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A mathematical model for the study of lipid accumulation in oleaginous microorganisms. II. Study of cellular lipids of *Mucor circinelloides* during growth on a vegetable oil.

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RESUMEN

Modelo matemático para el estudio de la acumulación de lípidos en microorganismos oleaginosos. II. Estudio de lípidos celulares de *Mucor circinelloides* durante el crecimiento sobre un aceite vegetal.

La producción de aceites microbianos a partir de materiales grasos de origen animal o vegetal ha sido objeto de investigación e interés industrial durante muchos años. En el proceso de crecimiento microbiano/acumulación de reservas grasas, los fenómenos dominantes que definen la composición de grasa endocelular son, primero, el proceso específico de incorporación de ácidos grasos como sustratos en la célula microbiana y, segundo, los cambios endocelulares de ácidos grasos definidos por las capacidades enzimáticas de los microorganismos. Los ácidos grasos serán o bien degradados para necesidades de crecimiento o actuarán como sustratos de procesos de biotransformación endocelular, desembocando en cambios en la concentración y producción de ácidos grasos "nuevos" que previamente no existían en el sustrato.

El objeto de este trabajo, es estudiar los lípidos endocelulares de *Mucor circinelloides CBS 172-27* en crecimiento sobre aceite de girasol. El modelo matemático descrito en la parte I se aplicó en orden a investigar:

a. Especificidad de microorganismos en la incorporación de ácidos grasos como sustratos.
b. Especificidad de microorganismos en la degradación de ácidos grasos presente en la grasa de reserva.
c. Posibilidades de biotransformaciones endocelulares durante el crecimiento microbiano.

En conclusión este trabajo está dirigido hacia el desarrollo de una expresión cuantitativa de parámetros que definan la composición de lípidos de reservas grasas. El modelo matemático propuesto puede ser usado no sólo para la selección de cepas microbianas que tengan potencial enzimático específico sino también para la selección de sustrato.

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. II. Study of cellular lipids of *Mucor circinelloides* during growth on a vegetable oil .

Microbial oil production from fatty materials of animal or plant origin has been an object of research and industrial interest for many years. During the process of microbial growth/accumulation of fat reserves, the dominating phenomena that define the composition of endocellular fat are, first, the specific process of incorporation of substrate fatty acids into the microbial cell and, second, the endocellular changes of fatty acids defined by the enzymic capabilities of the microorganism. The fatty acids will either be degraded for growth needs or act as substrate of endocellular biotransformation processes, leading to concentration changes and production of "new" fatty acids which did not previously exist in the substrate.

The purpose of the present work is to study the endocellular lipids of *Mucor circinelloides* CBS 172-27 grown on sunflower oil. The mathematical model, described in part I, was applied in order to investigate the following:

a. Microorganism specificity in the incorporation of substrate fatty acids.
b. Microorganism specificity in the degradation of fatty acids present in the reserve fat.
c. Possibilities of endocellular biotransformations during the microbial growth.

In conclusion, this work is aimed at developing a quantitative expression of parameters defining the lipid composition of fat reserves. The proposed mathematical model can be used not only for selection of microbial strains having specific enzymic potential but also for substrate selection.

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

1. INTRODUCTION

Microorganisms that can grow on culture media having fats or oils as sole carbon and energy source, are able to hydrolyse the substrate and uptake the hydrolysis products. It has already been shown that the incorporation of fatty acids is not random but specific for each particular fatty acid (Aggelis, 1989). After entering the microbial cell, fatty acids undergo a series of biochemical changes. They are either degraded for biomass production or used for lipid reserve formation. The latter sometimes has an interesting composition.

The following examples are very characteristic: *Torulopsis* spp cultured on substrates containing esters of stearic and/or palmitic acid accumulate triglycerides having structures similar to those of cocoa butter (Fuji Oil Co., 1979). *Candida lipolytica* cultured on vegetable oils or soaps show an important Δ-9 desaturase activity on incorporated palmitic and stearic acids. Δ-11 eicosenoic and erucic fatty acids are shortened to oleic acid (Montet et al., 1985). Moulds *Aspergillus versicolor* and *Aspergillus ustus* cultured on myristic and palmitic acid, respectively, synthesize appreciable amounts of arachidonic acid (Radwan and Soliman, 1988). *Mortierella ramanniana* var. angulispora desaturates linoleic acid to GLA in the exponential growth phase (Kamisaka et al., 1990). *Mucor circinelloides* CBS 172-27 cultured on sunflower oil accumulates 65% oil containing 17.4% of GLA (Aggelis et

al., 1991). Mould *Mortierella alpina* 1S-4, an arachidonic acid-producing fungus, produces large quantities dihomo-gamma-linolenic acid on a growth media containing sesame oil (Shimizu et al., 1989a). Another strain of the same mould seems to be able to biotransform alpha linolenic acid, which is found in abundance in linseed oil, into eicosapentaenoic acid (Shimizu et al., 1989b). *Nocardia cholosterolicum* NRRL 5667 has the ability to biotransform linoleic and linolenic acid to unsaturated hydroxy fatty acids (Koritala and Bagdy, 1992). A mutant of *Motierella alpina* 1S-4 defective in Δ-12 desaturase, was shown to be a novel patent producer of mead acid (5,8,11- cis-eicosatrienoic acid, 20:3ω9). The addition of 2% coconut oil in the culture medium increased the production of mead acid three fold without oil supplementation (Jareonkitmongkol et al., 1992).

It becomes apparent that the composition of cellular lipids, during microbial growth on a culture media having as carbon source fats, is connected first with the specificity of the incorporation of substrate fatty acids and second with enzymic processes taking place in the microbial cell. In the present work, the proposed mathematical model (part I of this work) is applied for the study of endocellular biotransformations taking place during the growth of *Mucor circinelloides* CBS 172-27 on sunflower oil.

Model application

As it has already been mentioned, microorganisms do not uptake the various substrate fatty acids at the same rate. There are many reasons for this and possibly some are not known yet. It is certain that differentiations in the composition of substrate fatty acids, observed during the growth stage, are associated with the lipolytic enzymes specificity (stereo-, type- specificity) (Aggelis et al., 1993; Spyed Rahmatullah et al., 1994a; 1994b) and the selective permeability of cellular membranes (Aggelis, 1989).

During the period of lipid reserve accumulation the substrate fatty acids contribute variably. This contribution is greatly influenced by the concentration of the fatty acids but it is not necessarily proportional to their concentration in the exocellular environment.

By applying equation:

$$L = L_0 \cdot e^{-k_2 \cdot t} \quad *$$

for every fatty acid it is possible to investigate the microorganism's specificity on the incorporation of substrate fatty acids in the microbial cell.

This equation can be transformed to:

$$(FA)_L = (FA)_{L_0} \cdot e^{-k_2 \cdot t} \quad (1)$$

where $(FA)_L$ concentration of substrate fatty acids in mg/l at time t and $(FA)_{L0}$ concentration at time t=0. The specific rate of incorporation of every fatty acid is expressed by coefficient k_2 (h^{-1}).

After entering the microbial cell, fatty acids undergo a series of biochemical changes. These changes lead either to degradation for biomass production or to formation of "new" fatty acids that did not previously exist in the substrate. Mutual biotransformations of various fatty acids are also possible and result in a different participation of each fatty acid in the lipid reserve.

Let us assume that a fatty acid, entering the microbial cell, does not participate in any biotransformation. Instead it is degraded to produce fat-free biomass or participates in the formation of lipid reserve.

The microorganism's specificity on this particular fatty acid can be expressed by equation:

$$X_L + L = X_{L_0} + L_0 - \frac{\ln x - \ln x_0}{k_1} \quad ** .$$

This equation can be transformed to:

$$(FA)_{x_L} + (FA)_L = (FA)_{x_{L_0}} + (FA)_{L_0} - \frac{\ln x - \ln x_0}{k_1} \quad (2)$$

where $(FA)_{x_L}$ is the concentration (mg/l) of the specific fatty acid of lipid reserve at time t, $(FA)_L$ its concentration (mg/l) in the substrate, $(FA)_{x_{L_0}}$ its concentration (mg/l) in lipid reserve at time t= 0, and $(FA)_{L_0}$ its concentration (mg/l) in the substrate at time t = 0. The constant k_1 ($(mg/l)^{-1}$) is an expression of the degradation rate of fatty acid.

By combining equations (1) and (2) it is possible to have important information in regard to microorganism's specificity, not only on the incorporation of substrate fatty acids but also on the degradation of fatty acid present in the lipid reserve. So, by combining equations (1) and (2) the values of $(FA)_{x_L}$ can be expressed as follows:

$$(FA)_{x_L} = (FA)_{x_{L_0}} + (FA)_{L0} \cdot (1 - e^{-k_2 \cdot t}) - \frac{\ln x - \ln x_0}{k_1} \quad (3)$$

Coefficients k_1, k_2 express the nutritional preferences of microorganism relative to the specific fatty acid.

The basic assumption made is that substrate fatty acids will degrade for formation of fat-free biomass or will contribute to the formation of lipid reserves. In other words, a satisfactory fitting of the curve derived from equation (3) on experimental data shows that the basic assumption is true. The specific fatty acid does not take place in endocellular biotransformations either as substrate or as product of some enzymic reactions.

On the contrary, deviation of experimental data from theoretical values calculated from equation (3), shows participation of the fatty acid under investigation in endocellular biotransformations. More specifically, if the

* verified in Part I of this paper. L= exocellular oil at time t; L_0 = exocellular oil at time t=0; k_2 = incorporation constant of oil.

** verified in Part I of this paper. X_L = endocellular lipids at time t; X_{L_0} = endocellular lipids at time t=0; X= biomass at time t; X_0 =biomass at time t=0; k_1 =degradation constant of oil.

curve of experimental data is above the curve derived from equation (3) it implies that the concentration of the fatty acid is enhanced by biotransformations of other fatty acids. On the other hand, if the curve of experimental data is below the curve calculated from equation (3) it implies that the fatty acid not only is degraded for biomass production but it also acts as substrate for production of other fatty acids.

Determination of coefficients k_1 and k_2.

Coefficient k_2 is statistically determined from equation (1). Values of FA(mg/l) are based on experimental data.

For the determination of coefficient k_1 it is assumed that during the first stage of growth, biotransformations of various fatty acids, in relation to the amounts entering the microbial cell, are quantitatively insignificant. So it is assumed that during the first stages of growth experimental data are in a considerable degree independent of biotransformations that can put some limitations in the use of equation (2). Consequently, by applying equation (2) for every individual fatty acid and using the values of x and $(FA)_L$ from the first stages of growth it is possible to determine coefficient k_1 for each fatty acid separately.

After calculation of coefficients k_1 and k_2 it is possible to use equation (3). Values of $(FA)x_L$ calculated from this equation represent expected values provided that the fatty acid $(FA)x_L$ had not taken part in endocellular biotransformation either as substrate or as product.

2. MATERIAL AND METHODS

Cellular and non-consumed lipids (see part I) were esterified according to the AFNOR (1984) method . The methyl esters produced were then analysed in a gas chromatograph Hewlett-Packard 5700A equipped with FID dual detector. Separation of methyl esters was effected on a DEGS column (1.83mx3mm) 10% on Chromosorb WHP 100-200 mesh. Peak identification was effected by use of standard methyl ester solutions and comparison of the respective retention times. Conditions used were as follows: Oven temperature 190 *°C*; injector and detector temperature 250 *°C;* carrier gas helium; flow 25 ml/min.

3. RESULTS AND DISCUSSION

Table I shows the lipid composition during the various periods of growth. As regard the composition of the oil present in the substrate, it does not remain constant. (Table Ia). More specifically, a gradual reduction of linoleic acid concentration with subsequent increase of stearic and oleic acids was observed. The concentration of palmitic acid was increasing for the first 120 h and then it decreased gradually.

Biomass composition (Table Ib) was continuously changing during microbial growth. Even from the first stages of growth, a spontaneous influx of substrate fatty acids into mycelium was observed. Thus, with exception of

Table I

Composition of substrate (Ia) and biomass (Ib) oil during growth of *Mucor circinelloides* CBS 172-27 on sunflower oil.

t\F.A.	C16:0	C18:0	C18:1	C18:2	other
0	6.4	3.8	24.9	64.3	0.6
28	7.0	4.8	27.7	58.6	1.9
49	7.5	5.0	27.9	58.4	1.2
80	7.6	5.4	27.8	58.2	1.0
120	7.8	5.6	27.3	58.2	1.1
180	6.5	7.8	28.0	56.5	1.2
240	6.2	9.3	28.9	55.6	1.0

Table Ia

t\F.A.	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3γ	other
0	3.1	31.4	2.7	4.2	40.5	11.3	6.6	0.2
28	0.2	7.4	0.5	4.0	25.3	58.3	2.9	1.4
49	0.3	10.7	0.9	4.2	27.1	52.7	3.2	0.9
80	0.4	9.5	1.0	4.4	27.5	51.5	3.5	2.2
120	0.5	9.9	1.2	4.6	27.7	48.5	5.1	2.5
180	0.4	8.1	0.9	18.9	22.5	43.0	4.0	2.2
240	0.4	8.0	0.9	30.8	21.9	31.3	3.7	3.0

Table Ib

gamma linolenic acid, mycelium lipids quickly obtained a composition similar to that of substrate oil. During the first period of growth, $t \leq 49h$, substrate fatty acids contribute to a different degree in the building up of reserve lipids. This contribution is not necessarily proportional to their concentration in the exocellular environment. As can be concluded from the significant differences observed in the composition of lipid reserves at times $t = 28h$ and $t = 49h$, fatty acids of lipid reserves are degraded, creating obviously fat-free biomass. This phenomenon can be quantitatively detected only after complete exhaustion of exocellular oil.

During the second period, $t = 49$—120 h, the composition of lipid reserves is a result of two specific actions. First the specificity of incorporation of substrate fatty acids in the mycelium (phenomenon dominating the first stage) and second the enzymic specificity on lipid reserves (degradation, bioconversions). The latter is the dominating phenomenon during the third period which is characterised by exhaustion of exogenous carbon source. After time $t =$ 120 h, the observed changes in the composition of lipid biomass should be attributed to the preferential degradation of certain fatty acids and possibly to endocellular biotransformations.

In Table II quantitative changes of the main fatty acid of substrate and biomass, are given. It was observed that palmitic, oleic and linoleic acid present in reserve lipids follow a route similar to that of total reserve lipids (see part I, Table I). More specifically, for these fatty acids three characteristic periods are observed eg.

accumulation, t = 0– 49h, degradation in parallel with uptake of traces of exocellular oil, t= 49-120 h and finally degradation after exhaustion of exocellular carbon source (t = 120– 240h).

On the other hand, stearic acid exhibited a different behaviour at least during the third period. More specifically, during that period, and contrary to expectations, a significant increase in the amount of stearic acid in the biomass was observed (Table II). This leads to the conclusion that under these conditions of growth, biosynthesis of this acid takes place. As regard gamma linolenic acid, not present in substrate, the following were observed. During the first 80 h, its concentration increased gradually. Then it started degrading to other fatty acids but stearic.

Table II
Quantitative changes of the main fatty acid of substrate *(L)* and biomass (X_L) during growth of *Mucor circinelloides* CBS 172-27 on sunflower oil

	Fatty acids (mg/l)								
t(h)	C16:0		C18:0		C18:1		C18:2		C18:3γ
	L	x_L	L	x_L	L	x_L	L	x_L	x_L
0	640.0	116.2	380.0	15.5	2490.0	149.9	6430.0	41.8	24.5
28	113.4	127.3	77.8	68.8	448.7	435.2	949.3	1002.8	49.9
49	31.5	214.0	21.0	84.0	117.2	542.0	245.3	1054.0	64.0
80	3.8	183.4	2.7	84.9	13.9	530.8	29.1	994.0	67.5
120	tr	123.8	tr	57.5	tr	346.3	tr	606.3	63.7
180	tr	76.1	tr	117.7	tr	211.5	tr	404.2	37.9
240	tr	72.8	tr	280.3	tr	199.3	tr	284.8	33.5

Incorporation constants for every individual substrate fatty acid is statistically calculated in Table III. Based on calculated values of k_2, microorganism specificity on the incorporation of substrate fatty acid has as follows:

$C18{:}2 > C18{:}1 > C16{:}0 > C18{:}0$.

Table III
Incorporation constants derived from equation $(FA)_L = (FA)_{L_0} \cdot e^{-k_2 \cdot t}$ and from data of Table II

	$(FA)_{L_0}$ (mg/l)	k_2	Standard error	R^2(%)
C16:0	667.4	-0.0639	1.28×10^{-3}	99.9
C18:0	409.1	-0.0619	1.69×10^{-3}	99.8
C18:1	2632.2	-0.0648	1.36×10^{-3}	99.9
C18:2	6407.8	-0.0673	5.37×10^{-4}	99.9

Conclusively, the microorganism incorporated more rapidly the most unsaturated fatty acid of the substrate. Degradation constants for every individual fatty acid present in the reserve lipid are given in Table IV. Based on k_1; values, microorganism specificity on the degradation of substrate fatty acids has as follows:

$C18{:}2 > C18{:}1 > C16{:}0 > C18{:}0$.

Table IV

Degradation constants derived from equation:

$$(FA)_{x_L} + (FA)_L = (FA)_{x_{L0}} + (FA)_{L_0} - \frac{\ln x - \ln x_0}{k_1}$$

and experimental data at time t=28h. (x=4720 mg/l x_0=1810 mg/l).

	$(FA)_{x_L}$	$(FA)_{L_0}$	$(FA)_L$	K_1
C16:0	127.3	640.0	113.4	1.8593×10^{-3}
C18:0	68.8	380.0	77.8	3.8509×10^{-3}
C18:1	435.2	2490.0	448.7	5.4583×10^{-4}
C18:2	1002.8	6430.0	949.3	2.1207×10^{-4}

The fitting of curve:

$$x_L = c_1 + c_2 \cdot \ln x + c_3 \cdot e^{c_4 \cdot t}, \quad c_1 = -\frac{1}{k_1} \text{ and } c_4 = -k_2$$

which is a mathematical form of equation (3), on experimental data of Table II, gave good results for oleic (R^2 = 89. 74%) and linoleic (R^2 = 96.18 %) acids (Figures 1,2). The opposite was observed for palmitic (R^2 = 61.76%) and stearic (R^2 = 55.93%) acids. This leads to the conclusion that accumulation of these two fatty acids is disturbed by endocellular biotransformations.

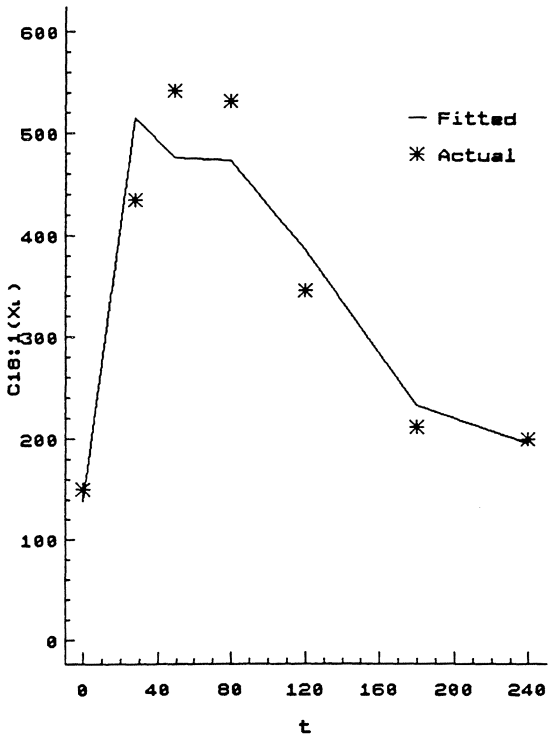

Figure 1
Curve fitted on the experimental data of oleic acid

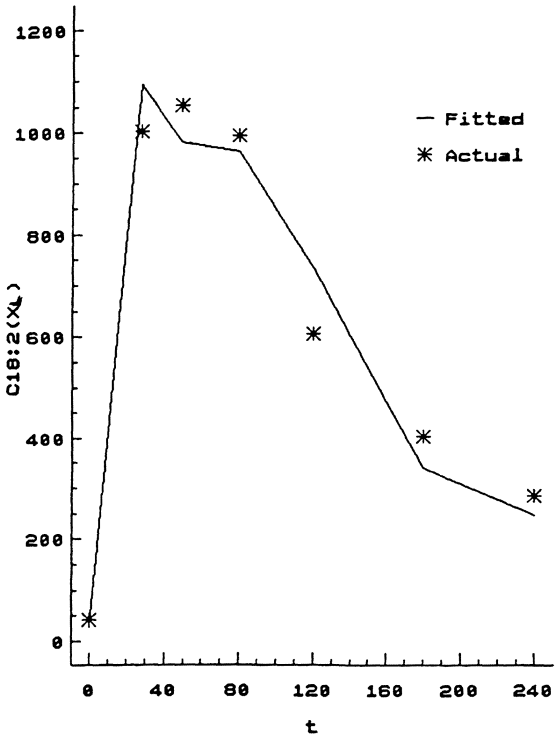

Figure 2
Curve fitted on the experimental data of linoleic acid

The predicted curves for these two fatty acids are given in Figures 3 and 4. During the first two periods of growth, the curve of experimental data for palmitic acid was above the predicted one. Therefore, that under these conditions the microorganism produced palmitic acid. During the third period the curves coincide. As regard to stearic acid, during the first period a coincidence of experimental and predicted curves was observed. Then the predicted curve was slightly over the experimental one and only during the third period the experimental curve was clearly above the predicted. The latter means that during that period the microorganism biosynthesises stearic acid.

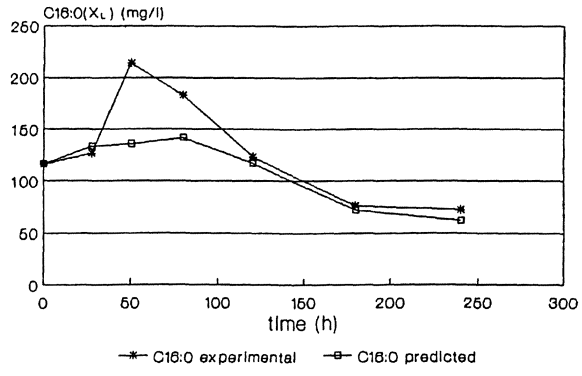

Figure 3
Predicted and experimental curves of palmitic acid

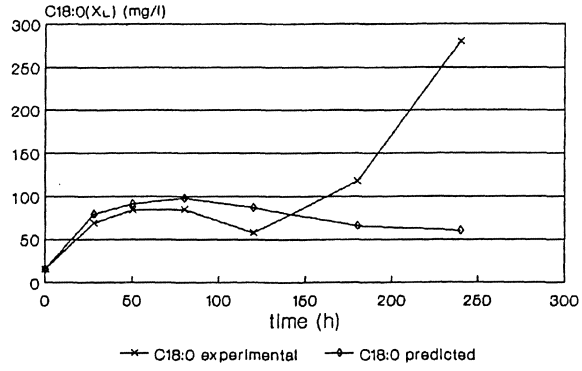

Figure 4
Predicted and experimental curves of stearic acid

4. CONCLUSION

The ability of some microorganisms to accumulate significant amounts of fat when cultured on substrates having as carbon and energy source fats, free fatty acids or soaps is of great interest.

The proposed model is possible to be used to clarify the capabilities of biotransformation of endocellular fatty acids of various oleaginous microorganisms.

Mucor circinelloides CBS 172-27 is used in the present work. During the first period of growth on sunflower oil this microorganism favours the production of palmitic and gamma linolenic acid. Then the mould shows a trend to petrify endocellular fat because of the enrichement of lipid reserves in stearic acid.

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