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# An L-amino acid electrode for monitoring beer fermentation

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A membrane covered amperometric L-amino acid electrode is described, employing L-amino acid oxidase immobilized on a Pt disc electrode with rabbit albumin and glutaraldehyde. The electrode response to a range of L-amino acids and a theoretical treatment for the rate determining step are presented. Results are also given for the application of the electrode in monitoring beer fermentations. Appropriate amino acid utilisation is vital for both yeast cell growth and beer flavour development.

**List of symbols**

| | |
|---|---|
| $A$ | electrode area |
| $D$ | diffusion coefficient |
| $e'$ | reduced enzyme concentration |
| $e_\Sigma$ | total enzyme concentration |
| $F$ | Faraday constant |
| $i$ | electrode current |
| $i_D$ | $= i/A$ |
| $I$ | $= l/k'_{ME}$ |
| $j$ | flux |
| $L$ | thickness of the electrolyte layer |
| $L_M$ | thickness of the membrane |
| $k_{cat}$ | rate constant for enzyme/substrate reaction |
| $k'$ | rate constant for electrode reaction |
| $k'_{ME}$ | electrochemical rate constant for the enzyme reaction |
| $k'_S$ | mass transfer rate constant for substrate in membrane |
| $K$ | membrane constant |
| $K_S$ | partition coefficient of substrate in membrane |
| $K_M$ | Michaelis constant |
| $n$ | number of electrons |
| $S$ | substrate |
| $\rho$ | $= Ii_D/[S]$ |
| $y$ | $= (\rho^{-1} - 1)/[S]$ |

## 1. Introduction

In our previous paper [1] we described the application of electrochemical sensors for carbon dioxide, oxygen and glucose in the monitoring of beer fermentation. During fermentation the concentrations of these analytes change and the rate of change gives a measure of yeast vitality [2, 3]. In this paper we describe an amperometric enzyme electrode for L-amino acids for use in a similar fashion. Amino acids are the major source of nitrogen for yeast cell growth [4, 5], and are also important in the proper development of beer flavour [5, 6]. The sensor utilizes the enzyme L-amino acid oxidase (L-AAO) and is selective to a range of L-amino acids, mainly those having hydrophobic side chains. The overall reaction of the enzyme with an amino acid is

$$\overset{+}{N}H_3CH(R)CO_2^- + O_2 + H_2O \xrightarrow{\text{L-AAO}}$$

$$RCOCO_2^- + NH_4^+ + H_2O_2$$

L-AAO was immobilized on to a Clark-type oxygen electrode [7] and the current generated by the oxidation of $H_2O_2$ used to give a measure of the amino acid concentration. The response of the electrode to a range of common L-amino acids was tested and evidence obtained for the identity of the rate determining step in the response.

Beer fermentations were carried out in 1 $dm^3$ tall tube fermenters with samples being taken at regular intervals amino acid analysis. Yeast cells metabolise L-amino acids in synthesizing proteins and therefore by following the uptake rate of amino acids an assessment of yeast vitality can be made.

## 2. Theory

Figure 1 illustrates the reaction scheme of the L-amino acid enzyme electrode used in our study. In a previous paper [8] we described a model for amperometric enzyme electrodes. A full mathematical treatment is given and its application to enzyme electrodes is also described [9]. The analysis is used to determine the rate determining step and the electrochemical rate constant for the enzyme electrode.

In this paper we shall use the model with the following assumptions:

(i) No concentration polarization exists in the electrolyte layer.
(ii) Oxygen concentration does not limit the sensor

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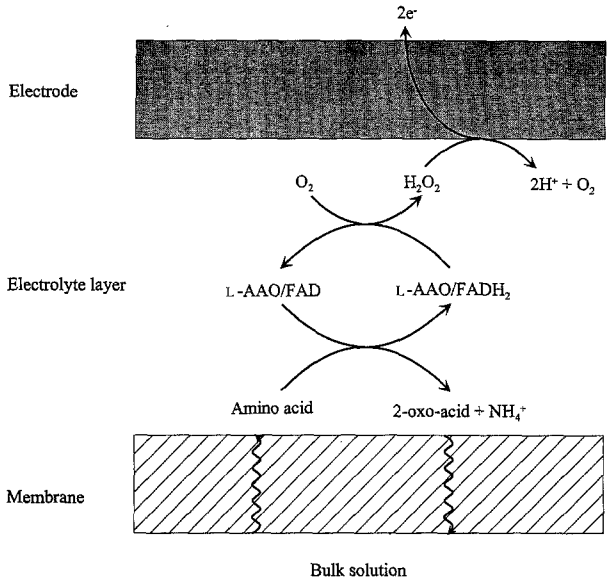


Fig. 1. Enzyme electrode.

response (achieved by saturating the solution with $O_2$).

(iii) There is no product inhibition.

The response of the electrode is limited by one of three possible kinetic steps: (a) the diffusion of substrate through the membrane, (b) the enzyme reaction, and (c) the electrode reaction.

The slowest step is rate determining. The current response of the electrode is given by [8]

$$i = nFAj \tag{1}$$

where the symbols have the meanings already described.

The current response for an electrode where transport through the membrane is rate limiting is given by

$$i = nFA\frac{[S]KD}{L_M} \tag{2}$$

where $K$ is the partition coefficient of the substrate through the membrane, $D$ is the diffusion coefficient of substrate through the membrane and $L_M$ is the membrane thickness.

When the enzyme reaction is rate limiting and the enzyme is unsaturated the current is given [8] as

$$i = \frac{nFALk_{cat}[S]e_\Sigma}{K_M} \tag{3}$$

where $k_{cat}$ is the rate constant for reaction of the saturated enzyme, $K_M$ is the Michaelis constant and $e_\Sigma$ is the total enzyme concentration. When the enzyme is saturated [8],

$$i = nFALK_{cat}e_\Sigma \tag{4}$$

Finally, the response where the electrode reaction is rate limiting is

$$i = nFAk'e_\Sigma \tag{5}$$

where $k'$ is the rate constant for the electrode reaction and the enzyme will nearly all be in the reduced state.

## 3. Experimental details

### 3.1. *Chemicals and solutions*

L-amino acid oxidase (L-AAO, EC 1.4.3.2, activity 7 U mg$^{-1}$, Boehringer Mannheim) from *Crotalus Durissus* venom suspended in 3.2 mol dm$^{-3}$ ammonium sulphate, was purified in a micro dialysis cell by repeated washing with pH 6 phosphate buffer to remove ammonium sulphate. Rabbit albumin and glutaraldehyde were purchased from Sigma Chemical Co. All other commercially supplied chemicals were of AnalaR grade. Solutions were made up with deionized water from a MilliQ system (Millipore). Stock solutions of L-amino acids were generally made at 0.1 mol dm$^{-3}$ concentration in the same buffer solution used for testing the electrode response, were stored at 4° C and discarded after one week.

### 3.2. *Electrode construction*

The working electrode was a platinum disc (7 mm diameter) which was polished to a mirror finish using 0.3 $\mu$m alumina (Buehler Ltd). Enzyme was immobilized on the Pt surface using a similar technique described by Kusano [10]. 3 mg rabbit albumin was first suspended in 30 $\mu$l dialyzed enzyme solution (0.03 mg). 1 $\mu$l of a 25 vol % glutaraldehyde was added and the solution mixed well. 10–20 $\mu$l of this solution was dropped onto the surface of the electrode and allowed to cure for 30 min at room temperature in a high humidity chamber. A dialysis membrane (Medicell Ltd, MWCO 14 000), previously soaked in buffer, was placed over the electrode and secured using a rubber 'O' ring. The electrode was prevented from drying out by storing in pH 6.5 phosphate buffer at 4° C when not in use.

Ale yeast was grown aerobically from cultured stock supplied by Whitbread plc. Brewers wort was also supplied by Whitbread of approximately 1040 specific gravity.

### 3.3. *Apparatus and procedures*

A typical three electrode electrochemical cell arrangement was used. The counter electrode was a Pt gauze and the reference electrode was a saturated calomel electrode (SCE). The enzyme electrode was potentiostated at +450 mV vs SCE and the cell volume was 20 cm$^3$. Oxygen levels were kept saturated by bubbling 100% oxygen (BOC Ltd) through the solution. Electrode potentials were controlled and currents measured using the Imperial College microprocessor unit (ICCMU) serial interface rack.

The electrode response was determined for a range of common amino acids. Solutions were generally prepared by the injection of 0.1 mol dm$^{-3}$ stock solution into standard pH 7.4 buffer. All solutions were stirred using a magnetic stirrer and amino acid additions and subsequent electrode response were obtained at room temperature.

### 3.4. *Beer fermentations*

Ale fermentations were carried out in glass tall tube fermenters. 500 cm$^3$ of aerated wort was placed in

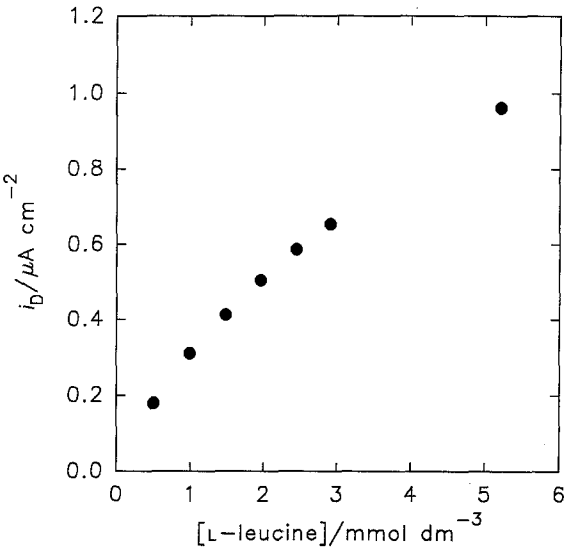


Fig. 2. Response of electrode to L-leucine. The enzyme was immobilized on the electrode surface using rabbit albumin/glutaraldehyde.

the tube. Yeast was pitched (added) at 2 g $dm^{-3}$ and the tube shaken to given uniform distribution of yeast. Samples were drawn at regular intervals and frozen immediately for future analysis.

Frozen samples were thawed out and centrifuged at 10 000 rpm for 10 min to remove yeast cells. The electrode response to amino acid levels in the beer was determined by adding 200 $\mu$l of beer to 20 $cm^3$ of standard buffer. Frequent calibration of the electrode was performed using 0.25 mmol $dm^{-3}$ L-leucine. In each case recorded currents were corrected for background signal in the absence of beer or L-leucine standard.

## 4. Results and discussion

### 4.1. *Immobilized enzyme electrode response to L-amino acids*

Figure 2 shows a calibration curve for L-leucine when enzyme was immobilized in a rabbit albumin/glutaraldehyde matrix. In this experiment no dialysis membrane was fitted over the electrode end, in this case the electrode response is governed by enzyme kinetics. A value of $K_M$ for the enzyme can be determined from a Michaelis–Menten plot of $i_D^{-1}$ against [L-leucine]$^{-1}$ (Fig. 3) [11]. The intercept with the $x$-axis ($-K_M^{-1}$) gives $K_M$ = 3.6 mmol $dm^{-3}$ for L-leucine as substrate. This value is in close agreement with other reported values [12].

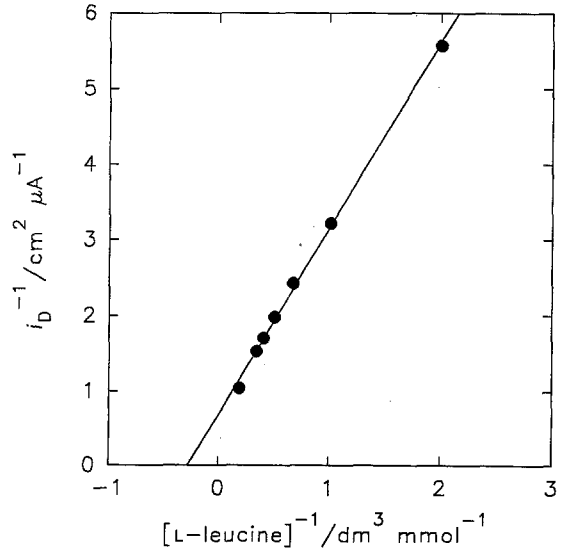


Fig. 3. Plot of $i_D^{-1}$ against [L-leucine]$^{-1}$ (Lineweaver–Burk) for data shown in Fig. 2.

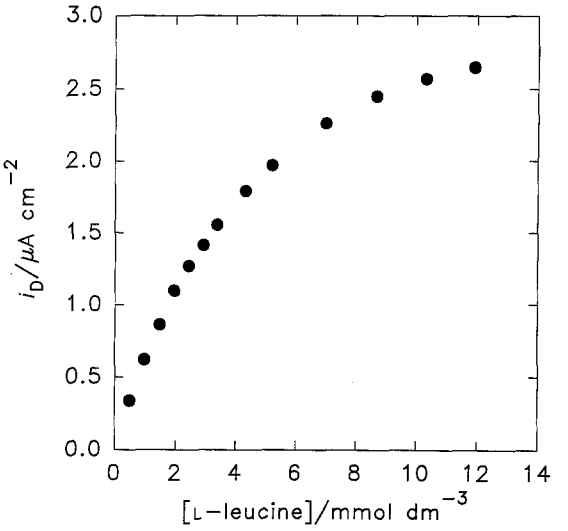


Fig. 4. Response of membrane covered electrode to L-leucine as substrate.

### 4.2. *Membrane electrode response to amino acids*

Figure 4 shows a typical response of the membrane covered electrode using L-leucine as substrate. The data in this figure can be analysed as previously described [8, 9]. The first step in the analysis is to make a Hanes plot [13] of $nF$[L-leucine]/$i_D$ against [L-leucine]. The plot for data in Fig. 4 is shown in Fig. 5. The intercept with the $y$-axis, $I$, gives the electrochemical rate constant, $k'_{ME}$, where [8]

$$\frac{1}{k'_{ME}} = \frac{K_M}{e_\Sigma L k_{cat}} + \frac{1}{k'_S} = I \qquad (6)$$

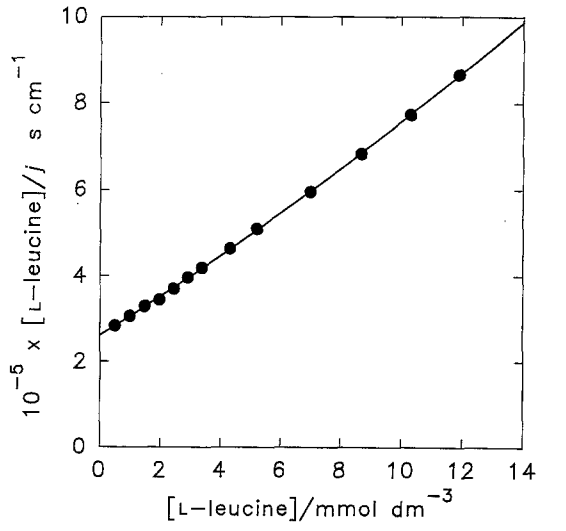


Fig. 5. Hanes plot of data shown in Fig. 4.

and,

$$k'_S = \frac{K_S D}{L_M} \tag{7}$$

where $K_S$ is the partition coefficient of the substrate in the membrane.

The next step in the analysis is the rho plot. The parameter $\rho$ is calculated using

$$\rho = \frac{I i_D}{[S]} \tag{8}$$

From Equation 24 in [8] $y$ is plotted against $\rho$, where

$$y = \frac{\rho^{-1} - 1}{[S]} = \frac{1}{K_{ME}}\left[1 - \frac{\rho k'_{ME}}{k'_S}\right] \tag{9}$$

and

$$K_{ME} = \frac{K_M (L k_{cat})^{-1} + e_\Sigma (k'_S)^{-1}}{(L k_{cat})^{-1} + (k')^{-1}} \tag{10}$$

A plot of Equation 9 for L-leucine is shown in Fig. 6. The horizontal line shows [8] that enzyme kinetics are rate limiting and that the data in Fig. 4 obey Michaelis–Menten kinetics. A value for $K_M$ can be determined, as before, from the Lineweaver–Burk plot $i_D^{-1}$ against [L-leucine]$^{-1}$. This plot is shown in Fig. 7 and we obtain $K_M = 5.4\,\text{mmol dm}^{-3}$ for L-leucine as substrate. This value is in good agreement with other values [12].

The response of the electrode to a range of amino acids, $r$, was determined and Table 1 shows these responses relative to L-leucine. The times to 90% total response, $t_{90}$, are also reported.

### 4.3. *Reproducibility and lifetime of sensor*

The electrode was used on a continuous basis for amino acid analysis and stored in buffer at 4 °C when not in use. The electrode response to 250 $\mu$mol dm$^{-3}$ L-leucine over a period of three weeks is shown in Fig. 8. It is clear that even after three weeks the electrode response was as much as 50% of the original signal. There is an initial increase in response during the first three days and this is consistent with the observation made by other workers using similar systems [14, 15]. Explanations for this observation are the establishment of diffusion channels in the immobilized layer, and changes in the enzyme conformation into a more reactive form. We conclude that the electrode has a useful lifetime of over a month but must be calibrated daily.

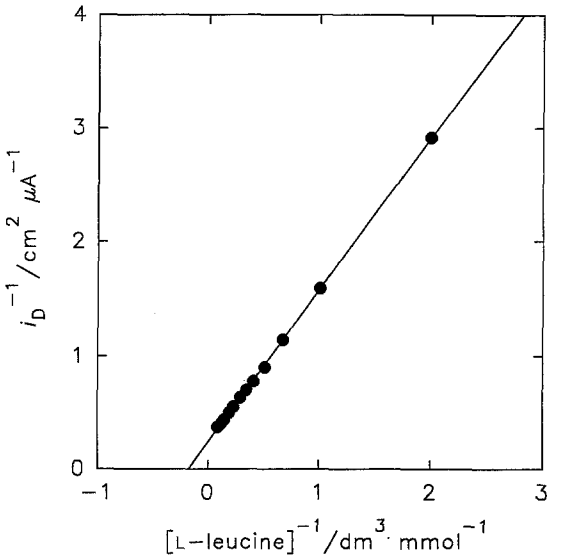


Fig. 7. Plot of $i_D^{-1}$ against [L-leucine]$^{-1}$ (Lineweaver–Burk) for data shown in Fig. 4.

### 4.4. *Beer fermentations*

Yeast cells use nitrogen in the form of ammonium ions, proteins, peptides and amino acids as fuel for growth. Wort contains a mixture of different amino acids which are listed in Table 1 in descending order of electrode response. Amino acids are taken up by yeast cells at different rates. Amino acids can be classified into four groups depending on the rate of utilization [6]. Group A amino acids are absorbed rapidly, typically within 20 h after starting the fermentation,

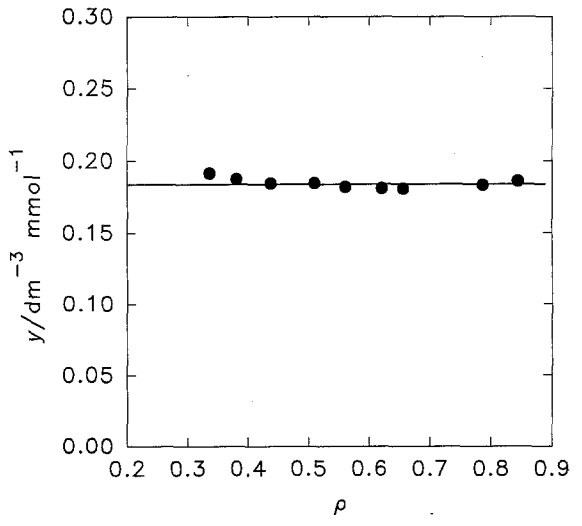


Fig. 6. Rho plot of data shown in Fig. 4.

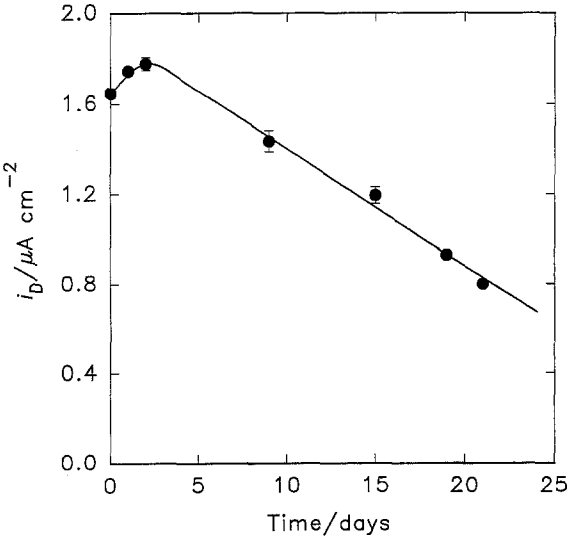


Fig. 8. Long term stability of sensor.

*Table 1. Summary of electrode response to amino acids*

| *L-Amino acid* | *Electrode response, r** | $t_{90}$ /s | *Typical amount in wort, p/%* | Ω | *Yeast activity [4]* |
|---|---|---|---|---|---|
| Phenylalanine | 1.19 | 120 | 5.5 | 0.79 | C |
| Methionine | 1.18 | 130 | 0.6 | 0.09 | B |
| Leucine | 1.00 | 174 | 8.3 | 1.00 | B |
| Iso-leucine | 0.59 | 250 | 4.2 | 0.30 | B |
| Histidine | 0.5 | 420 | 1.1 | 0.07 | B |
| Arginine | 0.18 | 565 | 3.4 | 0.07 | A |
| Valine | 0.11 | 370 | 7.1 | 0.09 | B |
| Glutamine | 0.11 | 415 | 1.6 | 0.02 | A |
| Asparagine | 0.04 | 450 | 2.4 | 0.01 | A |
| Lysine | 0.01 | 490 | 2.5 | 0.00 | A |
| Serine | <0.01 | – | 5.2 | <0.01 | A |
| Threonine | <0.01 | – | 3.7 | <0.01 | A |
| Aspartic acid | <0.01 | – | 2.4 | <0.01 | A |
| Proline | 0.00 | – | 26.9 | 0.00 | D |
| Alanine | 0.00 | – | 10.6 | 0.00 | C |
| Glycine | 0.00 | – | 4.3 | 0.00 | C |
| Glutamic acid | 0.00 | – | 2.3 | 0.00 | A |
| Tryptophan | insoluble | | 3.8 | | C |
| Cysteine | insoluble | | | | |

* Relative to L-leucine.
† Determined for wort supplied by Whitbread plc using HPLC.

group B amino acids are removed rather more slowly, and group C are taken up once all group A acids have been utilized. Proline the only acid in group D, is only absorbed very slowly and is present in large amounts at the end of fermentation. Each group represents about a quarter of the total amino acid content. The reason for different uptake rates is that there are two transport mechanisms in yeast cell walls for amino acid uptake. These mechanisms are described in detail elsewhere [16].

To determine which amino acids the sensor response will be due to during a beer fermentation we calculate Ω, where;

$$\Omega = \frac{pr}{8.3} \tag{11}$$

where $p$ is the typical percentage of each amino acid present in wort and 8.3 is the typical percentage of L-leucine in wort. The Ω values are listed in Table 1. We find that the sensor response will primarily come from L-leucine ($\Omega = 1.00$) followed by L-phenylalanine ($\Omega = 0.79$) and L-isoleucine ($\Omega = 0.30$). The sum of the Ω values, ΣΩ, for each group are; $\Sigma\Omega_A = 0.10$, $\Sigma\Omega_B = 1.55$ and $\Sigma\Omega_C = 0.79$. We conclude that the sensor response will primarily be attributed to amino acids in groups B and C.

Figure 9 shows a plot of an amino acid concentration versus time profile for an ale fermentation carried out in a tall tube glass fermenter. Error bars are included where multiple analyses were carried out. The current response of the sensor is expressed in terms of equivalent L-leucine concentration. In phase X, group A amino acids are utilized but this is not seen by the sensor. In phase Y, group B amino acids are absorbed, and in phase Z we see residual group C amino acids present in the wort.

The group D amino acid, proline, which typically forms 50% of the total amino nitrogen in wort, is not absorbed, and at the end of fermentation it represents over 90% of the total amino acid content. The sensitivity of the electrode towards more important amino acids, though they are present in smaller concentrations, is increased, due to the lack of response to the unreactive amino acid proline.

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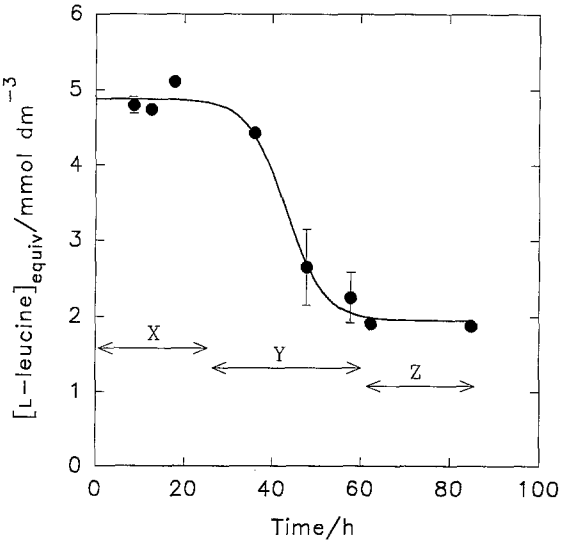


Fig. 9. Amino acid levels in an ale fermentation. Amino acid concentration given in terms of equivalent [L-leucine].

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