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A mathematical model for the study of lipid accumulation in oleaginous microorganisms. I. Lipid accumulation during growth of *Mucor circinelloides* CBS 172-27 on a vegetable oil.

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RESUMEN

Modelo matemático para el estudio de la acumulación de lípidos en microorganismos oleaginosos. I. Acumulación de lípidos durante el crecimiento de *Mucor circinelloides* CBS 172-27 sobre un aceite vegetal.

Durante muchos años la acumulación de lípidos en microorganismos desarrollados en medio de cultivo, tomando como única fuente de carbono y energía grasas vegetales o animales, ha sido objeto de investigación e interés industrial.

Interesadamente, la grasa acumulada tiene a menudo una composición y estructura muy diferente de la que tiene la grasa presente en el sustrato.

El presente trabajo describe una aproximación matemática a la acumulación de grasa por microorganismos oleaginosos en crecimiento en medios que contienen aceite vegetal como fuente de carbono. Se propone un modelo matemático que correlaciona la acumulación de grasa de reserva con el crecimiento de la población microbiana y la cantidad disponible de grasa exocelular. Este modelo es verificado por datos experimentales tomados del cultivo de *Mucor circinelloides* CBS 172-27 en aceite de girasol.

El modelo propuesto es descrito por la ecuación:

$$x_L = x_{Lo} + L_0(1 - e^{-k_2 t}) - \frac{\ln x - \ln x_0}{k_1}$$

donde x_L(mg/l) es la concentración de lípidos de reserva a un tiempo t(h), x_L (mg/l) la concentración de lípidos de reserva a un tiempo t=0, L_0(mg/l) la concentración inicial de grasa exocelular (a t=0), x(mg/l) la concentración de biomasa libre de grasa a un tiempo dado t y x_0 la concentración de biomasa libre de grasa a un tiempo t=0; k_1 y k_2 constantes.

PALABRAS-CLAVE: Aceite de girasol — Lípido (acumulación) — Modelo matemático — Mucor circinelloides CBS 172-27.

SUMMARY

A mathematical model for the study of lipid accumulation in oleaginous microorganisms. I. Lipid accumulation during growth of *Mucor circinelloides* CBS 172-27 on a vegetable oil.

The accumulation of lipids in microorganisms cultivated in growth media having as sole carbon and energy source vegetable or animal fat has been an object of research and industrial interest for many years. Interestingly, the accumulated fat often has a composition and structure much different from that of the fat present in the substrate.

The present work describes a mathematical approach to the accumulation of fat by oleaginous microorganisms growing on medium containing vegetable oil as carbon source. A mathematical model, correlating the accumulation of reserve fat with the growth of microbial population and the available quantity of exocellular fat, is proposed. This model is verified by experimental data taken by cultivation of *Mucor circinelloides* CBS 172-27 on sunflower oil.

The proposed model is described by the equation:

$$x_L = x_{Lo} + L_0(1 - e^{-k_2 t}) - \frac{\ln x - \ln x_0}{k_1}$$

where x_L(mg/l) the concentration of reserve lipids at time t(h), x_L(mg/l) the concentration of lipid reserves at time t=0, L_0(mg/l) the initial concentration of exocellular fat (at t=0), x(mg/l) the concentration of fat-free biomass at a given time t and x_0 the concentration of fat-free biomass at time t=0; k_1 and k_2 constants.

KEY-WORDS: Lipid (accumulation) — Mathematical model — Mucor circinelloides CBS 172-27 — Sunflower oil.

1. INTRODUCTION

The improvement of physicochemical characteristics and fatty acid composition of oils and fats by biotechnological means is of great interest. Compared with chemical methods used in the industry of fats and oils, biotechnological processes offer many advantages. Among the various methods proposed so far, modification of common fats by fermentation appears to be a simple, rapid and effective method.

Some microorganisms when cultivated on growth media containing as sole carbon source vegetable or animal fat, use part of the substrate for growth. The remaining fat is accumulated in the cell, after being modified in composition and/or structure level. This process is dependent on the enzymatic specificity shown by the microorganism (Fuji Oil Co. Ltd., 1979; Glatz et al., 1984; Montet et al., 1985; Aggelis et al., 1987; 1991a; 1991b; Radwan and Soliman, 1988; Kamisaka et al., 1990).

When the substrate is not completely exhausted by the microorganism, the unconsumed lipid displays a composition different from that of the original and, sometimes this may prove to be of special interest (Koritala et al., 1987, Aggelis 1989).

The aim of this work is to approach theoretically the process of lipid accumulation by microorganisms cultivated in media containing vegetable oil as sole carbon and energy source. A mathematical model, verified by experimental data, is also proposed.

Model description

Microbial growth on a culture medium containing triglycerides as carbon source takes place in three stages:

First stage. The fatty material present in the medium (*L* mg/l) is hydrolysed, and the fatty acids produced are then

used for production of fat-free biomass (x_L mg/l) and lipid reserves ($x_L$mg/l):

$$-\frac{dL}{dt} = \frac{dx}{dt} \cdot \frac{1}{Y_{x/L}} + \frac{dx_L}{dt} \quad (1)$$

where $Y_{x/L}$ is the yield in biomass of fatty substrate *(mgx/ mgL).*

In equation (1) it is assumed that oleaginous microorganisms cultured on media containing fats as carbon source accumulate reserve lipids by mechanisms different from those encountered when glucose is used as substrate. In the case of glucose and other carbohydrates, the accumulation of reserve fat starts after depletion from culture medium of certain nutrients e.g. nitrogen (Glatz et al., 1984; Botham and Ratledge, 1984). Earlier studies by Aggelis et al., 1991a; 1991b, have shown that some oleaginous microorganisms (e.g. *Mucor* spp) accumulate significant quantities of fat when growth occurs on culture media containing vegetable oils as carbon source, regardless of the nitrogen concentration in the medium. Therefore, in equation (1) it is assumed that accumulation of endocellular fat is governed by the concentration of fat in the growth medium.

Second stage. When the concentration of fat in the substrate reaches a critical point and the biosynthetic abilities of the microbial population cannot be supported any further, the carbon pool must be supplemented by biodegradation of the lipid reserves:

$$-\left(\frac{dL}{dt} + \frac{dx_L}{dt}\right) = \frac{dx}{dt} \cdot \frac{1}{Y_{x/L+x_L}} \quad (2)$$

where $Y_{x/L+x_L}$ is the yield in biomass of fatty substrate and reserve fat ($mgx / mg\ L + x_L$).

Third stage. It has already been shown that oleaginous yeast entering a period of carbon starvation, degrade reserve lipids and produce new biomass (Holdsworth and Ratledge, 1988). The same phenomenon has been also observed in strains of *Mucor species* (Aggelis, 1989).

The last stage is characterised by complete exhaustion of the exocellular oil. Any further growth of the microbial population is exclusively based on the degradation of reserve fat:

$$-\frac{dx_L}{dt} = \frac{dx}{dt} \cdot \frac{1}{Y_{x/x_L}} \quad (3)$$

where Y_{x/x_L} is the yield of the reserve fat in biomass (mgx/ mgx$_L$).

Equation (2) can better describe the three phases provided that another equation, which can describe the changes of the yield coefficient during the growth period, could be found. This is because the yield coefficient does not remain constant during the stages of gradual changes of carbon source from exo- to endo-cellular. This has always been related to gradual change of fat metabolism in the microbial cell (accumulation- degradation).

During the first stage (equation 1) the yield coefficient should be less than the other two. This is because part of the energy is consumed for biosynthesis of lipolytic enzymes and a significant quantity is consumed for incorporation of fatty acids and formation of lipid reserves. During the second stage, accumulation of lipid reserves gradually decreases and reaches zero, or even takes negative values ($dx_L/dt < 0$, equation 2). This phenomenon is accompanied by increase of the yield coefficient in the fat-free biomass. During the third stage (equation 3) phenomena of transport of exocellular fatty acids in the mycelium completely disappear and the microorganism starts degrading lipid reserves, a process which is more favoured in energy and yield terms.

Given that the transition from one stage to another is a gradual process it is concluded that the yield coefficient is dependent on the fat-free biomass not only in the intermediate stages of the various phases ($Y_{x/L} < Y_{x/L+x_L} < Y_{x/x_L}$) but also in each phase separately. Thus,

$$Y = k_1 \cdot x \quad (4)$$

where Y a general term for description of the complete biotransformation of oil to fat-free biomass and k_1 degradation constant of fat.

From equation (2) and (4) a new relation is derived:

$$-\frac{d(L + x_L)}{dt} = \frac{1}{k_1 x} \cdot \frac{dx}{dt}$$

Integration of the last equation gives equation (5):

$$-K_1(L + x_L) = \ln x + c \quad (5)$$

At time $t = 0$ equation (5) takes the form:

$$c = -k_1(L_0 + x_{Lo}) - \ln x_0 \quad (6)$$

where L_0 and x_{Lo} the values of L and x_L at time $t = 0$.

From equation (5) and (6) a new equation (7) is derived:

$$x_L + L = x_{Lo} + L_0 - \frac{\ln x - \ln x_0}{k_1} \quad (7)$$

On the other hand, the rate of change in the concentration of exocellular oil, in the absence of other carbon sourses, is proportional to the concentration of L:

$$\frac{dL}{dt} = -k_2 L \quad (8)$$

where k_2 constant related with fat incorporation in the microbial cell. After integration of equation (8), the concentration of exocellular oil can be expressed as:

$$L = L_0 \cdot e^{-k_2 \cdot t} \qquad (9)$$

Finally, by combining equation (7) and (9) a new formula is produced:

$$x_L = x_{Lo} + L_0 \cdot (1 - e^{-k_2 \cdot t}) - \frac{\ln x - \ln x_0}{k_1} \qquad (10)$$

The last equation $x_L = f(x, t)$ shows that, in culture media containing as sole carbon source a vegetable oil, the concentration of lipid reserves depends on the concentration of fat-free biomass and the available amount of exocellular fat ($L_0 \cdot (1 - e^{-k_2 \cdot t})$).

2. MATERIAL AND METHODS

The verification of the mathematical model was based on data derived from cultivation of *Mucor circinelloides* CBS 172-27. Verification of the model in a chemostat presents problems which may compromise the reliability of our study. This is because of the dissimilar mould growth in broth culture media (pellets formation) and mainly because of the heterogeneous composition of the growth medium having vegetable oil as carbon source. For the verification of the various equations, accurate measurements of biomass (x, mg/l), lipid reserves (x_L, mg/l) and exocellular oil (L, mg/l) are necessary. However, in the final equation (10), only the concentrations of biomass appears. This shows the versatility of the model.

Moulds were cultivated in a 50 ml conical flask containing 10 ml of nutrients (peptone 5g/l; yeast extract 1g/l; glucose 5 g/l) sterilised at 120°C for 20 min. Flask were incubated in a rotary shaker (200 rpm) at 28±1°C. No phenomenon of dimorphism (yeast-like morphogenesis) was observed during these cultures, that means that flask aeration meets the oxygen needs for aerobic growth of microorganism. At the end of the growth period, detectable from the exhaustion of glucose in the culture medium, the biomass was transferred to 500 ml conical flasks containing 100 ml growth media having the following composition:

KH_2PO_4 8g / l; $NaCl$ 0.1g / l; $MgSO_4 \cdot 7H_2O$ 0.5 g / l; $CaCl_2$ 0.1 g / l; $(NH_4)_2 SO_4$ 2g / l; yeast extract 1 g / l; pH 5.2. To each flask 1g of sunflower oil was added and the flasks were sterilised in autoclave at 121°C for 20 min. Flask were left in a rotary shaker (200 rpm) at 28±1°C.

Chemical analysis

Samples drawn at several time intervals from a culture growing for 10 days were filtered through Whatman no 541 paper. The collected cells were washed three times with n-hexane and then with deionised water. This treatment resulted in the removal of external lipids which were absorbed on the mycelium surface (Montet et al., 1985). To verify the complete removal of lipids from the mycelium surface the last hexane elute was checked by TLC (60G silica gel, Merck). The development solvent used was composed of petroleum ether-diethyl ether-acetic acid 70:30:1 (v/v/v), and visualisation was effected by exposing the plate to iodine vapour.

The collected biomass was lyophilised and the mycelium lipids were quantitatively extracted according to the Folch et al., 1957 method. Non-consumed lipids were extracted three times with petroleum ether, after acidification of growth media with equal volume of 4N hydrochloric acid.

The organic solvent was then dried over anhydrous sodium sulphate. Finally, the organic solvent was removed, prior to taking the lipid weight.

3. RESULTS AND DISCUSSION

As can be seen in Table I, the rapid exhaustion of substrate was accompanied by accumulation of significant quantities of reserve lipids. Lipid accumulation in the biomass can take place provided that the concentration of exocellular oil is more than 420 mg/l. As evidenced by the reduction of lipid reserves, it seems that no further microbial growth based solely on exocellular carbon source had occured. The lipid reserves have been complementarily used by the microbial cell for its growth needs and maintenance. Therefore, any further growth of microbial population was exclusively based on the degradation of lipid reserves.

Table I

Experimetal data showing the growth of *Mucor circinelloides* CBS 172-27 in growth medium containing as sole carbon source sunflower oil (x:fat-free biomass, x_L:endocellular oil, L: exocellular lipids)

$t(h)$	$x(mg/l)$	$x_L(mg/l)$	$L(mg/l)$
0	1810	370	10000
28	4720	1720	1620
49	5440	2000	420
80	5630	1930	50
120	5930	1250	tr
180	6450	940	tr
240	6580	910	tr

Figure 1 shows the three periods of growth. During the first period a dual use of exocellular oil was observed. It was used for production of fat-free biomass (x) and lipid reserves (x_L). During the second period ($t > 50$ h) the uptake of the remaining exocellular oil was followed by degradation of lipid reserves. From the middle of the second period a coincidence of curves $x_L + L = f(t)$ (curve show the decrease in carbon source in the system) and $x_L = f(t)$ was observed. This suggests that the growth was mainly or exclusively based on the degradation of the

reserve lipids of microorganism. These data demonstrated the correctness of the assumptions made for the derivation of the mathematical model.

Model verification

Equation 7 has the form:

$$y = \alpha + \frac{1}{\beta} \ln x$$

where $y = x_L + L$, $\alpha = x_{Lo} + L_0 + \frac{1}{k_1} \cdot \ln x_0$ and $\beta = -k_1$.

This equation was well fitted to the experimental data, with a coefficient of determination $R^2 = 99.76\%$ and $\beta = -k_1 = -1.3 \cdot 10^{-4}$ (standard error $< 5 \cdot 10^{-6}$).

Equation 9 has the form:

$$y = e^{\alpha+\beta x}$$

where y=L, $\alpha = \ln L_0$ and $\beta = -k_2$. This equation fitted well to the experimental data. In this case, the coefficient of determination was $R^2 = 99.97\%$ and $\beta = -k_2 = -0.0661$ (standard error 0. 000744).

Using the constants k_1, k_2 and the values of t, x and L (Table I) it was possible to calculate the theoretical values of x_L derived from the mathematical model (equation 10). On the other hand, equation 10 can be transformed as follows:

$$x_L = c_1 + c_2 \cdot \ln x + C_3 \cdot e^{c_4 t}$$

where $c_2 = -\frac{1}{k_1}$ and $c_4 = -k_2$

Non linear regression analysis (Neter and Wasserman, 1974), resulted in a very good fitting ($R^2 = 92.64\%$.) Figures 2, 3 show the fitted curve and the plot of predicted values respectively.

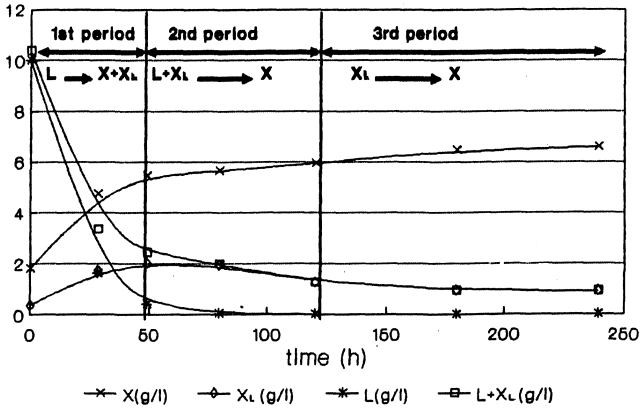

Figure 1
Periods of *Mucor circinelloides* CBS 172-27 growth on sunflower oil.

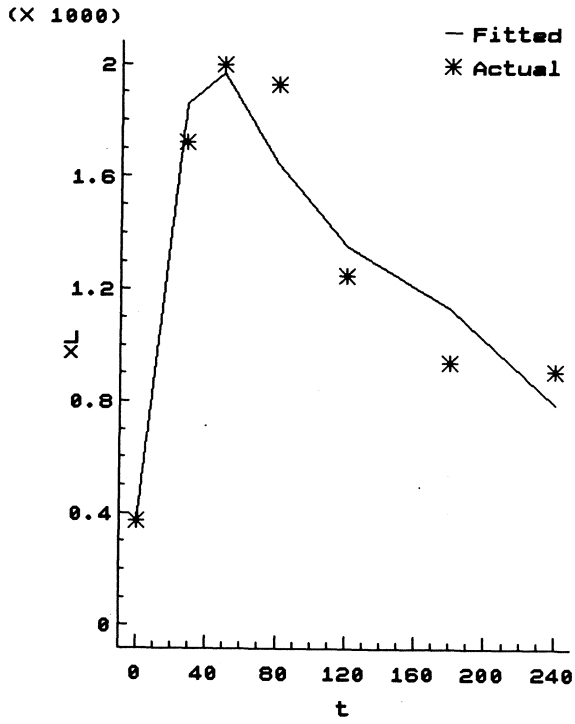

Figure 2
Curve fitted to the model $x_L = C_1 + C_2 \cdot \ln x + C_3 \cdot e^{C_4 \cdot t}$

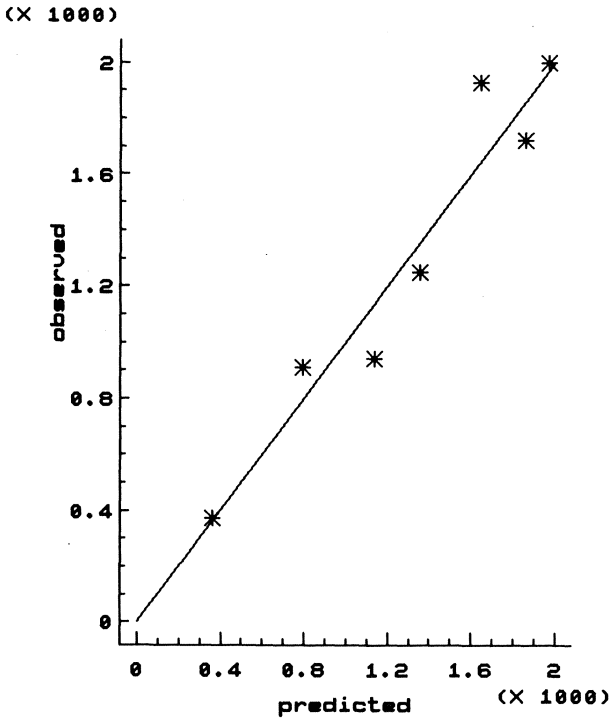

Figure 3
Plot of predicted values.

4. CONCLUSION

The proposed mathematical model correlates the growth of microbial population and the accumulation of lipid reserves in the microbial cell. It has been shown that fat accumulation takes place during the first stages of growth when the exocellular fat is present in significant amounts. Reduction of exocellular fat below certain limits results in degradation of stored lipids that are used to cover the needs of any further growth. Therefore, in culture media having as carbon and energy source fats, it is possible to increase the yield in endocellular fat by increasing the concentration of fat in the growth medium. Interruption of growth, when the concentration of exocellular fat is minimal, impedes the degradation of accumulated fat.

However, future prospects for microbial oils lie not exclusively on lipid accumulation in the microbial cell but also on the production of modified fats having different and more useful technological characteristics. For this reason, it would be interesting to investigate the possibility of fatty acid biotransformations, after their incorporation into the mycelium of *Mucor circinelloides.*

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REFERENCES

1. Aggelis, G., Pina, M., Ratomahenina, R., Arnaud, A., Graille, J., Galzy, P., Martin-Privat, P. et Perraud, J.P. (1987).- "Production d'huiles riches en acide gamma linolenique par diverses souches de Phycomycetes".- Oleagineux **42**, 379-386.
2. Aggelis, G. (1989).- "Etude de la production d'acide gamma linolenique par des phycomycetes (Mucorales)".- These de Doctorat en Biochimie. Université de Montpellier II - USTL.
3. Aggelis, G., Komaitis, M., Dimitroulias, G., Pina, M. et Graille, J. (1991a).- "Possibilité de production d'acide gamma linolenique par culture de *Mucor circinelloides* CBS 172-27 sur quelques huiles vegetales".- Oleagineux **46**, 208-212.
4. Aggelis, G., Balatsouras, G., Komaitis, M., Anagnostopoupou, G., Dimitroulias, G., Pina, M. et Graille, J., (1991b).- "Production d'acide gamma linolenique par bioconversion de l'acide linoleique de quelques huiles vegetales".- Revue Francaise des Corps Gras **38**, 95-101.
5. Botham, P. A. and Ratledge, C. (1979).- "A biochemical explanation for lipid accumulation in *Candida* 107 and other oleaginous micro-organisms"- Journal of General Microbiology **114**, 361-375.
6. Fuji Oil Co. Ltd. (1979).- "Method for producing cacao butter substitute".- Br. Pat. 1 555 000.
7. Glatz, B.A., Hammond, E.G., Hsu, K.H., Baehman, L., Bati, N., Bednarski, W., Brown, D. and Floetenmeyer, M. (1984).- "Production and modification of fats and oils by yeast fermentation".- In "Biotechnology for the oils and fats industry" (Ratledge, C., Rattray, J.B.M. and Dawson, P.S.S., eds.).- American Oil Chemists' Society, 163-176.
8. Holdsworth, J.E. and Ratledge, C. (1988).- "Lipid turnover in oleaginous yeasts".- Journal of General Microbiology **134**, 339-346.
9. Folch, J., Lees, M. and Sloan-Stanley, G.H. (1957).- "A simple method for the isolation and purification of total lipid from animal tissues".- Journal of Biological Chemistry **226**, 497-509.
10. Kamisaka, Y., Yokochi, T., Nakahara, T. and Suzuki, O. (1990).- "Incorporation of linoleic acid and its conversion to γ-linolenic acid in fungi".- Lipids **25**, 54-60.
11. Koritala, S., Hesseltine, C.W., Pryde, E.H., and Mounts, T.L. (1987).– "Biochemical modification of fats by microorganisms: A preliminary survey".- JAOCS **64**, 509-513.
12. Montet, D., Ratomahenina, R., Galzy, P., Pina, M. and Graille, J. (1985).- "A study of the influence of the growth media on the fatty acid composition in *Candida lipolytica* Diddens and Lodder".- Biotechnology Letters **7**, 733-736.
13. Neter, J., Wasserman, W. (1974).- "Applied linear statistical models".- (R. D. Irwin Inc., Homewood eds.). Illinois, U.S.A.
14. Radwan, S.S. and Soliman, A. H. (1988).- "Arachidonic acid from fungi utilizing fatty acids with shorter chains as sole sources of carbon and energy".- Journal of General Microbiology **134**, 387-393.

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