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Mathematical modelling of lipid production by oleaginous yeasts in continuous cultures

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Abstract. A mathematical model was constructed to describe the influence of the carbon to nitrogen ratio (C/N-ratio) of the growth medium on lipid production by oleaginous yeasts. To test this model and to determine some relevant model parameters, the oleaginous yeast *Apiotrichum curvatum* ATCC 20509 was grown in continuous cultures at various C/N-ratios and dilution rates. It appeared that when nitrogen is limiting for the formation of biomass, the remaining glucose can be converted to storage carbohydrate and storage lipid. No clear dependence of carbohydrate yield on the C/N-ratio could be demonstrated, but lipid yield increased gradually with increasing C/N-ratios.

The maximal dilution rate for lipid producing yeast cells appeared to be optimal at relatively low C/N-ratios. It can be concluded that the experimental results fitted well with the mathematical model. By using this model, lipid yield and lipid production rate can be calculated at any C/N-ratio of the growth medium and optimum operation conditions can be predicted for the production of microbial lipids.

INTRODUCTION

Oleaginous yeasts can accumulate large quantities of lipid when grown under nitrogen limited conditions. These lipids usually comprise more than 80% triacylglyceride with a fatty acid composition similar to many plant seed oils. Although there is an increasing interest in the production of 'single cell oil', thus far the economics of the process are unfavourable, and further optimizing fermentation conditions will be of paramount importance if oil fermentations are to be economically feasible.

The principal factors that affect the process economics of microbial oil fermentations are the *yield coefficient* (defined as the amount of lipid product produced per unit weight of nutrient utilized), and the *fermentor productivity* (defined as the amount of lipid product produced per unit of fermentor volume and per unit time).

It is well established that the yield coefficient of oleaginous yeasts is high when these organisms are grown in a medium with a high carbon to nitrogen ratio (C/N-ratio). After nitrogen is exhausted, the excess of carbon substrate is con-

verted to lipid and the yeast cells become filled with lipid droplets (Gill, Hall & Ratledge 1977).

In batch cultures of oleaginous yeasts, the specific rate of lipid synthesis appeared to be rather low (Kessel 1968), and in nitrogen limited continuous cultures it was demonstrated that no efficient lipid production could be achieved at dilution rates higher than about 0.04–0.06h^{-1} (Evans & Ratledge 1983). Consequently, fermentor productivity will be low in growth media with high C/N-ratios.

From the facts stated above, it will be apparent that the C/N-ratio of the growth-medium will have a considerable influence on the process economics of oil fermentations.

In order to predict the influence of the C/N-ratio on lipid yield and fermentor productivity, a mathematical model was constructed. This model can be used to optimize conditions for microbial oil production. Experimental results of continuous cultures of the oleaginous yeast *Apiotrichum curvatum* are presented to determine some relevant model parameters and to test the practical use of the model.

DERIVATION OF A MATHEMATICAL MODEL FOR LIPID PRODUCTION IN OLEAGINOUS YEASTS GROWN IN CONTINUOUS CULTURES

1. Relationship between the C/N-ratio of the growth medium and the lipid yield

The model is based on a stoichiometric balance equation for carbon, nitrogen, oxygen, and hydrogen. In this model the formation of an intermediate between substrate utilization and lipid production is postulated as suggested in literature (Boulton & Ratledge 1983). It is also assumed that the postulated intermediate contains no nitrogen. All substrates and products are converted to formulae which contain one mole of carbon. This leads to the following general balance equation for biomass, intermediate, and lipid production:

$$\underset{\text{substrate}}{CH_kO_l} + a\,NH_3 + b\,O_2 = \underset{\text{biomass}}{Y_x\,CH_pO_nN_q} + \underset{\text{intermediate}}{Y_iCH_tO_u} + \underset{\text{lipid}}{Y_lCH_rO_s} + c\,H_2O + d\,CO_2 \qquad [1]$$

In this equation CH_kO_l, $CH_pO_nN_q$, CH_tO_u, and CH_rO_s denote the elemental compositions of the organic substrate, microbial biomass, intermediate, and lipid product respectively. The values of the subscripts k, l, r, and s can be calculated from the molecular formulae of the organic substrate and lipid product (triacylalcohol). The values of p, n, and q, and of t and u, can be determined after an elemental analysis of the biomass and the intermediate respectively. From Eq. (1) the elemental balances of carbon and nitrogen can be derived (see Table 1 for nomenclature symbols):

Table 1. Nomenclature

C/N-ratio	carbon to nitrogen ratio of the growth medium (C-mol/N-mol)
D	dilution rate = volume of incoming medium per unit time/volume of medium in the culture vessel (h^{-1})
D_{max}	maximum dilution rate (h^{-1})
L	lipid yield coefficient (g lipid produced/g substrate consumed)
M_l	molecular weight of the lipid product (C-mol)
M_s	molecular weight of the substrate (C-mol)
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
q	nitrogen content of biomass (mol nitrogen/mol biomass)
q_L	specific lipid production rate (g lipid $\cdot m^{-3} \cdot h^{-1} \cdot$ g biomass^{-1})
Q_L	lipid production rate (kg lipid $\cdot m^{-3} \cdot hr^{-1}$)
[S]	substrate concentration (C-mol substrate $\cdot l^{-1}$)
[S*]	substrate concentration ($Kg \cdot m^{-3}$)
[X]	biomass concentration (C-mol biomass $\cdot l^{-1}$)
Y_x	molecular fraction of carbon substrate that is converted to biomass (C-mol biomass/C-mol substrate).
Y_{xs}	Maximal amount of biomass that can be produced per C-mol carbon substrate under carbon-limited conditions, when no lipid or intermediate formation occurs. (C-mol biomass/C-mol substrate)
Y_i	molecular fraction of carbon substrate, that is converted to intermediate (C-mol intermediate/C-mol substrate).
Y_{is}	maximal amount of intermediate that can be produced per mol carbon substrate (C-mol intermediate/C-mol substrate).
Y_l	molecular fraction of carbon substrate that is converted to storage lipid (C-mol lipid/C-mol substrate)
Y_{ls}	maximal amount of storage lipid that can be produced per mol carbon substrate (C-mol lipid/C-mol substrate)

$$\text{carbon: } Y_x + Y_i + Y_l + d = 1 \qquad [2]$$

$$\text{nitrogen: } Y_x q = a \qquad [3]$$

In the model presented here, it is assumed that intermediate and intracellular lipid will only accumulate under nitrogen-limited conditions. Consequently two different situations can be distinguished: (i) nitrogen is present in excess, and (ii) nitrogen is limiting for the formation of biomass.

(i) Nitrogen is present in excess. When nitrogen is present in excess, Y_i, and Y_l will be zero, and $Y_x = Y_{xs}$. From Eq. [1] it can be deduced that when $a > q.Y_{xs}$, no nitrogen limitation occurs. e.g. The C/N-ratio (mol/mol) of the growth medium must be greater than $1/q.Y_{xs}$ to induce nitrogen limitation. The value at which the C/N-ratio of the growth medium equals the value of $1/q.Y_{xs}$ will be called the critical C/N-ratio for lipid production:

$$C/N_{critical} = 1/q.Y_{xs} \quad [4]$$

or

$$Y_{xs} = 1/(q.\ C/N_{critical}) \quad [5a]$$

(ii) Nitrogen is limiting. When nitrogen is limiting, the value of Y_x from Eq. [1] will be dependent on the amount of nitrogen that is available and will be:

$$Y_x = 1/(q.C/N) \quad [5b]$$

The molecular fraction of substrate, needed for the production of Y_x mol biomass under nitrogen limiting conditions is: Y_x/Y_{xs}. The molecular fraction of substrate, needed for the production of intermediate is: Y_i/Y_{is}. The molecular fraction of substrate that remains for the production of lipid will be: $1-Y_x/Y_{xs}-Y_i/Y_{is}$. The amount of lipid that can be produced from the remaining substrate will accordingly be:

$$Y_l = Y_{ls}\,(1-Y_x/Y_{xs}-Y_i/Y_{is}) \quad [6]$$

Using Eq. [6], the amount of lipid (mol) per mol substrate can be calculated at any C/N-ratio of the growth medium when:

(i) the nitrogen content of the biomass is known (= the value of subscript q in Eq. [1])

(ii) the values of Y_{xs}, Y_i/Y_{is}, and Y_{ls} have been calculated or have been determined experimentally.

When the molecular weight of the substrate (M_s) and of the lipid product (M_l) have been determined, the yield coefficient (L) can be calculated, being:

$$L = (M_l/M_s)Y_l\ (\text{g lipid/g substrate}) \quad [7]$$

Assuming that, when substrate costs are concerned, the carbon source of the growth medium is the major component to take into account, substrate costs can be calculated directly from the yield coefficient when the price of the substrate is known.

The substrate costs ($\$_s$) per kg lipid produced are then:

$$\$_s = \frac{\text{price of carbon source per kg}}{\text{yield coefficient}} \quad [8]$$

2. Relationship between the C/N-ratio of the growth medium and the lipid production rate

In continuous cultures of oleaginous yeasts, only a reduced amount of biomass per mol carbon substrate can be formed under nitrogen-limited conditions. Therefore, compared to growth conditions with excess nitrogen, more time will

be required to consume the available carbon substrate completely. Consequently relative low dilution rates will be required to obtain efficient lipid production. This will be illustrated in the following mathematical model. In this model it is assumed that:

- To obtain optimal lipid production in a given growth medium, the carbon substrate must be fully consumed at the highest possible dilution rate.
- The overall rate of carbon substrate consumption is a linear function of the biomass concentration in the growth medium.
- The amount of substrate that is consumed for maintenance purposes is negligible.

The linear function of substrate consumption and biomass concentration can be written as follows:

$$d[S]/dt = \mu[X]_t/Y_{xs} \qquad [9]$$

The maximal dilution rate at which the carbon substrate can still be fully used, is given by:

$$(d[S]/dt)_{max} = (\mu_{max} \cdot [X]_t)/Y_{xs} = D_{max} \cdot [S] \qquad [10]$$

The relation between biomass concentration and carbon substrate concentration is:

$$[X]_t = Y_x \cdot [S] \qquad [11]$$

Combination of equations [10] and [11] results in:

$$D_{max} = \mu_{max} \cdot Y_x/Y_{xs} \qquad [12]$$

When nitrogen is present in excess, $Y_x = Y_{xs}$, and $D_{max} = \mu_{max}$. When nitrogen is limiting, Y_x will be smaller than Y_{xs} and the maximal obtainable dilution rate will be smaller than μ_{max}. Using equation [5a] and [5b], equation [12] can be transformed in:

$$D_{max} = \mu_{max} \cdot \{(C/N_{critical})/(C/N)\}\ (h^{-1}) \qquad [13]$$

Using this equation, the maximal dilution rate in continuous cultures of oleaginous yeasts can be calculated at any C/N-ratio, providing μ_{max} and the nitrogen content of the biomass of the yeast strain are known. In continuous cultures, the lipid production rate Q_L is given by:

$$Q_L = L \cdot D \cdot [S^*]\ (kg\ lipid/m^3 \cdot hr) \qquad [14]$$

Fermentation costs ($\$_f$) for microbial lipid production can be written as:

$$\$_f = \frac{\text{total fermentor costs per m}^3\text{ per hour}}{\text{lipid production rate}} \qquad [15]$$

in which total fermentor costs comprise all costs involved to keep a fermentor

operational including fixed (i.e. capital) costs. It will be clear that in order to minimize fermentation costs, the aim must be to maximize the lipid production rate. Using equations [6] and [13], the lipid production rate can be calculated at any C/N-ratio of the growth medium and fermentation costs can be estimated.

Since lipid yields increase and dilution rates decrease with increasing C/N-ratios, an optimal C/N-ratio for total lipid production costs is to be expected.

The determination of several model parameters and the practical applicability of the model will be demonstrated in the experimental section of this paper.

MATERIALS AND METHODS

Organism and growth media

The yeast strain *Apiotrichum curvatum* ATCC 20509 was used in all experiments. The growth medium contained ($g \cdot l^{-1}$): NH_4Cl, 0.673; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 . 7H_2O$, 1.5; yeast extract, 1.0; $CaCl_2 . 2H_2O$, 0.1; $FeCl_3 . 6H_2O$, 0.01; $ZnSO_4 . 7H_2O$, 0.001. Media with different C/N-ratios were made by varying the amount of added glucose. To prevent bacterial growth, the pH of the medium was adjusted at 2.5 and the medium was heat sterilized for 4 hr at 100 °C.

Continuous culture conditions

Cultures were grown continuously in a 1 liter fermentor vessel (working volume) at 30 °C; pH was maintained at a value of 4.5 by automatic addition of NaOH. Sterile filtered air was supplied to the fermentor at a rate of 60 l h^{-1}. Dissolved oxygen concentration was measured with a sterilized oxygen electrode and controlled at a preset value of 60% of air saturation by regulating the rotation speed of the stirrer. (Electronic Workshop, Biological Laboratory, Vrije Universiteit, Amsterdam, The Netherlands). To prevent foaming 0.4 ml l^{-1} silicone antifoam emulsion M-30 (Serva, Heidelberg, F.R.G.) was added to the medium.

Experimental system to enable a gradual increase of the C/N-ratio of the growth medium

To attain a gradient of the C/N-ratio in time, a system with two medium vessels was used: medium vessel A is connected in series with medium vessel B. Medium from vessel A is pumped into the culture vessel. Since vessel A is a part of a closed system, only connected with the culture vessel and medium vessel B, an underpressure arises in vessel A after switching on the pump. Vessel A will be refilled by medium from vessel B, resulting in a constant volume of vessel A and a decreasing volume in vessel B. Vessel A as well as vessel B contained

the growth medium described above, except for a difference in glucose concentration which was 4.0 g l^{-1} in vessel A and 50.0 g l^{-1} in vessel B. In this way the glucose concentration and consequently the C/N-ratio of the growth medium will gradually increase in time according to the following equation:

$$d[S]_A/dt = D_A[S]_B - D_A[S]_{A(t)} \quad [16]$$

in which $[S]_{A(t)}$ is the substrate (glucose) concentration in vessel A at time t, $[S]_B$ is the substrate concentration in vessel B and D_A is the dilution rate in vessel A. Equation [16] can be transformed in:

$$[S]_{A(t)} = [S]_B + \{[S]_{A(o)} - [S]_B\} \cdot e^{-D_A \cdot t} \quad [17]$$

in which $[S]_{A(o)}$ is the glucose concentration in the vessel A at t = o. The glucose concentration available for yeast growth in the culture vessel is described by:

$$d[S]_C/dt = D[S]_{A(t)} - D[S]_{C(t)} \quad [18]$$

in which $[S]_{C(t)}$ is the glucose concentration in the culture vessel and D is the dilution rate in the culture vessel. Using equation [17], equation [18] can be transformed in the following equation:

$$[S]_{C(t)} = [S]_B + \frac{D([S]_{A(o)} - [S]_B)}{D - D_A} \cdot e^{-D_A \cdot t} + \{[S]_{C(o)} - [S]_B - \frac{D([S]_{A(o)} - [S]_B)}{D - D_A}\} \cdot e^{-D \cdot t} \quad [19]$$

Analytical procedures.

Dry weight determination. Yeast dry weight was determined by filtration using a Stefi filter apparatus and membrane filters with a pore diameter of 0.45 μm (Schleicher & Schuell, Dassel, F.R.G.). 1–2 ml culture was passed through the filter which had been previously weighed. After washing the filter, the filter was dried to constant weight at 60 °C.

Determination of yeast lipid content. To determine the total lipid content, yeast cells were extracted with ethanol, n-hexane, and chloroform respectively. Subsequently the residual cell mass was refluxed with alcoholic KOH and extracted with ether. A complete description of this method is given by Hammond et al. (1981).

Determination of yeast carbohydrate content. The total carbohydrate content of a washed cell suspension was determined by the modified anthrone method as described by Mokrash (1954), using a saccharose solution (1 mg ml^{-1}) as a reference.

Determination of residual glucose. To determine if the glucose in the growth me-

dium was consumed completely, a glucose teststrip with a color indicator was used (Boehringer, Mannheim, F.R.G.).

Determination of residual nitrogen. Samples of yeast cultures were centrifuged at 20,000 × g for 5 minutes and the supernates were subsequently dried in preweighed vessels at 80 °C till constant weight. 1 mg of these dried supernates was analyzed in a Carbo Elba Elemental Analyzer 1106 as described by Kirsten (1979).

RESULTS

1. Relationship between the C/N-ratio of the growth medium and the lipid yield

The experiments were designed to test the theoretical equations relating the C/N-ratio of the growth medium and the lipid yield. To determine experimentally the model parameters $C/N_{critical}$ (Eq. [4] and [5a]), the value of q (Eq. [4]), and the values of Y_{xs}, Y_{ls}, and Y_{is} (Eq. [6]), the oleaginous yeast *Apiotrichum curvatum* was cultivated in a continuous culture in which the C/N-ratio of the growth medium was gradually increased from C/N = 7 to C/N = 58 in 240 hrs. This was accomplished by using a system of two medium vessels with different concentrations of glucose as described in the materials and methods section. The dilution rate of the culture vessel was maintained at 0.02 h^{-1} and all culture conditions were kept constant throughout the experiment except the glucose concentration of the incoming growth medium.

The critical C/N-ratio could be determined at the point at which no residual nitrogen was left in the culture fluid (Fig. 1) and corresponded with a value of 11 (g/g). The value of Y_{xs} could be determined at C/N-ratios smaller than 11 and assuming an average molecular weight of 25 for yeast biomass (Dekkers et al. 1981) the average value of Y_{xs} was estimated to be 0.66 mol biomass/mol glucose (= 0.55 g biomass/g glucose). From the experimentally determined values of $C/N_{critical}$ and Y_{xs}, the value of q (representing the nitrogen content of the biomass of *A. curvatum*) can be calculated to be 0.12 by using Eq. [5a].

Since the nitrogen content of the growth medium was kept constant, it must be assumed that the biomass concentration cannot exceed the value that is reached when $C/N = C/N_{critical}$. From about 30 hr after the initiation of the gradient (when $C/N_{critical}$ is reached) the biomass will thus remain constant at 0.172 mol/l (= 4.2 g/l). The observed increase in dry weight (Fig. 1) after this point therefore will be the result of the accumulation of intermediate and lipid product. In literature (Boulton & Ratledge 1983) it is assumed that under nitrogen-limited conditions glucose is converted to lipid via an intermediate. Boulton & Ratledge suggested that this intermediate was a carbohydrate compound. This

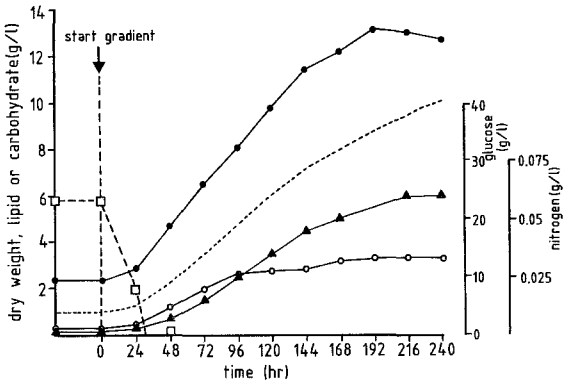


Fig. 1. Continuous culture of *A. curvatum* in which the glucose concentration in the culture vessel is gradually increased according to the system described in *Materials and methods*. Dry weight (•), lipid content (▲), carbohydrate content (○) and residual nitrogen (□) were determined every 24 hours; (---) theoretical glucose concentration in the culture vessel.

suggestion is confirmed by the significant increase in total carbohydrate content of the yeast cells that was observed after nitrogen depletion (Fig. 1). As expected, also the lipid content of the yeast cells increased with increasing glucose concentration (Fig. 1).

In the model it is assumed that when nitrogen is present in excess, no intermediate and lipid are produced. To calculate the model parameters Y_l, Y_{ls}, Y_i, and Y_{is}, it therefore will be necessary to correct the observed carbohydrate and lipid production for functional lipid and carbohydrate that is normally present in yeast biomass. The lipid content and the carbohydrate content of biomass was determined to be 10% and 16% respectively under steady state conditions of carbon- limited continuous cultures. The corrected values for carbohydrate intermediate and lipid product at different C/N-ratios are presented in Fig. 2a and 2b. Fig. 2c shows the courses of biomass and dry weight with increasing C/N-ratio.

In Fig. 3 the experimental yield of dry weight, carbohydrate intermediate and lipid per g glucose are presented as a function of the C/N-ratio. The biomass yield per g glucose is calculated from Eq. [5a] (excess nitrogen) and Eq. [5b] (limited nitrogen).

Using the data presented in Fig. 3 and assuming a carbohydrate molecular weight (C-mol) of 30.0 and a lipid molecular weight of 15.6, the model parameters Y_x, Y_i, and Y_l can be calculated at different C/N-ratios. By introducing these values into Eq. [6] of the model, the best fitting values for Y_{ls}, and Y_{is} can be calculated, using the already determined value of 0.66 mol/mol for Y_{xs}. In this way a best fitting value for Y_{ls} of 0.41 mol/mol and for Y_{is} of approximately 1.0 mol/mol can be derived. The experimental form of Eq. [6] can then be written as:

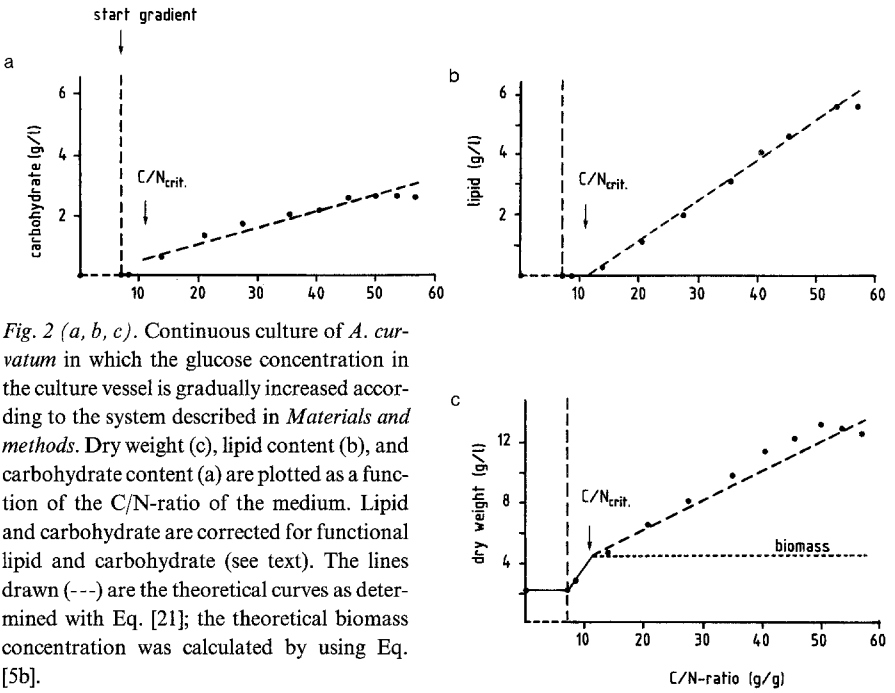


Fig. 2 (a, b, c). Continuous culture of *A. curvatum* in which the glucose concentration in the culture vessel is gradually increased according to the system described in *Materials and methods*. Dry weight (c), lipid content (b), and carbohydrate content (a) are plotted as a function of the C/N-ratio of the medium. Lipid and carbohydrate are corrected for functional lipid and carbohydrate (see text). The lines drawn (---) are the theoretical curves as determined with Eq. [21]; the theoretical biomass concentration was calculated by using Eq. [5b].

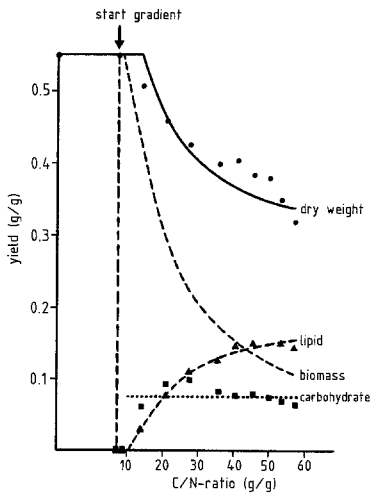
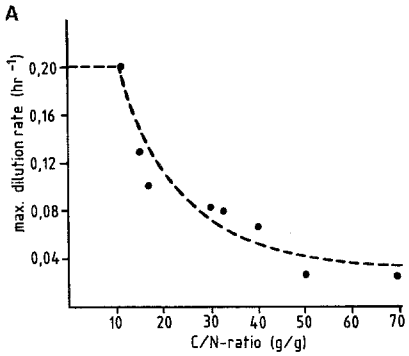
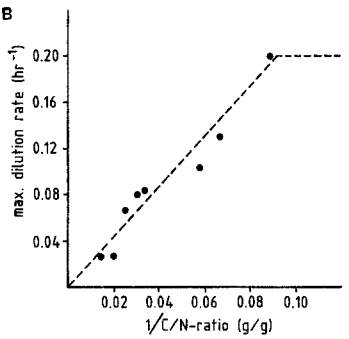


Fig. 3. Dry weight yield (•), lipid yield (▲) and carbohydrate yield (■), determined in a continuous culture of *A. curvatum* in which the glucose concentration is gradually increased, as a function of the C/N-ratio of the medium. Lipid and carbohydrate yield are corrected for functional lipid and carbohydrate (see text). The lines drawn (---) are theoretical curves determined with Eq. [21].





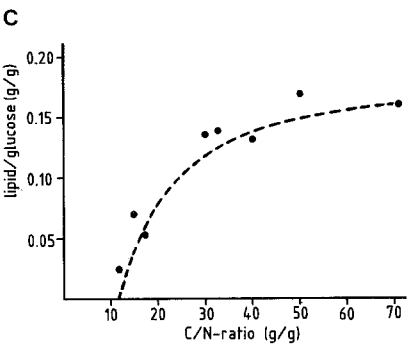


Fig. 4a. Maximum dilution rates, determined in continuous cultures with different C/N-ratio's of the growth media. The striped line is the theoretical curve, calculated with the parameters collected in Table 2.

Fig. 4b. Plot of the reciprocal of the C/N-ratio of the growth medium against the maximum dilution rate (D). The striped line is the theoretical curve calculated with the parameters of Table 2.

Fig. 4c. Influence of the C/N-ratio of the growth medium on the lipid yield in continuous cultures at maximum dilution rates. Lipid yield is already corrected for functional lipid (see text).

$$Y_l = 0.41\,(1 - Y_x/Y_{xs} - Y_i)\ \text{mol/mol} \qquad [20]$$

Using Eq. [5a] and [5b], Y_x/Y_{xs} can be written as $(C/N_{critical})/(C/N)$. Since no obvious trend in carbohydrate yield could be observed (Fig. 3) after nitrogen depletion, an average value for Y_i of 0.078 mol/mol can be used which is independent of the C/N-ratio. Eq. [20] subsequently can be transformed in:

$$Y_l = 0.41\{1 - 11.0/(C/N) - 0.078\}\ \text{mol/mol} \qquad [21]$$

From this equation the lipid yield L can be calculated at any C/N-ratio above $(C/N_{critical}$ (by using Eq. [7]).

2. *Relationship between the C/N-ratio of the growth medium and the lipid production rate*

When nitrogen is present in excess, the maximum dilution rate (D_{max}) is equal to the maximum specific growth rate (μ_{max}). This maximal specific growth rate of *A. curvatum* was determined in a continuous culture with a C/N-ratio of 10.6 which is slightly smaller than the $C/N_{critical}$. The μ_{max} appeared to be 0.20 hr^{-1} (Table 2). When steady state was reached, the nitrogen content of the supernate

Table 2. Estimates of the model parameters for *A. curvatum* growing in glucose in continuous culture, and their comparison with literature values

Parameter	Estimation	Literature value
$C/N_{critical}$	12.83 (mol/mol)	
μ_{max}	0.20 (hr^{-1})	0.2–0.3 (Evans & Ratledge 1983)
		0.19 (Glatz et al. 1984)[b]
q	0.12 (mol/mol)	0.12–0.20 (Dekkers et al. 1981)[a]
		0.15 (Oura 1972)[a]
Y_{xs}	0.66 (mol/mol)	0.79 (Glatz et al. 1984)[b]
		0.60 (Evans & Ratledge 1983)
		0.60–0.72 (Ratledge 1982)
Y_{ls}	0.41 (mol/mol)	0.42 (Ratledge 1982)
		0.55 (Glatz et al. 1984)[b]
Y_{is}	1.0 (mol/mol)	
Y_i	0.078 (mol/mol)	

[a] determined for *Saccharomyces cerevisiae*

[b] determined for *A. curvatum* in whey permeate in batch culture

was determined, and, as expected, some nitrogen could be detected (0.019 g/l).

Under nitrogen-limited conditions the maximal achievable dilution rate was determined at 7 different C/N-ratios by checking the culture fluid for the absence of residual glucose. In Fig. 4a the experimental results are compared with the theoretical values, calculated by using Eq. [13].

When D_{max} is plotted against the reciprocal value of the C/N-ratio, a straight line is expected that goes through the origin with a slope that equals the value of the product of $C/N_{critical}$ and μ_{max}. In Fig. 4b the line obtained after linear regression of the experimental values is depicted. The experimental line fitted well with the theoretical model showing only a very small intercept and a slope of 2.14 while theoretically a slope of 2.20 was calculated.

Lipid yields of continuous cultures grown at 7 different C/N-ratios were determined after steady state was reached. These lipid yields were compared with the yields that can be calculated from Eq. [21] which is shown in Fig. 4c.

The model parameters, determined in the experiments described above, are summarized in Table 2. Using these parameter-values, the course of substrate costs (Eq. [8]) and fermentation costs (Eq. [15]) can be estimated at any C/N-ratio. This is illustrated in Fig. 5a and Fig. 5b respectively, where arbitrary values for price of carbon source per kg and overall fermentor costs per $m^3 \cdot h$ have been taken.

These figures show that substrate costs will be most favourable at the highest C/N-ratios and fermentation costs will be minimal at a relatively low C/N-ratio of about 25 with a corresponding dilution rate of 0.09.

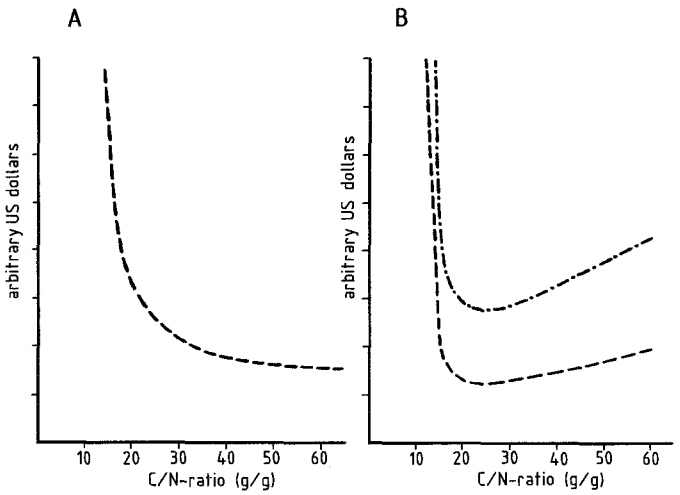


Fig. 5. Relationship between the C/N-ratio of the growth medium and substrate costs (a) and fermentor costs (b). Fermentor costs are shown for media with different substrate concentrations: (–·–·–) = 20 gr/l, (– – –) = 45 gr/l.

DISCUSSION

Lipid accumulation of oleaginous yeasts growing in continuous culture has been described repeatedly (Gill et al. 1977; Ratledge & Hall 1977; Boulton & Ratledge 1981; Yoon & Rhee 1983 Evans & Ratledge 1983; Floetenmeyer et al. 1985). These studies indicated that lipid concentrations could be achieved similar to these observed in batch cultures providing the medium has a high C/N-ratio and a dilution rate of about 0.02–0.06 h^{-1}.

In this paper it is demonstrated that lipid production is not simply a matter of high C/N-ratios and low dilution rates. Actually, it is shown that a rather broad range of lipid yields and dilution rates can be traversed by varying the C/N-ratio of the growth medium. Even in recent studies concerning the efficiency of lipid synthesis of different yeast species (Eroshin & Krylova 1983; Pan & Rhee 1986) the significant influence of the C/N-ratio of the growth medium on biomass yields and energetic yields has been completely overlooked.

By using a mathematical model, it is demonstrated that the lipid yield of a given yeast strain can be predicted at any C/N-ratio of the growth medium when some relevant growth parameters of the yeast strain are known, being: (i) the nitrogen content of the yeast biomass; (ii) the maximal growth yield on a certain carbon source and yield constants for (iii) carbohydrate intermediate, and (iv) lipid product.

Nitrogen content (i), and maximal growth yield (ii) can be considered as real strain-specific constants which can easily be determined in carbon-limited cultures with excess nitrogen. Determination of the yield constant for a carbohy-

drate intermediate (iii) may be complicated since the amount of carbohydrate that is stored in the yeast cell may depend on various growth conditions such as carbon source and growth rate.

Boulton & Ratledge (1983) suggested that intracellular carbohydrate must be regarded as a 'short term' storage product that will be converted to lipid later on. So the rather high carbohydrate content that was observed in the non-steady state experiments (Fig. 3) may be the consequence of a too rapid addition of extra glucose to the culture, leaving not enough time for the conversion of intracellular carbohydrate to lipid. However, also in steady state cultures (Fig. 4) a significant intracellular accumulation of carbohydrate could be observed (results not shown), indicating a more permanent nature of this storage product under these conditions. Whether at lower dilution rates the carbohydrate completely can be converted to lipid is not known at this moment.

Since the accumulation of considerable amounts of carbohydrate by oleaginous yeasts certainly will be disadvantageous for the lipid yield, more research concerning the biochemistry and the regulation of carbohydrate storage is required to further optimize lipid production.

As for the lipid yield constant Y_{ls} (iv), it can be calculated from biochemical pathways that the biosynthesis of 1 mol triacylglycerol requires 26 mol acetyl-CoA, 23 mol ATP, 45 mol NADPH, and 1 mol alpha-glycerol-phosphate. The production of acetyl-CoA from glucose is maximal in the Embden-Meyerhof-Parnas pathway + ATP:citrate-lyase (Ratledge 1982), while the production of NADPH is maximal in the pentose phosphate pathway. When the metabolism of glucose is optimally balanced for triacylglycerol biosynthesis, it can be calculated that 15.5 mol glucose is required for the formation of 1 mol triacylglycerol. It is assumed here that transhydrogenase is absent in yeast (Bruinenberg et al. 1985) and that the efficiency of oxidative phosphorylation is 2 mol ATP per mol NADH oxidized (P/O = 2). When a P/O of 3 or 1 is assumed, 15 and 17 mol glucose are required respectively for the production of 1 mol triacylglycerol. These calculations result in theoretical values of $Y_{ls} = 0.55$ (P/O = 1), $Y_{ls} = 0.59$ (P/O = 2) and $Y_{ls} = 0.60$ (P/O = 3) respectively. The experimental value of $Y_{ls} = 0.41$ indicates that glucose metabolism is not optimally balanced for triacylglycerol biosynthesis.

In this paper also a model is presented to predict the maximal dilution rate for lipid production at different C/N-ratios of the growth medium. In this model it is assumed that the amount of substrate that is consumed for maintenance processes is negligible. This assumption seems to be justified by the good fit between experimentally determined maximal dilution rates and theoretical calculations (Figs 4a & 4b) and by the fact that lipid yields in continuous cultures under steady state conditions at different C/N-ratios and dilution rates do not diverge significantly from calculated values based on non steady state experiments.

An overview of the various model parameters that have been determined by using the mathematical model is given in Table 2. These values are compared with literature values. The parameters μ_{max}, q, Y_{xs} and Y_{ls} are in good agreement with literature values. Under conditions of excess nitrogen, when no intracellular product formation occurs, 10% of the dry weight consisted of lipid and 16% consisted of carbohydrate. These values for socalled functional lipid and functional carbohydrate are also reported by other investigators (Stouthamer & van Verseveld 1985). Under nitrogen-limited conditions cells of *A. curvatum* contained up to 20% carbohydrate and up to 50% lipid (g/g DW). (In Figs 2, 3 & 4 the lipid and carbohydrate yield are corrected for the functional lipid- and carbohydrate content.)

The intracellular accumulation of carbohydrate after nitrogen depletion did not show a clear dependence on the C/N-ratio. The experimental data could be fitted best with the model assuming a constant value of $Y_i = 0.078$ mol/mol and a value of Y_{is} approximating 1.0, indicating that little energy is required for the storage of carbohydrate.

Fig. 5 shows that the model can be used to predict the influence of the C/N-ratio on substrate and fermentation costs. Since substrate costs will be most favourable at the highest C/N-ratios and fermentation costs will be minimal at rather low C/N-ratios, one must compromize between optimal lipid yield and optimal fermentor productivity to minimize total production costs. Ultimately the most favourable C/N-ratio will depend on the actual price of the carbon substrate per kg and the total fermentation costs per m^3h.

REFERENCES

Boulton, C. A. & Ratledge, C. (1981) Correlation of lipid accumulation in yeast with possession of ATP:citrate lyase. J. Gen. Microbiol. 127: 169–176

Boulton, C. A. & Ratledge, C. (1983) Use of transition studies in continuous cultures of *Lipomyces starkeyi,* and oleaginous yeast, to investigate the physiology of lipid accumulation. J. Gen. Microbiol. 129: 2871–2876

Bruinenberg, P. M., van Dijken, J. P. & Scheffers, W. A. (1983) An enzymic analysis of NADPH production and consumption in *Candida utilis*. J. Gen. Microbiol. 129: 965–971

Dekkers, J. G. J., de Kok, H. E. & Roels, J. A. (1981) Energetics of *Saccharomyces cerevisiae* CBS 426: comparison of anaerobic and aerobic glucose limitation. Biotechnol. Bioeng. 23: 1023–1025

Eroshin, V. K. & Krylova, N. I. (1983) Efficiency of lipid synthesis by yeasts. Biotech. Bioeng. 25: 1693–1700

Evans, C. T. & Ratledge, C. (1983) A comparison of the oleaginous yeast, *Candida curvata,* grown on different carbon sources in continuous and batch culture. Lipids 18: 623–629

Floetenmeyer, M. D., Glatz, B. A. & Hammond, E. G. (1985) Continuous culture fermentation of whey permeate to produce microbiol oil. J. Dairy Sci. 68: 633–637

Gill, C. O., Hall, M. J. & Ratledge, C. (1977) Lipid accumulation in an oleaginous yeast (*Candida* 107) growing on glucose in single-stage continuous culture. Appl. Environ. Microbiol. 33: 231–239

Glatz, B. A., Hammond, E. G., Hsu, K. H., Baehman, L., Bati, N., Bednarski, W., Brown, D.

& Floetenmeyer, M. (1984) Modification of fats and oils. In: C. Ratledge, P. Dawson & J. Rattray (eds), Biotechnology for the oils and fats industry, pp. 163–176. Amer. Oil Chemists' Soc., Monograph 11. AOCS, Illinois.

Hammond, E. G., Glatz, B. A., Choi, Y. & Teasdale, M. T. (1981) Oil production by *Candida curvata* and extraction, composition and properties of the oil. E. H. Pryde, L. H. Princen & K. D. Mukerjee (eds), New sources of fats and oils, pp. 171–187. Amer. Oil. Chemists' Soc., Monograph 9. AOCS, Illinois.

Kessel, R. H. J. (1968) Fatty acids of *Rhodotorula gracilis:* fat production in submerged culture and the particular effect of pH value. J. Appl. Bacteriol. 31: 220–231

Kirsten, W. J. (1979) Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen and sulfur, and for sulfur alone in organic and inorganic materials. Anal. Chem. 51: 1173–1179

Mokrasch, L. C. (1954) Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. J. Biol. Chem. 208: 55–59

Oura, E. (1972) Reactions leading to the formation of yeast cell material from glucose and ethanol. Alkon Keskus Laboratorio Report 8078, Helsinki

Pan, J. G. & Rhee, J. S. (1986) Biomass yields and energetic yields of oleaginous yeasts in batch culture. Biotech. Bioeng. 28: 112–114

Ratledge, C. (1982) Microbiol oils and fats : an assessment of their commercial potential. Prog. Indust. Microbiol. 16: 119–206

Ratledge, C. & Hall, M. J. (1979) Accumulation of lipid by *Rhodotorula glutinis* in continuous culture. Biotechnol Lett. 1: 115–120

Ratledge, C., Boulton, C. A. & Evans, C. T. (1984) Continuous culture studies of lipid production by oleaginous microorganisms. In: A. C. R. Dean, D. C. Ellwood, C. G. T. Evans (eds), Continuous culture, Vol. 8: Biotechnology, Medicine and the environment, pp. 272–291. Society of Chemical industry, London/Ellis Horwood Ltd., Chichester

Stouthamer, A. H. & Verseveld, H. W. van (1985) Stoichiometry of microbiol growth. In: C. L. Cooney & E. A. Humphrey (eds), Comprehensive Biotechnology, pp. 215–238 Pergamon Press, Oxford/New York.

Yoon, S. H. & Rhee, J. S. (1983) Lipid from yeast fermentation: Effects of cultural conditions on lipid production and its characteristics of *Rhodotorula glutinis.* J. Amer. Oil Chem. Soc. 60: 1281–1286

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