric acid.

Citric acid synthesis from saccharose can be represented by the equation

$$\mathrm{CH_{11/6}O_{11/12}} + 1/4\mathrm{O_2} = \mathrm{CH_{4/3}O_{7/6}} + 1/4\mathrm{H_2O} \quad (B)$$

The above reaction shows that citric acid overproduction is not accompanied with carbon dioxide evolution [7]. CO_2 is exclusively connected with precursors formation of biomass synthesis and the catabolism of sucrose. These processes can be reflected by the equation

$$x'\mathrm{CH_{11/6}O_{11/12}} + y'\mathrm{O_2} + z\mathrm{NH_4^+} + w\mathrm{PO_4^{3-}} \rightarrow \mathrm{CH_aO_bN_cP_d} + u\mathrm{CO_2} + v'\mathrm{H_2O} \quad (C)$$

Thus, the amount of oxygen consumed by *Aspergillus niger* (Q_{O_2}) is divided into 2 components according to the expression

$$Q_{O_2} = Q_{O_2(X)} + Q_{O_2(CA)} \quad (3)$$

The amount of oxygen connected with biomass growth processes and cell maintenance ($Q_{O_2(X)}$) can be described depending on respiratory quotient for biomass growth (RQ_X) in the following way

$$Q_{O_2(X)} = Q_{CO_2}/RQ_X \quad (4)$$

The oxygen uptake responsible for product formation ($Q_{O_2(CA)}$) is expressed as

$$Q_{O_2(CA)} = Q_{O_2} - Q_{CO_2}/RQ_X \quad (5)$$

Respiratory quotient during growth phase in fermentation on carbohydrates as a sole carbon source equals 1.0, but in an intensive phase of proteins synthesis, RQ_X increases and reaches values equal to 1.2 [8]. During citric acid accumulation phase, where both processes, *i.e.* growth of biomass and CA excretion run simultaneously, the overall RQ value is dropping down as a result of CA synthesis according to reaction (B). These circumstances hide the true value of respiratory quotient for biomass growth, thus a proportion of $Q_{O_2(X)}$ to Q_{CO_2} is difficult to establish. In consequence, $Q_{O_2(X)}$ values are often calculated on the basis of $RQ_X = 1.0$ [9]. The data from Table 1 show deviations of yield of product on oxygen ($Y_{P/O}$) from the theoretical values formulated in eqn (B) on the assumption that RQ_X can take 1.0 or 1.2 values. The possibility of RQ_X deviation during fermentation from 1.0 was also suggested by other authors [10].

From eqn (B) it results that $Y_{P/O}$ equals 128 g CA per 1 mol of oxygen assimilated by fungus and connected with citric acid synthesis.

Taking into consideration expression (4), the estimated volumetric product formation rate (Q^K_{CA}/(g dm^{-3} h^{-1})) can be described by the equation

$$Q^K_{CA} = Y_{P/O}\cdot Q_{O_2(CA)} \quad (6)$$

The product increase in time dt equals

$$\mathrm{d}P = Q^K_{CA}\cdot\mathrm{d}t = Y_{P/O}\cdot Q_{O_2(CA)}\cdot\mathrm{d}t \quad (7)$$

Therefore, the actual estimated citric acid concentration is expressed as

$$P_t = P_0 + Y_{P/O}\int_0^t Q_{O_2(CA)}\cdot\mathrm{d}t \quad (8)$$

Figs. 1 and 2 show the correlation between CA synthesis and its estimation based on exhaust gas analysis on both synthetic and molasses media on the assumption that biomass is growing with RQ_X values equal to 1.2.

Estimated volumetric product formation rate from eqn (6) reflects the actual acidogenesis activity of mycelium, and can be continuously measured during fermentation. Fig. 3 represents the Q^K_{CA} data obtained through this software probe. The Q^K_{CA} measurement can be additionally calibrated by introduction to eqn

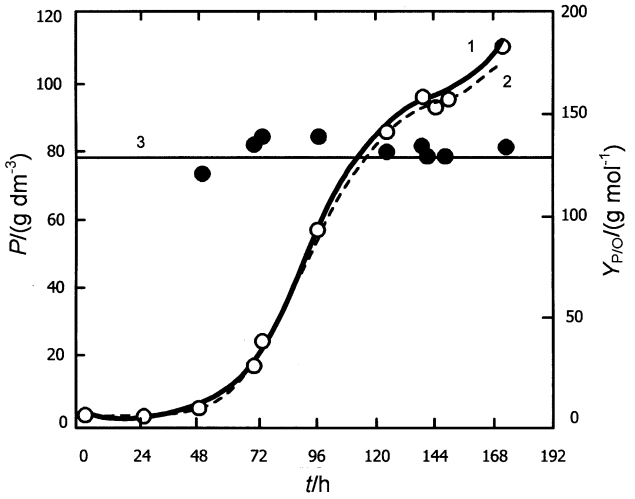

Fig. 1. Correlation between CA synthesis and its estimation based on exhaust gas analysis on synthetic media. Product estimation based on the conditions: *1.* $RQ_X = 1.2$ and $Y_{P/O} = 134$ g mol^{-1} calculated from real values of O_2 and CO_2; *2.* $RQ_X = 1.2$ and theoretical value of $Y_{P/O} = 128$ g mol^{-1}, ○ product concentration, ● changes of $Y_{P/O}$ real values during fermentation; *3.* theoretical value of $Y_{P/O} = 128$ g mol^{-1}. Run *2* (see Table 1).

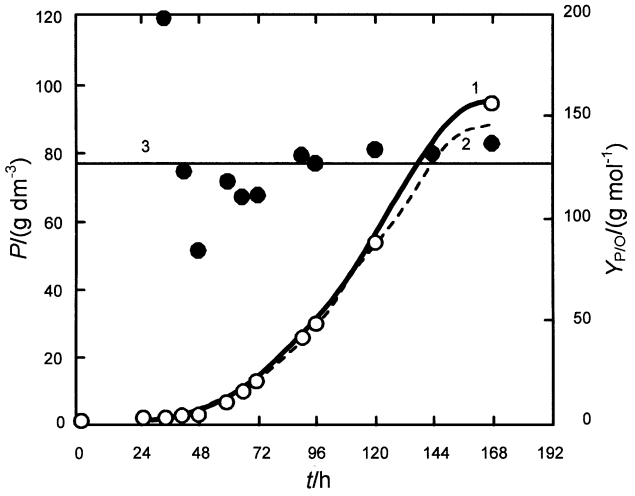

Fig. 2. Correlation between CA synthesis and its estimation based on exhaust gas analysis on molasses media. Product estimation based on the conditions: *1.* $RQ_X = 1.2$ and $Y_{P/O} = 138$ g mol^{-1} calculated from real values of O_2 and CO_2; *2.* $RQ_X = 1.2$ and theoretical value of $Y_{P/O} = 128$ g mol^{-1}, ○ product concentration, ● changes of $Y_{P/O}$ real values during fermentation; *3.* theoretical value of $Y_{P/O} = 128$ g mol^{-1}. Run *7* (see Table 1).

(*6*) an empirical correction factor obtained during the fermentation course in order to minimize errors derived from a specific accuracy of the equipment.

The second process that strongly influences the citric acid production is biomass formation (eqn (*B*)). A filamentous growth of *Aspergillus niger* causes difficulties in continuous determination of cell mass and applying specific rates to on-line process control. Searching for suitable parameters for biomass growth control, the share of the oxygen uptake rate connected with cells growth and maintenance was taken into account. $Q_{O_2(X)}$ can be expressed as

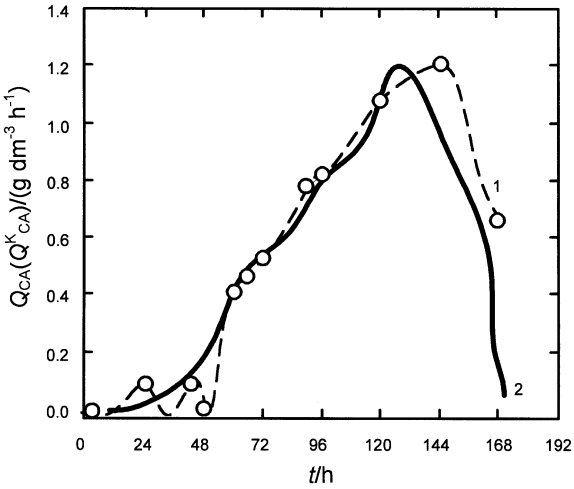

Fig. 3. Correlation between the CA production rate and its estimation based on exhaust gas analysis on molasses media. Estimation based on $RQ_X = 1.2$ and $Y_{P/O} = 138$ g mol^{-1} calculated from real values of O_2 and CO_2. *1.* Q_{CA}, 2. Q^K_{CA}. Run *7* (see Table 1).

$$Q_{O_2(X)} = Q_{O_2(X)m} + Q_{O_2(X)g} \qquad (9)$$

The oxygen uptake rate connected with cells maintenance ($Q_{O_2(X)m}$) is described by the equation

$$Q_{O_2(X)m} = X \cdot m_0 \qquad (10)$$

The share of oxygen uptake connected with the biomass growth can be characterized by the expression

$$Q_{O_2(X)g} = \frac{1}{Y_{X/O}} \cdot \frac{dX}{dt} \qquad (11)$$

Thus, the oxygen balance equation connected with biomass growth and maintenance processes takes the following form

$$Q_{O_2(X)} = X \cdot m_0 + \frac{1}{Y_{X/O}} \cdot \frac{dX}{dt} \qquad (12)$$

From eqn (*12*) a relationship for biomass calculation can be derived

$$X_t = X_0 + Y_{X/O} \int_0^t (Q_{O_2(X)} - X \cdot m_0) dt \qquad (13)$$

$Q_{O_2(X)}$ is expressed according to eqn (*5*) as Q_{CO_2}/RQ_X.

Table 1. Yield of Product on Oxygen in Dependence on Substrates and Respiratory Quotient for Biomass Growth

Run	Substrates	$Y_{P/O}$* ($RQ_X = 1.0$)	$Y_{P/O}$* ($RQ_X = 1.2$)	Deviation of real $Y_{P/O}$ values from theoretical = 128 g mol^{-1} calculated at $RQ_X = 1.2$	
		g mol^{-1}	g mol^{-1}	δ / g mol^{-1}	δ_r / %
1	Saccharose	173	158	30	23.4
2	Saccharose	147	134	6	4.7
3	Saccharose	154	130	2	1.6
4	Saccharose	156	138	10	7.8
5	Saccharose	147	130	2	1.6
6	Saccharose	164	148	20	15.6
7	Beet molasses	164	138	10	7.8
8	Beet molasses	156	136	8	6.3
9	Beet molasses	147	122	−6	4.7
10	Beet molasses	182	150	22	17.2
Mean		159	138	10	7.5
Standard deviation		11.8	10.8	–	–
Significance level ($\alpha = 0.05$)		8.4	7.7	–	–

* Calculation based on CA determination, total oxygen consumed and carbon dioxide evolved during fermentation.

The possibility of continuous biomass estimation during fermentation processes based on eqn (*13*) depends on the knowledge of $Y_{X/O}$, m_0, and RQ_X values and the assumption that all these parameters are constant. This is an essential prerequisite for real microorganisms growth control based on the biomass content calculation. Both the values of the *yield of biomass* on oxygen and *maintenance coefficient* presented in different publications are reported as a constant. $Y_{X/O}$ are about 0.05 g mol^{-1} and differences in each individual report are not significant. The differences between m_0 values are higher and show a discrepancy in the range 0.5×10^{-3}—1.0×10^{-3} mol g^{-1} h^{-1} [11, 12]. This suggests the nonconstant m_0 values, which is especially probable during batch cultivation processes [11, 13]. In the present work, the experiments with both synthetic and molasses media confirmed these observations (Fig. 4). Nonconstant value of maintenance coefficient can be explained, among other things, as a result of cell damage by lysis and shear forces as well as other processes occurring in the fermentation [14].

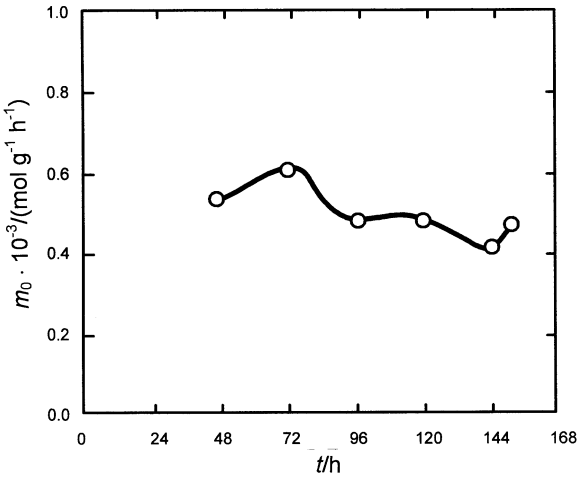

Fig. 4. Variation of maintenance coefficient during fermentation. Run *9* (see Table 1).

Apart from $Y_{X/O}$ and m_0 the third parameter important for biomass growth control is RQ_X that is reported also as a constant value [7]. The data obtained in this work (Table 1) and relatively high correlation between theoretical and real values of $Y_{P/O}$ proved that RQ_X is stable during fermentation, but differs from the value equal to 1.0. This phenomenon was also observed by other authors [10].

Taking into account the above observation, estimation of biomass content based on eqn (*15*) is possible [15] but should be based on the known course of m_0 changes. This can be realized for example in production scale where processes are repeated many times. Any deviations from normal conditions in a large extent eliminate this parameter (*i.e.* estimated biomass content) as a parameter for biomass growth control.

It was found that the most adequate and suitable parameter for cells growth optimization during citric acid biosynthesis is the control of $Q_{O_2(X)}$ values. This parameter according to eqn (*12*) entirely reflects the main processes connected with biomass life, *i.e.* a biomass increase and cells maintenance.

Eqn (*4*) shows that at constant values of RQ_X, $Q_{O_2(X)}$ can be adequately represented by Q_{CO_2}. Relatively high correlation between oxygen consumption and product formation (Figs. 1 and 2) calculated based on formula (*8*) indirectly pointed at the accuracy of CO_2 measurement, which allowed to reasonably precisely reflect all processes connected with biomass growth and its activity by Q_{CO_2} measurement during fermentation.

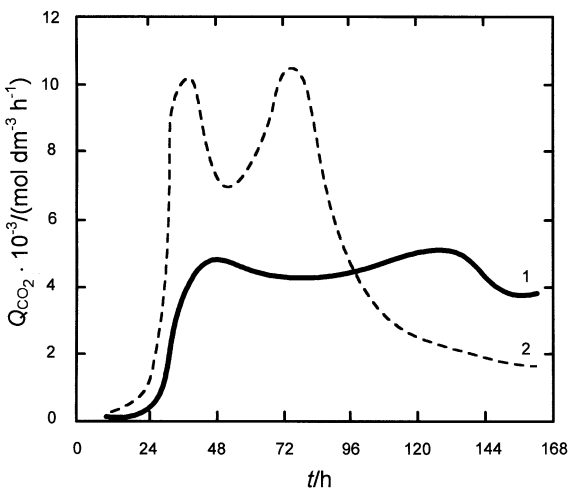

Fig. 5. Carbon dioxide evolution rates during fermentation on beet molasses. *1.* High CA yield, *2.* low CA yield.

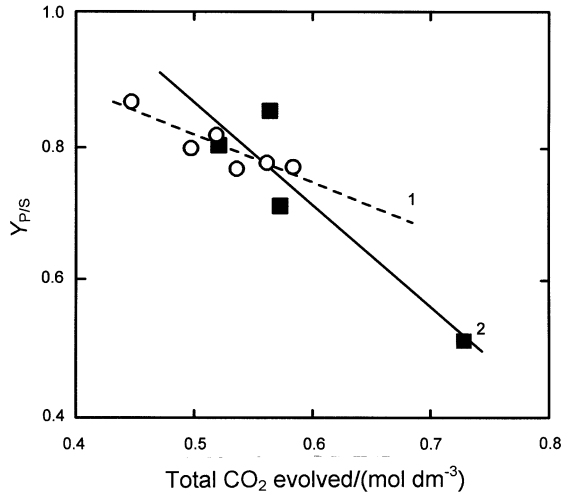

Fig. 6. Dependence of yield of product on substrate from carbon dioxide evolved during fermentation. ○ Synthetic medium, ■ molasses medium. Trends: *1.* synthetic and *2.* molasses media.

Since Q_{CO_2} controls a biomass formation, its course profile has to be optimized. This can be realized by optimization of aeration by controlling both air flow and agitation rates during CA fermentation. Fig. 5 shows CO_2 evolution rates controlled in the optimal range 5—6 mmol dm^{-3} h^{-1} that promoted 80 % of CA yield ($Y_{P/S}$), and in nonoptimal conditions resulting in CA yield reduction to 40 %. In consequence, it can be concluded that citric acid yield depended on the total carbon dioxide evolved during fermentation (Fig. 6).

CONCLUSION

Control parameters directly connected with metabolic state of cells that reflect the actual activity of microorganisms in CA biosynthesis, can be chosen based on the mass balance of the process. The parameters like the estimated volumetric product formation rate could be continuously measured and employed to on-line control of citric acid overproduction as it properly reflects actual acidogenesis activity of the mycelium. The correlation between gas exchange rates and the product formation also allows to calculate the actual concentration of citric acid in the medium. The estimation of product synthesis cannot be used instead of a quantitative determination of citric acid but it gives a possibility of on-line monitoring of product formation. For biomass on-line control, the most suitable parameter is carbon dioxide evolution rate, which comprehensively reflects all processes connected with biomass formation and cell maintenance.

SYMBOLS

m_0	maintenance coefficient	mol g^{-1} h^{-1}
P	product concentration	g dm^{-3}
Q_{CA}	volumetric product formation rate	g dm^{-3} h^{-1}
Q_{CO_2}	carbon dioxide evolution rate	mol dm^{-3} h^{-1}
Q_{CA}^{K}	estimated volumetric product formation rate	g dm^{-3} h^{-1}
Q_{O_2}	oxygen uptake rate	mol dm^{-3} h^{-1}
$Q_{O_2(CA)}$	oxygen uptake rate for citric acid formation	mol dm^{-3} h^{-1}
$Q_{O_2(X)}$	oxygen uptake rate for biomass growth and maintenance	mol dm^{-3} h^{-1}
$Q_{O_2(X)g}$	oxygen uptake rate for biomass growth	mol dm^{-3} h^{-1}
$Q_{O_2(X)m}$	oxygen uptake rate for biomass maintenance	mol dm^{-3} h^{-1}
RQ	respiratory quotient	
RQ_X	respiratory quotient connected with biomass growth	
t	time	h
V_m	air molar volume	dm^3 mol^{-1}
$\dot{V}_P$	air flow-rate	dm^3 h^{-1}
V_r	medium volume	dm^3
X	concentration of the biomass (d.m.)	g dm^{-3}
$Y_{P/O}$	yield of product on oxygen	g mol^{-1}
$Y_{P/S}$	yield of product on substrate	
$Y_{X/O}$	yield of biomass on oxygen	g mol^{-1}
φ_{CO_2}	carbon dioxide volume fraction in fermentation gases	
φ_{CO_2P}	carbon dioxide volume fraction in air	
φ_{O_2}	oxygen volume fraction in fermentation gases	
φ_{O_2P}	oxygen volume fraction in air	

x, y, z, w, a, b, c, d, u, w, v, s, g, x', y', v' factors

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