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Optimization of lipid production in the oleaginous yeast *Apiotrichum curvatum* in wheypermeate

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Summary. Lipid production of the oleaginous yeast *Apiotrichum curvatum* was studied in wheypermeate to determine optimum operation conditions in this medium. Studies on the influence of the carbon to nitrogen ratio (C/N-ratio) of the growth medium on lipid production in continuous cultures demonstrated that cellular lipid content in wheypermeate remained constant at 22% of the cell dry weight up to a C/N-ratio of about 25. The maximal dilution rate at which all lactose is consumed in wheypermeate with excess nitrogen was found to be 0.073 h^{-1}. At C/N-ratios higher than 25–30 lipid content gradually increased to nearly 50% at C/N = 70 and the maximal obtainable dilution rate decreased to 0.02 h^{-1} at C/N = 70. From these studies it could be derived that maximal lipid production rates can be obtained at C/N-ratios of 30–35 in wheypermeate. Since the C/N-ratio of wheypermeate normally has a value between 70 and 101, some additional nitrogen is required to optimize the lipid production rate. Lipid production rates of *A. curvatum* in wheypermeate were compared in four different culture modes: batch, fed-batch, continuous and partial recycling cultures. Highest lipid production rates were achieved in culture modes with high cell densities. A lipid production rate of nearly 1 g/l/h was reached in a partial recycling culture. It was calculated that by using this cultivation technique lipid production rates of even 2.9 g/l/h may be reached when the supply of oxygen can be optimized.

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Nomenclature: C/N-ratio, carbon to nitrogen ratio of the growth medium (g/g); C/N_{crit}, C/N-ratio at which there is just enough nitrogen to allow all carbon source to be converted to biomass; 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}); DW, cell dry weight; L, lipid yield (g storage lipid/g carbon source); μ, specific growth rate (h^{-1}); μ_{max}, maximum specific growth rate (h^{-1}); Q_L, lipid production rate (g/l/h); Y_i, molecular fraction of carbon substrate that is converted to storage carbohydrate (C-mol/C-mol); Y_{ls}, maximal amount of storage lipid that can be produced per mol carbon source (C-mol/C-mol)

Introduction

Some yeasts are able to accumulate storage lipid up to 60% of their dry weight, when grown under nitrogen-limited conditions. These lipids usually consist of 80%–90% triacylglycerols with a fatty acid composition similar to many plant seed oils. The possibility of producing lipids by using oleaginous yeasts for industrial purposes has been considered a number of times (Radledge 1984; Floetenmeyer et al. 1985). The process economics of microbial oil fermentations are mainly determined by substrate costs and fermentor costs. Substrate costs depend on the price of the carbon source and the lipid yield (defined as the amount of lipid produced per unit weight of carbon source utilized). Fermentor costs are strongly dependent on the lipid production rate (defined as the amount of lipid produced per unit of fermentor volume per unit time). Thus, in optimizing lipid production of yeast, lipid yield and lipid production rate should be as high as possible and an inexpensive carbon source should be used.

Recently, a mathematical model was constructed that describes the influence of the carbon to nitrogen ratio (C/N-ratio) of the growth medium on lipid yield and lipid production rate by oleaginous yeasts (Ykema et al. 1986). The model was tested by growing the oleaginous yeast *Apiotrichum curvatum* in continuous cultures in semi-

defined medium with various C/N-ratios and dilution rates. It appeared that when nitrogen is limiting for the production of biomass, the remaining carbon source can be converted to storage lipid and storage carbohydrate and that lipid yield increases with increasing C/N-ratios. On the other hand, the maximum achievable dilution rate at which all carbon source is consumed (D_{max}) decreases with increasing C/N-ratios of the growth medium. Since lipid yields increased and dilution rates decreased with increasing C/N-ratios, an optimal C/N-ratio for the lipid production rate could be determined.

Another important factor, next to lipid yield and D_{max}, that affects the lipid production rate is the culture mode. Evans and Ratledge (1983) and Floetenmeyer et al. (1985) showed that lipid production rates are faster in continuous cultures than in batch cultures. However, highest lipid production rates will be achieved in culture modes, that enable the cultivation at high cell densities like fed-batch cultures as reported for *Lipomyces starkeyi* by Yamauchi et al. (1983) and for *Rhodotorula glutinis* by Pan et al. (1986).

Wheypermeate is an inexpensive growth medium containing about 45 g/l of lactose as the main carbon source. Lipid production of *A. curvatum* in wheypermeate was described earlier by Moon et al. (1978) and Floetenmeyer et al. (1985). However, no clear account of the influence of the C/N-ratio was given and culture modes with high cell densities were not reported before for *A. curvatum* in wheypermeate.

In this paper experimental results are described which enable the estimation of optimal culture conditions for the production of lipids by *A. curvatum* in wheypermeate. It is shown that high lipid production rates can be reached when a) NH_4Cl is added to this medium to obtain an optimal C/N-ratio and b) culture modes are applied that enable cultivation at high cell densities.

Materials and methods

Organism and growth media

The yeast strain *Apiotrichum curvatum* ATCC 20509 was used in all experiments. Wheypermeate was used as a growth medium and was prepared as follows: dried whey (63.5 g per liter demineralized water) was deproteinized by precipitation at 80° C, pH 4.5 (Meyrath and Bayer 1979). To prevent bacterial growth, the pH of the medium was adjusted at 2.5 and the medium was further sterilized by filtration on an Amicon ultra filtration system (cut off 50000 D, ∅ 150 mm). The desired C/N-ratio was obtained by adding the appropriate amount of NH_4Cl. To prevent foaming 0.5 ml · l^{-1} silicone antifoam emulsion M-30 (Serva, Heidelberg, W. Germany) was added to the medium. Semidefined growth medium contained (g/l): NH_4Cl, 0.673; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.001. In this case media with different C/N-ratios were made by varying the amount of added glucose or NH_4Cl, assuming 10.5% nitrogen and 26% carbon in yeast extract. Fermentor cultures (1 l working volume) were inoculated with 50 ml shake cultures that had been grown over night in 500 ml flasks at 30° C in the same medium as used in the fermentor.

Fermentor modes

All cultures were grown in a 2-liter fermentor vessel with a working volume of 1 l at 30° C; pH was maintained at a value of 4.8 by automatic addition of KOH. Sterile filtered air was supplied to the fermentor at a rate of 40 to 100 l · h^{-1}. Dissolved oxygen was measured with a sterilized oxygen electrode and kept at least above 10% of air saturation by varying the agitation speed and the rate of air supply. Fed-batch cultures were simulated by operating in the recycling mode. Therefore the chemostat was modified by attaching one (or if necessary, two parallel) 0.2 µm polycarbonate membrane filtration unit(s) that continuously returned cells to the culture vessel while removing filtered broth at a rate equal to the rate of fresh medium input (Chesbro et al. 1979). Medium provision rate and filtration rate were automatically controlled by means of computer guided pumps and weight balances. Partial recycling was carried out by adding an extra pump withdrawing culture at a rate regulated by another mass/flow controller, measured as weight increase upon an extra weight balance. Growth rates were calculated as described by Damiano et al. (1985).

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 as described before (Ykema et al. 1986). Shortly: vessel A is connected in series with vessel B. Medium from vessel A is pumped into the culture vessel ($D = 0.02\ h^{-1}$). Since vessel A is 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 (in this case 0.5 l) and a decreasing volume in vessel B. Vessel A as well as vessel B contain wheypermeate, except for a difference in NH_4Cl concentration which resulted in an initial C/N-ratio = 5 in vessel A and a C/N-ratio = 93 in vessel B (wheypermeate without NH_4Cl). In this way the NH_4Cl concentration in vessel A will gradually decrease according to the following equation:

$$N_{A(t)} = N_B + [N_{A(o)} - N_B] \cdot e^{-D_A \cdot t}$$

in which $N_{A(t)}$ is the nitrogen concentration in vessel A at time t; $N_{A(0)}$ the nitrogen concentration on t = 0, N_B the nitrogen concentration in vessel B, and D_A is the dilution rate in vessel A.

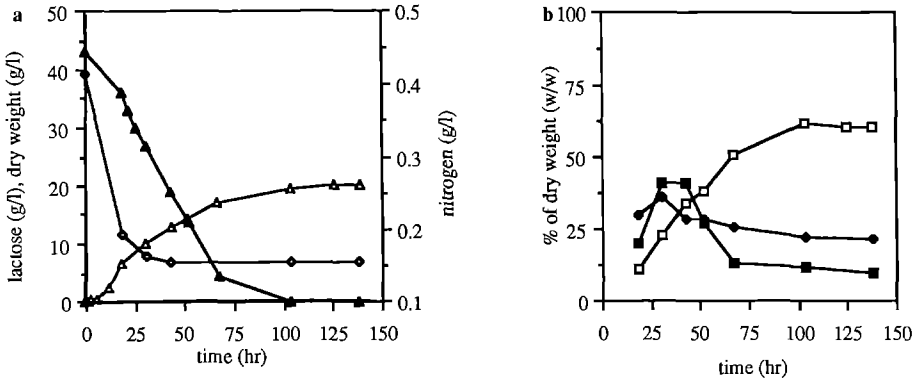

Fig. 1. Growth and utilization of medium components **(a)** and cell composition of *A. curvatum* **(b)** in batch culture on wheypermeate without any additional nitrogen. (△) cell dry weight; (▲) residual lactose; (◇) residual nitrogen; (□) lipid; (■) carbohydrate; (◆) protein

The gradual increase of the NH_4Cl concentration in the culture vessel is given by the next equation:

$$N_{C(t)} = N_B + \frac{D[N_{A(o)} - N_B]}{D - D_A} \cdot e^{-D_A \cdot t} + \left\{ N_{C(o)} - N_B - \frac{D[N_{A(o)} - N_B]}{D - D_A} \right\} \cdot e^{-D \cdot t}$$

in which $N_{C(t)}$ is the nitrogen concentration in the culture vessel at time t, and D the dilution rate in the culture vessel.

Analytical procedures

Yeast dry weight was determined by filtration on membrane filters as described before (Ykema et al. 1986). Yeast lipid content was determined by extracting yeast cells with ethanol, n-hexane and chloroform respectively. Subsequently the residual cell mass was refluxed with alcoholic KOH and extracted with diethylether as described by Hammond et al. (1981). Yeast carbohydrate content was determined by the modified anthrone method as described by Mokrash (1954), using a saccharose solution (1 mg/ml) as a reference. Nitrogen determinations were as described previously (Ykema et al. 1986). Protein was determined by the Folin method (Lowry et al. 1951) with bovine serum albumine as standard. For lactose determinations lactose was first converted to glucose, using β-galactosidase according to Kurtz et al. (1970). Then glucose was determined using a standard kit from Boehringer (Mannheim, FRG). A lactose solution (0.5 mg/ml) was used as reference

Results

Optimization of the lipid yield

Recently it was shown that there is a direct relationship between the C/N-ratio of semidefined growth medium and the lipid yield of *A. curvatum* (Ykema et al. 1986). In order to find out how lipid production of *A. curvatum* is influenced by the C/N-ratio of wheypermeate, *A. curvatum* was first cultivated in batchculture in wheypermeate without any additional nitrogen. The C/N-ratio of wheypermeate is very high and varies, depending on the prepared sample from 69.6 to 101.0 (43–48 g lactose/l and 0.20–0.26 g available nitrogen/l). The results of this experiment are presented in Fig. 1 and can be summarized as follows: (i) Before all available nitrogen is exhausted, lipid is already synthesized up to 22% of the cell dry weight. (ii) The lipid content in the cells increases (up to 60% of the cell dry weight) untill all lactose is consumed. (iii) In the first 30 h much carbohydrate is found intracellular (40% of the cell dry weight) which diminished to 10% of the cell dry weight at the end of the culture. (iv) The total amount of protein (in g/l, results not shown) increased until $t = 66$ h although there was no available nitrogen left in the wheypermeate after $t = 43$ h.

In examining more precisely the relationship between the C/N-ratio of wheypermeate on the lipid yield, *A. curvatum* was cultivated in a continuous culture in which the C/N-ratio of the wheypermeate was gradually increased from C/N = 5 to C/N = 76 in 240 h. This was accomplished by using a system of two medium vessels with different concentrations of nitrogen as described in *"Materials and methods"*. The dilution rate of the culture vessel was maintained at 0.02 h^{-1}, to ensure that even at high C/N-ratios all lactose would be consumed. All culture conditions were kept constant except the NH_4Cl concentration of the incoming growth medium. This experiment clearly showed a remarkable relationship between the C/N-ratio of wheypermeate and lipid production (Fig. 2). Figure 2, and also Fig. 1, show, in contrast to our experiments in semidefined growth medium, that before all nitrogen is exhausted, lipid and carbohydrate are accumulated

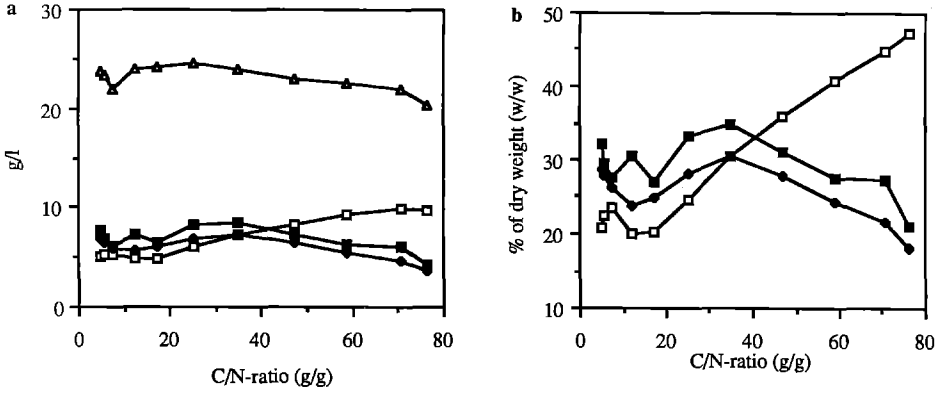

Fig. 2. Growth **(a)** and cell composition **(b)** of *A. curvatum* as a function of the C/N-ratio of the medium, determined in a continuous culture in which the nitrogen concentration in wheypermeate was gradually decreased as described in *Materials and methods*. (△) cell dry weight; (□) lipid; (■) carbohydrate and (◆) protein

in the yeast cells. Lipid droplets and carbohydrate granules could also be detected microscopically. At C/N-ratios > about 25, lipid content in the cells increases from an average of 22% to 50% of the cell dry weight at C/N = 76. Because all conditions and the composition of the growth medium were kept constant with the exception of the NH_4Cl concentration, it can be concluded that the increase of the lipid content in the cells is the result of nitrogen-limitation. Cell dry weight and the amount of protein and carbohydrate decreased slowly at C/N-ratios > 25–30.

In Fig. 3 lipid yields of *A. curvatum* in wheypermeate and in semidefined growth medium are compared. Lipid yields appeared to be higher in wheypermeate at all C/N-ratios.

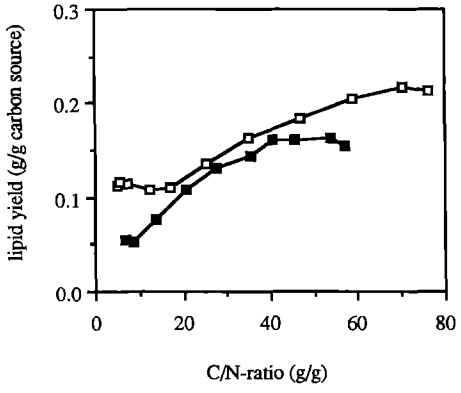

Fig. 3. Lipid yields (□) of *A. curvatum* as a function of the C/N-ratio of wheypermeate, determined in a continuous culture in which the nitrogen concentration was gradually decreased as described in *Materials and methods*. For comparison lipid yields (■) of a similar experiment in semidefined medium are added (Ykema et al. 1986)

Relationship between the C/N-ratio of the growth medium and maximum dilution rates

In semidefined growth medium it is found that the maximum dilution rate at which all lactose is still consumed (D_{max}) in continuous cultures of *A. curvatum* is equal to the maximum specific growth rate (μ_{max}), when nitrogen is present in excess ($C/N = < C/N_{crit}$, in which the C/N_{crit} is defined as the C/N-ratio of the medium at which just enough nitrogen allows all carbon to be converted to biomass). The μ_{max} appeared to be 0.20 (h^{-1}). Under nitrogen-limited conditions ($C/N > C/N_{crit}$), D_{max} values can be predicted by using the equation:

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

(Ykema et al. 1986). D_{max} values determined in wheypermeate at different C/N-ratios are compared with D_{max} values observed in semidefined growth medium in Fig. 4. It appeared that at $C/N < 30$, D_{max} values in wheypermeate are significantly lower than D_{max} values in semidefined growth medium. At $C/N > 30$ there is no reason to assume that D_{max} values in wheypermeate differ from D_{max} values in semidefined growth medium and thus from the theoretical curve, calculated with Eq. (1).

Optimization of fermentation conditions

In optimizing fermentation conditions four different culture modes were compared: batch, fed-batch, continuous and partial recycling cultures.

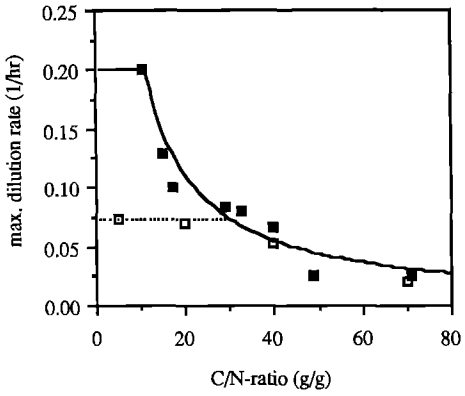

Fig. 4. Maximum dilution rates of *A. curvatum*, determined in continuous cultures with different C/N-ratios in wheypermeate (□). A hypothetical line for maximum dilution rates in wheypermeate with a C/N-ratio <30 is drawn (····). For comparison maximum dilution rates, determined in semidefined medium (■) and a theoretical curve (—) as determined with Eq. (1) are added (Ykema et al. 1986)

The fed-batch mode was simulated by a batch culture, in which the cell dry weight was increased by operating in the recycling mode, because of difficulties with concentrating wheypermeate. The key parameter to be determined, when comparing different culture modes, is the lipid production rate (Q_L). Lipid production rates (g/l/h) for batch and fed-batch cultures can be calculated by dividing the amount of lipid (g/l) by the number of hours needed to run a complete fermentation (i.e. from inoculation till all lactose is consumed), and for continuous and partial-recycling cultures by multiplying the amount of lipid (g/l) by the dilution rate (h^{-1}) or by the bleed flow rate ($l \cdot h^{-1}$) respectively.

In Table 1 some experimental lipid production rates obtained at different C/N-ratios and in different culture modes are shown. From Table 1 it can be concluded that the culture mode is a very important factor for the lipid production rate. Continuous cultures yielded higher lipid production rates than batch cultures as observed earlier by Evans and Ratledge (1983). A fed-batch culture at C/N=40, when harvested at a dry weight of 85 g/l, yielded a lipid production rate equal to a continuous culture at C/N=40. Highest lipid production rates were obtained in partial recycling cultures. A lipid production rate of nearly 1 g/l/h was reached in a partial recycling culture at a dilution rate of 0.033 h^{-1} at C/N=40 at a dry weight of 91 g/l.

In Fig. 5 theoretical maximum lipid production rates, calculated on basis of experimental results, are presented as a function of the C/N-ratio of wheypermeate. Very high lipid production rates should be obtainable in partial recycling systems (up to 2.8 g/l/h) in which high cell densities are combined with a continuous output of lipid. It is apparent that for all culture systems the calculated lipid production rates, based on total lipid production, are highest at C/N=30–35.

Discussion

In order to optimize lipid production of *A. curvatum* in wheypermeate the influence of the C/N-ratio of the growth medium on lipid yield and lipid production rate has been investigated and the productivity of different culture modes was compared.

In batch cultures in wheypermeate without additional nitrogen a significant and temporary ele-

Table 1. Experimental lipid production rates of *A. curvatum* in wheypermeate

Culture mode	C/N-ratio	Time (h)	D (h^{-1})	DW (g/l)	Lipid content (%)	Q_L (g/l/h)
Batch	25	27		23.2	18	0.155
Batch	40	39		21.6	36	0.199
Batch	70	93		19.7	58	0.123
Fed-batch[a]	40	70		85.0	35	0.372
Continuous	20		0.07	21.0	20	0.294
Continuous	40		0.053	20.0	36	0.382
Partial recycling[b]	40		0.033[c]	91.4	33	0.995

[a] A recycle unit was attached to a batch culture after all lactose was consumed (total feed flow rate = 0.15 $l \cdot h^{-1}$)

[b] A recycle unit was attached to a batch culture after all lactose was consumed (total feed flow rate = 0.15 $l \cdot h^{-1}$); when a dry weight of about 90 $g \cdot l^{-1}$ was reached an extra pump withdrawing culture (bleed flow rate = 0.033 $l \cdot h^{-1}$) was added. A sample was taken when steady state was reached

[c] In this case the bleed flow rate ($l \cdot h^{-1}$) is equal to the growth rate (h^{-1}), because the volume of the culture was 1 l

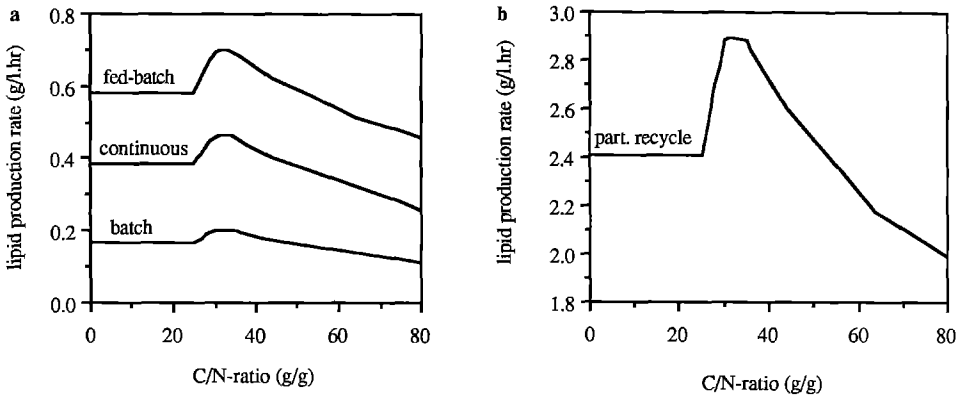

Fig. 5. Theoretical maximum lipid production rates on wheypermeate in batch, continuous, fed-batch and partial recycling cultures as a function of the C/N-ratio. In the calculations it is assumed that the relationship between the C/N-ratio of wheypermeate and lipid content, as drwan in Fig. 2b, is valid for all culture modes. Cell dry weights in batch and continuous cultures for all C/N-ratios are derived from Fig. 2a. Up to C/N = 25 an average cell dry weight of 24 g/l and an average of 22% lipid is assumed. In fed-batch and partial recycling cultures a maximum cell dry weight of 150 g/l is used in the calculations. Maximum dilution rates in continuous and partial recycling cultures are taken from Fig. 4, supposing a D_{max} of 0.073 h^{-1} at C/N < 30 and using Eq. (1) for C/N > 30

vation of the cellular carbohydrate content was observed at the early stages of growth (Fig. 1). This observation strongly suggests that intracellular carbohydrate fulfils the role of an intermediate between sugar utilization and lipid formation. This view is supported in literature by Glatz et al. (1984) and Boulton and Ratledge (1983) who suggested the occurence of an intermediate on theoretical and experimental grounds respectively.

It was further observed that in batch cultures the amount of protein (in g/l) increased even after all available nitrogen has been consumed. This may probably be due to the existence of a nitrogen pool in *A. curvatum* in the form of amino acids in vacuoles as reported before for *Saccharomyces cerevisiae* and *Candida utilis* (Middelhoven 1968; Wiemken and Durr 1974).

In marked contrast to experiments in a semidefined growth medium with glucose as carbon source (Ykema et al. 1986), lipid is already accumulated up to 22% of the cell dry weight in wheypermeate under circumstances where nitrogen is still present in excess. Several experimental results from continuous cultures in which the C/N-ratio was gradually increased, strongly suggest that another factor in wheypermeate is limiting for biomass synthesis at C/N-ratios < 25–30, and that the effect of the nitrogen-limitation is only visible at higher C/N-ratios: i) lipid content is relatively constant up to C/N = about 25 and increasing gradually at higher C/N-ratios (Fig. 2b); ii) cell dry weight is relatively constant up to C/N = 25–30 and decreasing at higher C/N-ratios (Fig. 2a); iii) the maximum dilution rate is relatively constant up to C/N = 25–30, however at C/N-ratios higher than 25–30 the maximum dilution rate decreases slowly (Fig. 4).

The concept of another limiting factor, apart from nitrogen, is further supported by the observation that in a batch culture in wheypermeate supplemented with 3.38 g/l yeast extract, and adjusted to C/N = 5 with NH_4Cl only 10% lipid was found in the cell dry weight after all lactose was consumed and lipid droplets could not be detected microscopically (results not shown).

Recently, a mathematical model was constructed which made it possible to predict the lipid yield L (expressed as g storage lipid/g carbon source) at any C/N-ratio in a semidefined growth medium (Ykema et al. 1986). Although somewhat troubled by the occurence of the growth limitation at C/N-ratios below 25–30, the same mathematical model can be applied when wheypermeate is used as a growth medium. Using the data presented in Figs. 2 and 3, the relationship between lipid yield and C/N-ratio could be deduced and some relevant model parameters could be determined. The results of these calculations are shown in Table 2 and are compared with corresponding data obtained in semidefined growth medium. Figure 6 shows the lipid yields corrected for functional lipids in wheypermeate and semidefined media as a function of the C/N-ratio. The lines drawn are the theoretical curves determined with Eq. (2) and (3) in Table 2. The calculated Y_{ls} and Y_i values of wheypermeate and semidefined growth medium reflect the observed differences between the two growth media: lipid

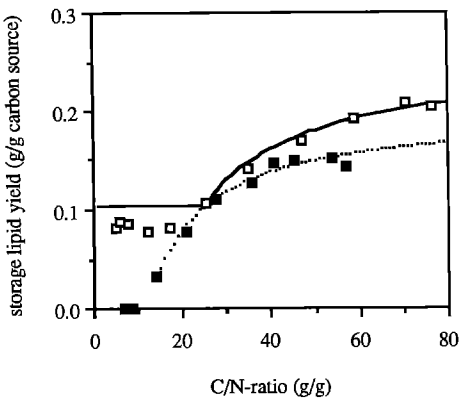

Fig. 6. Lipid yields corrected for functional lipids in wheypermeate (□) and semidefined medium (■) as a function of the C/N-ratio of the medium. The lines drawn are theoretical curves determined with Eq. (2) for wheypermeate (—) and Eq. (3) (in Table 2) for semidefined medium (· · · ·)

yield and carbohydrate yield are highest in wheypermeate. These differences are probably due to the difference in carbon source (lactose in wheypermeate and glucose in semidefined growth medium). Evans and Ratledge (1983), who compared lipid production by *A. curvatum* in a semidefined growth medium on five different carbon sources, reported significantly higher lipid yields and dry weight yields for lactose when compared with glucose.

Lipid production rates in batch and fed-batch cultures are directly dependent on the fermentation time, and fermentation times can be strongly influenced by the inocula. In this study fermentor cultures were inoculated with a 50 ml (5%; v/v) overnight shake culture grown at the same C/N-ratio as the fermentor culture. By inoculating with a bigger inoculum of cells grown at a low C/N-ratio, fermentation times will be shorter and thus lipid production rates will be even higher in batch and fed-batch cultures as those presented in Fig. 5.

The advantage of partial recycling cultures over batch and fed-batch cultures is, that partial recycling cultures allow continuous operation and increased productivity by eliminating batch downtime. The use of cell recycling systems has already been described for ethanol production from whey by *Kluyveromyces lactis* (Janssens et al. 1984) and the production of citric acid by *Saccharomycopsis lipolytica* (Enzminger and Asenjo 1986).

In the labscale fermentor used in this report about 90 g/l was the highest achievable stable cell density. At higher cell densities problems with O_2 transfer arose and recycling filters obstructed quickly. Pan et al. (1986) used O_2-enriched air and in this way they were able to reach cell dry weights of 185 g/l and a lipid production rate of 0.87 g/l/h in a fed-batch culture of *Rhodotorula glutinis*. Yamauchi et al. (1983) obtained high cell densities of *Lipomyces starkeyi* (153 g/l) and a lipid production rate of 0.59 g/l/h, by means of a microcomputer-aided fed-batch culture technique, in which the dissolved oxygen concentration in the broth could be maintained at a constant level.

In our laboratory an experimental lipid production rate of nearly 1 g/l/h was reached in a partial recycling culture with a cell dry weight of 91 g/l at C/N = 40, indicating the importance of this culture mode (Table 1). This is to our knowledge, the highest lipid production rate ever reported. It can be calculated that this high lipid production rate can even be improved by lowering the C/N-ratio of wheypermeate to C/N =

Table 2. Model equations and parameters for *A. curvatum* growing in wheypermeate and semidefined medium

Wheypermeate	Semidefined medium
L^a = 0.29·(0.882-13/[C/N]) (2)	L^a = 0.21·(0.922-11/[C/N]) (3)
$Y_{ls}{}^b$ = 0.53	$Y_{ls}{}^b$ = 0.41
$Y_i{}^c$ = 0.118	$Y_i{}^c$ = 0.078
$C/N_{crit}{}^d$ = 11–13	$C/N_{crit}{}^d$ = 11

[a] Lipid yield (g storage lipid/g carbon source)
[b] Maximal amount of storage lipid that can be produced per mol carbon substrate (C-mol/C-mol), assuming a molecular weight of 15.6, 28.5 and 30 for lipid, lactose and glucose respectively
[c] Molecular fraction of carbon substrate, that is converted to storage carbohydrate (C-mol/C-mol), assuming a molecular weight of 30 for carbohydrate
[d] C/N-ratio of the medium at which just enough nitrogen is available to allow all lactose to be converted to biomass

30–35 (Fig. 5), increasing the cell dry weight up to 150 g/l and increasing the bleed flow rate to 0.053 l/h at which rate still all lactose will be consumed (Fig. 4). In this way, with suitable fermentor equipment, lipid production rates of nearly 2.9 g/l/h can be reached (Fig. 5).

Since lipid yields were relatively high in wheypermeate and since whey is an inexpensive fermentable substrate, it can be concluded that this medium is very suitable for the production of lipids by *A. curvatum*. This is especially the case when some nitrogen is added and culture modes are applied that enable cultivation at high cell densities.

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