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Bioprocess parameters and oxygen transfer effects in the growth of *Pseudomonas dacunhae* for L-alanine production

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Abstract

Because alanine produced by most micro-organisms through fermentation is of the DL-racemate form and because it is too difficult to obtain L-alanine by fermentation, industrial production of L-alanine is accomplished by using L-aspartic acid as the reactant and L-aspartate β-decarboxylase (ADL) enzyme of resting *Pseudomonas dacunhae* cells as the biocatalyst. This work reports on the optimum growth conditions, i.e. optimum pH, temperature, agitation rate, substrate concentration and growth kinetics, of *P. dacunhae* investigated in shake flask cultures, as well as oxygen transfer kinetics studied in laboratory-scale bioreactors. Values of pH 7.0–7.5 and $T = 229$ K were optimal for both the growth and the enzyme activity. Although the specific growth rate μ increased with increasing agitation rate between 50 and 300 min^{-1}, the enzyme activity showed a maximum at an agitation rate of 150 min^{-1}. Substrate inhibition was observed for high glutamic acid concentrations, i.e. $C_{Go} > 10$ kg m^{-3}, which is used as the carbon source for the cultivation. The oxygen transfer kinetics were investigated under optimum conditions in a bioreactor of working volume 4×10^{-3} m^3 with temperature, pH, dissolved oxygen, stirring rate and foam control. The micro-organism concentration reached a plateau and the enzyme activity reached a maximum at a residence time of $t = 15$ h. The oxygen transfer coefficient $K_L a$ and oxygen uptake rate r_o, were maximum at residence times of $t = 10$ h and $t = 7$ h respectively. Increases in both the agitation rate and the oxygen feed rate increased the $K_L a$ values, with the agitation rate increase being much more effective.

Keywords: *Pseudomonas dacunhae*; L-aspartate β-decarboxylase enzyme; Growth kinetics; Oxygen transfer; Oxygen uptake rate; L-alanine production

1. Introduction

Because alanine produced by most micro-organisms through fermentation is of the DL-racemate form, industrial production of L-alanine—which is a speciality chemical used as a component of amino acid infusion and as a food additive because of its good taste—is accomplished from L-aspartic acid using the L-aspartate β-decarboxylase (L-aspartate 4-carboxy-lyase, EC 4.1.1.12) activity of immobilized *Pseudomonas dacunhae* cells, with the advantage of high yield and selectivity [1]. Although the use of different immobilizing agents [2–4], reactor types, and configurations and/or reactor–separator systems have been reported [5–8], the growth kinetics of the micro-organism, together with the related enzyme activity and oxygen transfer kinetics were not investigated.

This work reports on the optimum growth conditions of the micro-organism, considering both the micro-organism concentration and its enzyme activity. A growth model that involves substrate inhibition is given. To design, scale-up and operate the bioreactor with the adequate mass transfer, the oxygen consumption rates and oxygen transfer coefficients, which are the indicators of the mass transfer characteristics of a fermentation process, are required. However, as a result of the complex composition of the fermentation liquids, it can be difficult to predict these parameters with reasonable accuracy. Therefore, in this work, these parameters were investigated on the laboratory scale, along with the growth of *P. dacunhae* cells under optimum conditions, to determine their variation with the agitation and air feed rates. The experimental values of the mass transfer coefficient and oxygen uptake rate were compared with the available correlations in the literature.

2. Experimental details

2.1. Micro-organism and culture maintenance

The bacteria *P. dacunhae* (DSM 1455) was used as the microbial source of L-aspartate β-decarboxylase (ADL)

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enzyme in this study. The stock cultures were maintained on agar slants that contained 0.3% meat extract, 1.25% yeast extract, 0.5% peptone, 0.5% KH_2PO_4 and 1.5% agar, and their pH values were adjusted to 7.0 using 4 M KOH [9].

2.2. Media and bioreactors

The cells growing on the newly prepared slants were inoculated into the cultivation medium that contained 2.78% L-glutamic acid, 0.5% yeast extract, 0.05% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ [9], and the pH of the medium was adjusted to 7.5 using 25% NH_3 solution, unless otherwise stated. Small-scale batch experiments were conducted in orbital shakers under agitation and heating rate control, using air-filtered Erlenmeyer flasks 0.5×10^{-3} m^3 in size that had working volume capacities of 0.2×10^{-5}–2.0×10^{-4} m^3. The laboratory-scale batch bioreactor (Chemap CF 3000) consisted of a system of working volume 2.0×10^{-3}–4.0×10^{-3} m^3 with temperature, pH, foam, stirring rate and dissolved oxygen control, which was stirred with a six-blade Rushton turbine.

2.3. Measurement of the enzyme activity

The culture broth was harvested by centrifugation (Sorvall RC 28S) at 7000*g* at +4 °C for 15 min. After subsequent washing with 0.9% saline and water, the ADL activity of a defined amount of resting cells was measured as the initial L-alanine production rate (r_{P_0}) in moles of alanine (Ala) per cubic meter of solution per hour, with 5×10^{-6} m^3 of standard substrate solution containing 1 kmol m^{-3} L-aspartic acid, 1×10^{-3} kmol m^{-3} pyruvic acid, 1×10^{-4} kmol m^{-3} pyridoxal 5′-phosphate and 0.1% Tween 20. The pH of the bioreaction medium was adjusted to 5.5 using a 25% NH_3 solution.

2.4. Measurement of the micro-organism and amino acid concentrations

The micro-organism concentrations based on dry weights were measured with a UV–vis spectrophotometer (Schimadzu UV-160A), using the calibration curve obtained at 500 nm. The L-alanine concentrations were measured with a Waters amino acid analysis system, using the Pico Tag method [10]. This method is based on reversed-phase high pressure liquid chromatography (HPLC), using a precolumn derivation technique with a gradient program developed for L-alanine analysis [11].

3. Results and discussions

3.1. Effect of pH value

In the literature, there are no reports of investigations to find the optimum pH for growth and/or enzyme activity. However, Chibata et al. [12] cultivated a micro-organism at pH 7 for L-alanine production. Therefore, in this study, the effect of the initial pH on the micro-organism concentration at a residence time of $t = 24$ h and on the enzyme activity as the initial L-alanine production rate were investigated in shake flasks in the pH range 4.5–9.0, and the results can be seen in Fig. 1. It is clear from the figure that a pH value of 5.5–7.5 is optimal for growth, while the pH range 7.0–8.0 is optimal for the ADL activity. Therefore, the pH range 7.0–7.5 can be considered as being optimal for both growth and enzyme activity.

3.2. Effect of temperature

The effect of the temperature on the biomass concentration and on the enzyme activity were investigated in the temperature range $T = 297$–305 K. The results are shown in Fig. 2.

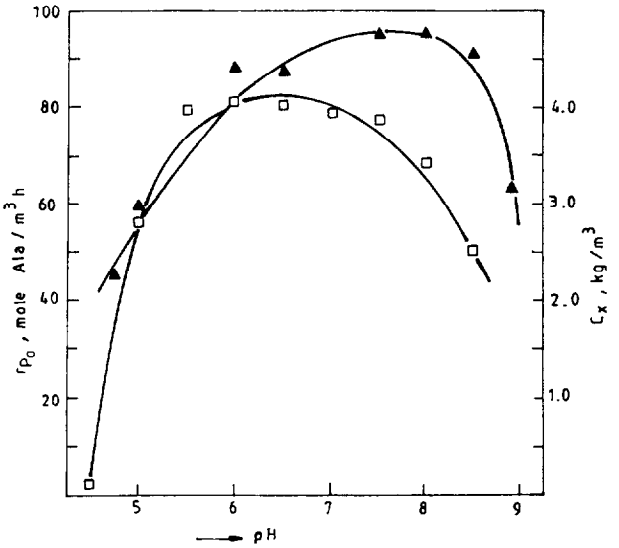

Fig. 1. Effect of the initial pH value on the biomass concentration (–□–) and enzyme activity (–▲–). $C_{Go} = 27.8$ kg m^{-3}, $T = 303$ K, $N = 105$ min^{-1}, $V = 0.2 \times 10^{-4}$ m^3, $t = 24$ h.

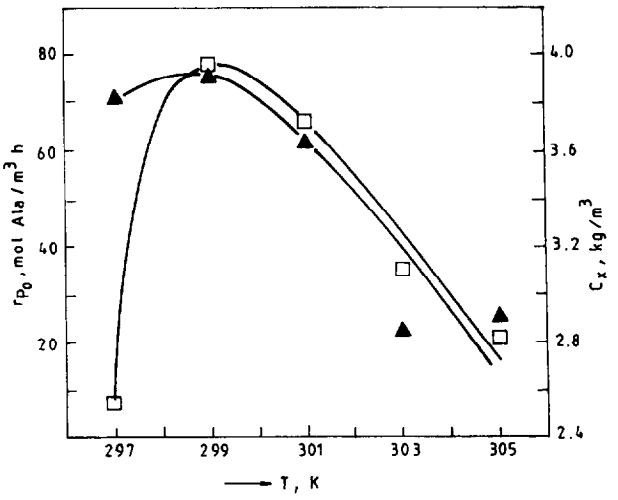

Fig. 2. Effect of the temperature on the biomass concentration (–□–) and enzyme activity (–▲–). $C_{Go} = 27.8$ kg m^{-3}, pH 7.5, $N = 105$ min^{-1}, $V = 0.2 \times 10^{-4}$ m^3, $t = 24$ h.

It is clear from the figure that the temperature is a very important bioreactor operation parameter, and that $T=299$ K is optimal for both growth and enzyme activity. However, Chibata et al. stated that $T=303$ K gives better results than does $T=299$ K for the enzyme activity, and employed 303 K for the growth [12]. This difference in the optimum temperature value probably results from differences in the sensitivity in temperature measurement, which can vary depending on the instrumentation, as well as differences in the sensitivity in the measurement of the ADL activity. Chibata et al. [12] measured the ADL activity by manometric measurement of the side-product CO_2 liberated from L-aspartic acid.

3.3. Effect of agitation rate

The effect of the agitation rate N in shake flasks was investigated in orbital shakers in the range $N=50$–300 min^{-1}. Biomass concentration vs. residence time curves can be seen in Fig. 3 for five different agitation rates. The biomass concentration and the relative enzyme activity corresponding to a residence time of $t=24$ h are plotted vs. the agitation rate in Fig. 4. It is clear from Fig. 4 that, although the biomass concentration increases with increasing agitation rate as a result of the decrease in mass transfer limitations, the enzyme activity shows a maximum at $N=150$ min^{-1}. Because ADL is an intracellular enzyme, this result is probably not related to the negative effect of shear stress on the protein molecule, but probably relates to the difference in the dissolved oxygen concentration caused by the different agitation rates, which could cause an optimum for the enzyme production. Another possibility is that the enzyme production could be related to the growth rate of the cells.

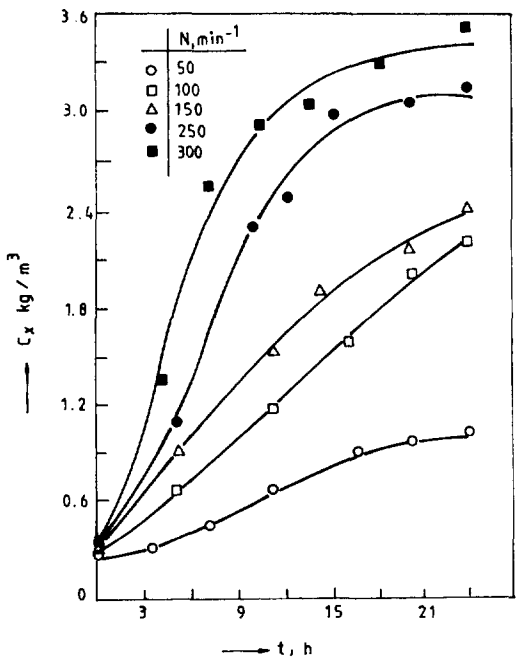

Fig. 3. Effect of the agitation rate and residence time on the biomass concentration. $C_{Go}=10.0$ kg m^{-3}, pH 7.5, $T=303$ K, $V=0.2\times10^{-3}$ m^3.

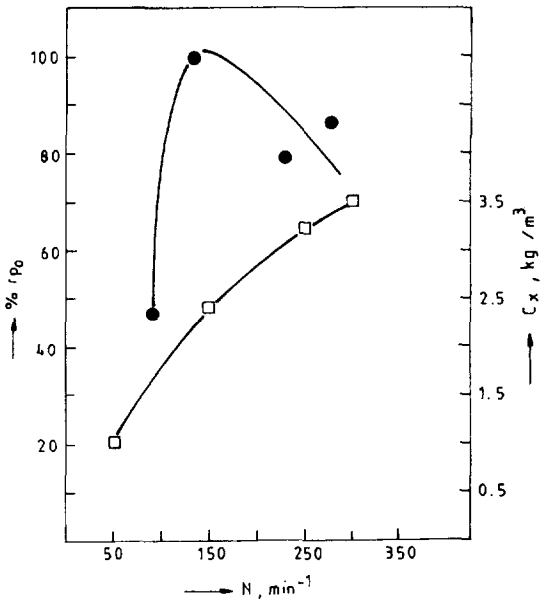

Fig. 4. Effect of the agitation rate on the biomass concentration (–□–) and relative enzyme activity (–●–). $C_{Go}=10.0$ kg m^{-3}, pH 7.5, $T=303$ K, $V=0.2\times10^{-3}$ m^3, $t=24$ h.

3.4. Effect of L-glutamic acid concentration and growth kinetics

L-glutamic acid is preferred as the carbon source for the micro-organism instead of glucose [13], because the amino acid stimulates ADL enzyme formation, while giving maximal growth. Takamatsu and Ryu [7] used 27.8 kg m^{-3} L-glutamic acid for the cultivation of the micro-organism, but did not give further details related to its effect when used in higher or lower concentrations. Therefore, in this study, the effect of the L-glutamic acid initial concentration was investigated in the range $C_{Go}=0$–40 kg m^{-3} range.

Biomass concentration vs. residence time plots can be seen in Fig. 5(a) and (b). $C_{Go}=10$ kg m^{-3} is optimal for growth and a further increase in the concentration decreased the biomass concentration, as a result of substrate inhibition. The variation of the biomass concentration and the enzyme activity with the L-glutamic acid concentration for a residence time of $t=25$ h can be seen in Fig. 6. It is clear from the figure that, although the range $C_{Go}=5$–10 kg m^{-3} gives better results for the biomass, the range $C_{Go}=10$–20 kg m^{-3} is optimal for the enzyme activity. Therefore, $C_{Go}=10$ kg m^{-3} should be chosen as the initial substrate concentration to cultivate the micro-organism, while achieving a higher enzyme activity and a higher concentration of the active enzyme.

The specific growth rate μ values obtained from the micro-organism concentration C_x vs. t plots are given in Fig. 7. The variation of μ with C_{Go} obeyed the substrate inhibition model given by

$$\mu=\mu_{max}\frac{C_{Go}}{K_1+C_{Go}+C_{Go}^2/K_2} \quad (1)$$

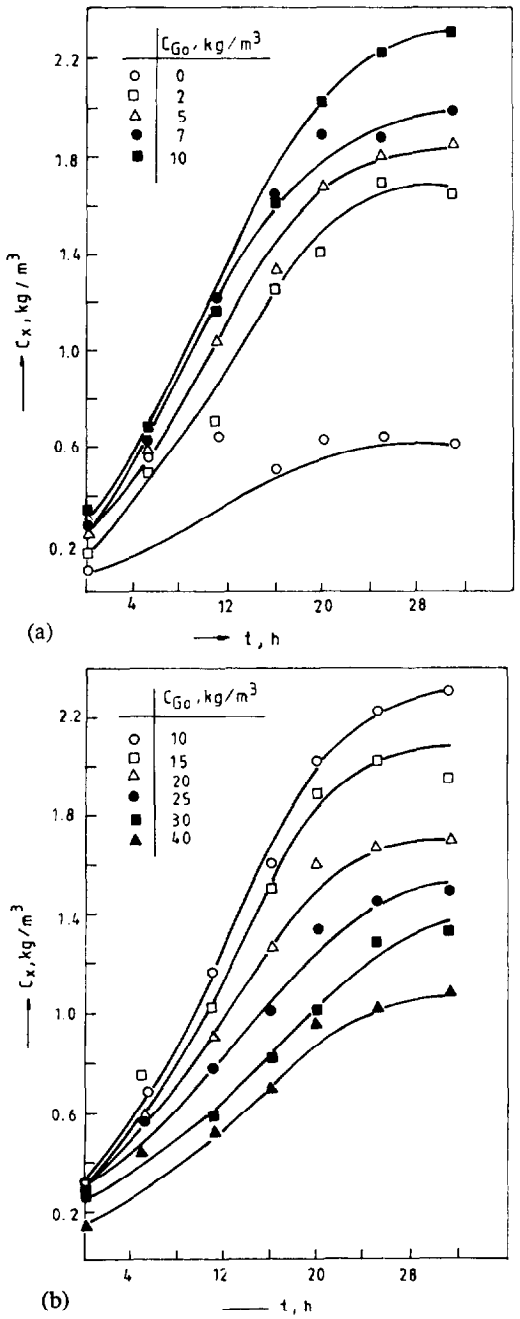

Fig. 5. (a) Effect of the initial glutamic acid concentration ($C_{Go} < 10$ kg m^{-3}) and residence time on the biomass concentration. pH 7.5, $T = 303$ K, $N = 105$ min^{-1}, $V = 0.2 \times 10^{-3}$ m^3. (b) Effect of the initial glutamic acid concentration ($C_{Go} > 10$ kg m^{-3}) and residence time on the biomass concentration. pH 7.5, $T = 303$ K, $N = 105$ min^{-1}, $V = 0.2 \times 10^{-3}$ m^3.

The kinetic parameters of the model were found with a non-linear regression analysis computer program based on the Marquardt method [14]: $\mu_{max} = 0.1143$ h^{-1}, $K_1 = 0.57$ kg m^{-3} and $K_2 = 55.7$ kg m^{-3}. The variation according to the model is shown by the full line in Fig. 7.

3.5. Scale-up and oxygen transfer kinetics

Oxygen transfer effects were investigated in the bioreactor of working volume 4×10^{-3} m^3 under optimum conditions

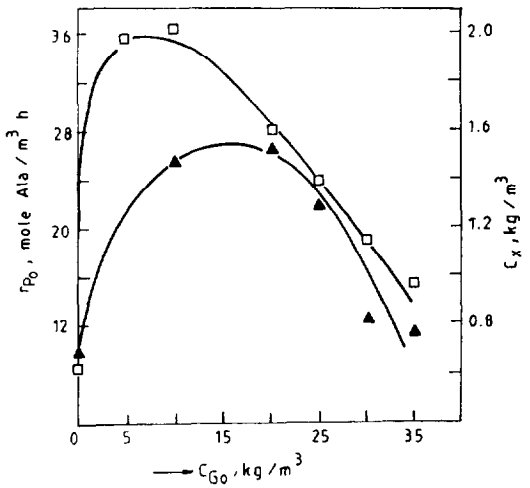

Fig. 6. Effect of the initial glutamic acid concentration on the biomass concentration (–□–) and enzyme activity (–▲–). pH 7.5, $T = 303$ K, $N = 105$ min^{-1}, $V = 0.2 \times 10^{-3}$ m^3, $t = 25$ h.

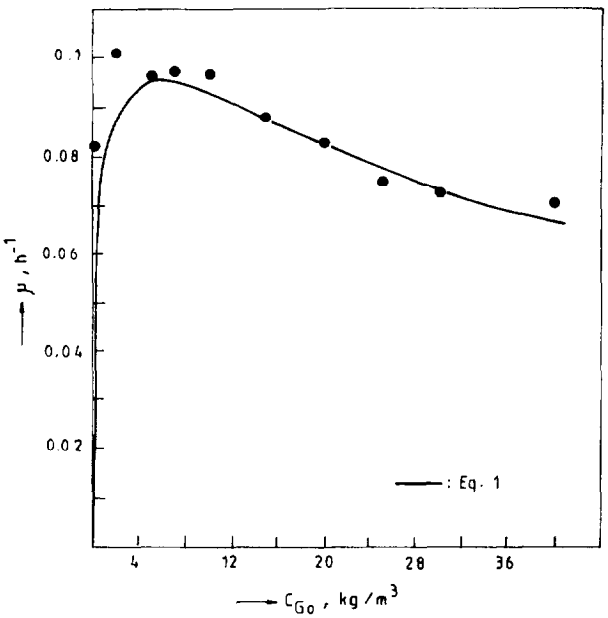

Fig. 7. Effect of the initial glutamic acid concentration on the specific growth rate. pH 7.5, $T = 303$ K, $N = 105$ min^{-1}, $V = 0.2 \times 10^{-3}$ m^3.

found in the shake-flask experiments. The pH was controlled using a 99.99% H_3PO_4 solution, with a sensitivity of pH ± 0.1, and the dissolved oxygen concentration was controlled using agitation and varying either the air or pure oxygen feed rate. Fig. 8 shows the variation of the agitation rate, volumetric oxygen flow rate, dissolved oxygen concentration and biomass concentration with the residence time. As is clear from the figure, the air or low oxygen feed rates were not sufficient to maintain higher dissolved oxygen concentrations in the logarithmic growth phase, and a further increase in the oxygen feed rate was found necessary. The micro-organism concentration reached a plateau at a residence time of $t = 15$ h. The enzyme activity of the cells was at a maximum at this residence time, with a value of 133 mol Ala m^{-3} h^{-1}.

The dynamic method was applied [15] to find the oxygen transfer coefficient $K_L a$ and oxygen uptake rate r_o values

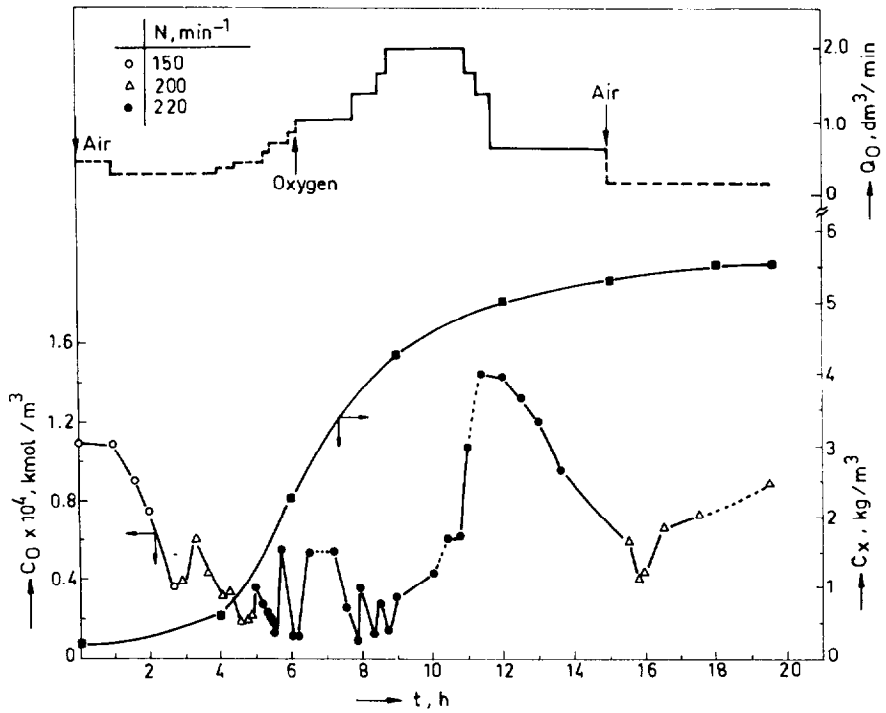

Fig. 8. Effect of the oxygen feed rate, agitation rate and residence time on the dissolved oxygen and biomass concentrations. The dotted line shows the measurement of the oxygen transfer coefficient and oxygen uptake rate by the dynamic method. $C_{Go} = 10$ kg m^{-3}, pH 7.5, $T = 299$ K, $V = 2.0 \times 10^{-3}$ m^3.

during the residence times corresponding to the dotted regions of the dissolved oxygen concentration vs. residence time curve, by adjusting the agitation rate and air feed rate to the desired values. During the application of the dynamic method, the transfer of oxygen from the headspace to the broth was prevented by reducing the agitation rate as soon as the gas flow was switched off.

At $t < 0$ h, the oxygen transfer coefficient $K_L a_o$ was measured in the medium in the absence of the micro-organism, and was found to be 0.0013 s^{-1}. The variation of $K_L a$ and of the enhancement factor E ($= K_L a / K_L a_o$) with the agitation rate and the air flow rate throughout the cultivation is indicated in Table 1. Their variations with the residence time at constant agitation and air flow rates can be seen in Fig. 9. As it is apparent from the figure, neither $K_L a$ nor r_o varies greatly with the residence time during the lag period ($t = 0$–3 h) or the stationary period ($t > 12$ h). However, during the log period $t = 4$–12 h, the variations of the mass transfer coefficient and oxygen consumption rate with the residence time are significant. The oxygen transfer coefficient is at a maxi-

Table 1
Variation of the oxygen transfer parameters with the agitation and air flow rates

t (h)	N (min^{-1})	Q_A (dm^3 min^{-1})	$K_L a$ (s^{-1})	r_o ($\times 10^7$ kmol m^{-3} s^{-1})	E
0	150	2.0	0.0017	0.648	1.28
0	150	4.0	0.0027	0.679	2.08
0	220	2.0	0.0034	0.713	2.62
5.5	220	4.0	0.0079	2.100	6.08
6.5	220	2.0	0.0135	7.992	10.38
6.5	250	2.0	0.0167	4.800	12.85
6.5	270	2.0	0.0186	5.280	14.30
6.5	220	4.0	0.0141	2.470	10.85
6.5	220	5.3	0.0152	2.520	11.69
7.0	220	2.0	0.0183	8.160	14.07
10.0	220	2.0	0.0480	3.910	36.92
13.0	220	2.0	0.0092	1.540	7.07
16.0	220	2.0	0.0060	0.700	4.60
18.0	220	2.0	0.0056	0.857	4.30
18.0	250	2.0	0.0109	0.600	8.38
18.0	270	2.0	0.0117	0.600	9.00
18.0	220	4.0	0.0058	0.864	4.46
18.0	220	5.3	0.0063	0.590	4.85
20.0	220	2.0	0.0024	0.640	1.87

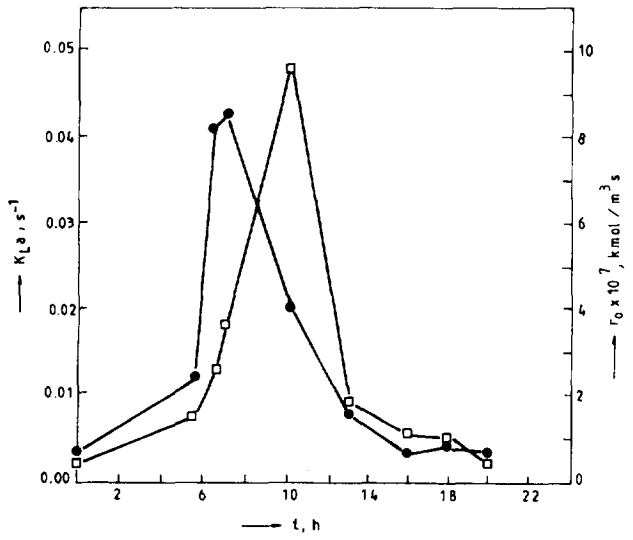

Fig. 9. Effect of the residence time on the oxygen transfer coefficient (–□–) and oxygen uptake rate (–●–). pH 7.5, $T=299$ K, $N=220$ min^{-1}, $Q_A=2.0$ dm^3 min^{-1}, $V=2.0\times10^{-3}$ m^3.

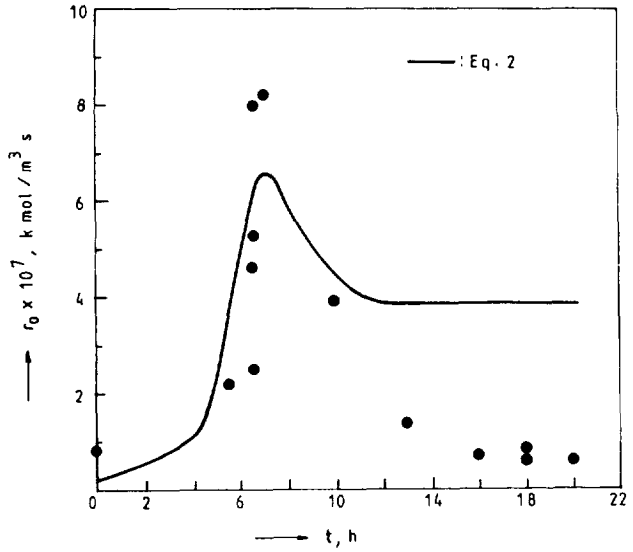

Fig. 10. Comparison of experimental and correlated values of the oxygen uptake rate. pH 7.5, $T=299$ K, $N=220$ min^{-1}, $Q_A=2.0$ dm^3 min^{-1}, $V=2.0\times10^{-3}$ m^3.

mum at $t=7$ h, while the oxygen uptake rate is at a maximum at $t=10$ h.

Increases in the rate of production of the biomass, in the biomass concentration and in the substrate consumption rate increase the oxygen uptake rate, which depends on the metabolic functions of the biomass. During the lag period, r_o tends to increase, mainly as a result of increases in the biomass production rate and biomass concentration. In the log period, r_o is high, as a result of the high substrate consumption and biomass production rates. As the substrate consumption rate and biomass production rate decrease, r_o also decreases. van't Reit [16] gave a correlation for r_o that depended on an elemental balance for the growth of a biomass grown on a specific media:

$$r_o=[(2.78\times10^{-4})(1/Y_{sx}-1.04)\mu+m_s]C'_x \quad (2)$$

To compare the experimental results with this correlation, C'_x values were calculated assuming that the *P. dacunhae* had the molecular formula $CH_{1.93}O_{0.55}N_{0.25}P_{0.021}$ [17]. Taking the Y_{sx} and m_s values as 0.85 and 0.2×10^{-5} kmol kmol^{-1} s^{-1}, respectively, r_o was plotted vs. time using Eqs. (1) and (2), and experimental values of C_{Go} and C'_x. This variation is shown in Fig. 10 as a full line, together with the experimental r_o values. There is notably good agreement between the model and the experimental results, except for the stationary phase, i.e. $t>12$ h, which probably results from the fact that the maintenance coefficient m_s would not retain its assumed constant value during this period.

As can be seen from Table 1, when compared with the gas flow rate, the agitation rate is a more effective parameter for increasing the oxygen transfer coefficient. The K_La values were also calculated from the correlations given for the stirred vessels (water with salts and non-coalescing bubbles) [16], gas side resistance to mass transfer was neglected as the solubility of the oxygen in the fermentation liquid is low. We have

$$K_La=0.002(P_g/V)^{0.7}u_g^{0.2} \quad (3)$$

The power input P_g in aerated stirred liquid was calculated from the correlation given by Nagata [18], i.e.

$$\log(P_g/P)=-192(D_i/D)^{4.38}\,Re^{0.115}\,Fr^{1.95D_i/D}A \quad (4)$$

The power input P in gas-free stirred liquid was calculated from the power number vs. Reynolds number graphical correlation [18]. Comparisons of the calculated K_La values with the experimental values that correspond to the beginning of the log period are given in Fig. 11. There is fairly good agreement between the experimental and correlated values in this period, which corresponds to enhancement factor E values of around 10 – 14. When the E value is much lower (in the lag and stationary periods) or much higher (towards the end of the log period) than this range, the difference between the experimental and correlated values increases, because of the

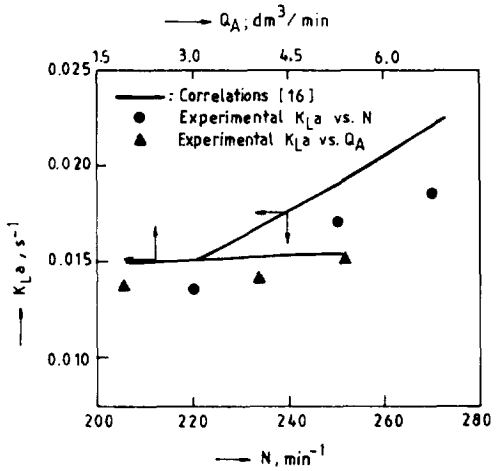

Fig. 11. Effect of the air feed rate and agitation rate on the oxygen transfer coefficient. pH 7.5, $T=299$ K, $V=2.0\times10^{-3}$ m^3, $t=6.5$ h. For K_La vs. Q_A, $N=220$ min^{-1}; for K_La vs. N, $Q_A=2.0$ dm^3 min^{-1}.

dynamic behavior of the complex fermentation media, which could not be correlated for the entire fermentation process.

The correlations given for the processes, where mass transfer is accompanied by a chemical reaction, incorporate the controlling resistance mainly in the mass transfer step. However, the mass transfer can be enhanced multi-fold as a result of several causes, two of which are the presence of catalyst particles in heterogeneous reactive systems, depending on the relative rates of mass transfer and chemical reaction; and the presence of particles in the mass transfer zone. Therefore, fine biocatalyst particles (biomass) which change in number and activity throughout the fermentation process should cause a disagreement between the observed and correlated values.

In gas–liquid mass transfer, the observed effects of the fine particles on K_La are quite complex. Nevertheless, it has been shown that, when the diameter of the particles is 1.0–0.5 μm, the liquid-side mass transfer coefficient first increases and then decreases with increasing particle concentration. Particles with a diameter somewhat greater than the thickness of the mass transfer layer also enhance the gas absorption, but the enhancement decreases with increasing particle diameter [19]. Therefore, along with the increase in the concentration of rod-shaped biocatalyst *P. dacunhae* cells that have dimensions 0.8 μm × 1.4–1.8 μm [20], the mass transfer coefficient could be different from the correlation values, depending on the oxygen uptake rate, diameter and concentration.

4. Conclusions

For the bioproduction of L-alanine from L-aspartic acid, the L-aspartate β-decarboxylase (ADL) enzyme activity of the resting *P. dacunhae* cells is used. The enzyme activity of the cells is an important parameter for the yield and productivity of industrial L-alanine production using the biotransformation process. Among the parameters that affect both the activity and the growth, the effects of the pH value, temperature, initial substrate concentration and agitation rate were investigated, and their optimum values were found to be pH 7.0–7.5, $T=299$ K, $C_{Go}=10$ kg m^{-3} and $N=150$ min^{-1}, respectively, in the shake flasks. The optimum values of the pH, temperature and initial substrate concentration were found to be different from the values used in the literature [7,12].

The oxygen transfer kinetics was investigated in a bioreactor of working volume 4×10^{-3} m^3 operated under the optimum conditions. The dissolved oxygen concentration was controlled by varying the agitation and air or oxygen flow rates. Although agitation is much more effective for control of the increase in the dissolved oxygen concentration, the agitation rate was not increased above 220 min^{-1} to avoid decreasing the enzyme activity.

The oxygen transfer coefficients and the oxygen consumption rates found by applying the dynamic method revealed that both parameters show maximum values during the log phase of the growth. The variation of the oxygen consumption rates throughout the fermentation is as expected and agrees with the related correlation. The experimental oxygen transfer coefficient increased significantly with the agitation rate rather than with the increase in the gas flow rate—which agrees with the correlations [16]—with slightly lower values of K_La for the initial periods of the log phase of the growth. As a result of the complex effect of fine biocatalyst particles, it was not possible to compare the experimental mass transfer coefficients with the correlations throughout the dynamic fermentation process.

Acknowledgements

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Appendix A. Nomenclature

A	aeration number ($=Q_A/(ND_I^3)$) (—)
ADL	L-aspartate β-decarboxylase
C_{Go}	initial glutamic acid concentration (kg m^{-3})
C_o	dissolved oxygen concentration (kmol m^{-3})
C_x	micro-organism concentration (kg dry cell m^{-3})
C'_x	micro-organism concentration (kmol m^{-3})
D	bioreactor diameter (m)
D_I	impeller diameter (m)
E	enhancement factor ($=K_La/K_La_o$); mass transfer coefficient with chemical reaction per physical mass transfer coefficient (—)
Fr	Froude number ($=D_IN^2/g$) (—)
K_La	overall liquid film mass transfer coefficient (s^{-1})
K_1	parameter in the model for growth; Eq. (1) (kg m^{-3})
K_2	parameter in the model for growth; Eq. (1) (kg m^{-3})
m_s	maintenance coefficient (kmol kmol^{-1} s^{-1})
N	agitation or shaking rate (min^{-1})
P	power input in gas-free stirred liquid (W)
P_g	power input in aerated stirred liquid (W)
Q_A	volumetric air flow rate (dm^3 min^{-1})
Q_o	volumetric oxygen feed rate (dm^3 min^{-1})
r_{Po}	initial L-alanine production rate (mol m^{-3} solution h^{-1})
r_o	oxygen uptake rate (kmol m^{-3} s^{-1})
Re	Reynolds Number ($=D_I^2N\rho/\mu$) (—)
t	bioreactor residence time (h)
T	bioreaction medium temperature (K)
u_g	superficial gas velocity (m s^{-1})
V	volume of the bioreaction media (m^3)
Y_{sx}	yield of biomass on substrate (—)

Greek symbols

ρ liquid phase density (kg m^{-3})
μ specific growth rate (h^{-1})
μ_{max} maximum specific growth rate (h^{-1})

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