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Effect of Solubilizing Agents on the *Candida lipolytica* Growth on *n*-Alkane Substrate

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ABSTRACT. The role of methanol, ethanol and sorbitol oleate on the growth of *Candida lipolytica* on *n*-alkane substrate was studied by changing the concentrations of these solubilizing or emulsifing agents. These three substances enhanced the yeast growth at low concentration ranges, but beyond a critical concentration, exhibited inhibitory effects.

At an optimal concentration of solubilizing agent, the maximum growth rate was multiplied by 1.95 (CH_3OH), 1.9 (CH_3CH_2OH) and 1.5 (sorbitol oleate). The stimulating effects may be due to increased solubilities of *n*-alkane substrate. The modelization of the growth enhancement (E) with the concentration of the solubilizing agent (P) is performed.

The kinetics of microbial culture on insoluble substrate, like *n*-alkane, is controlled by the transfer phenomena of the substrate from the dispersed phase (hydrocarbon drop) to the microorganism, especially when the productivity is high. The mechanism is as yet submitted to discussion (Miura 1978).

Predominantly, most of the authors consider that the direct contact between the tiny hydrocarbon droplets and the cells is the rate controlling factor in microbial utilization of the substrate (Erickson *et al.* 1969, Aiba *et al.* 1969, Moo-Young *et al.* 1971, Yoshida and Yamane 1974). According to Käppeli and Finnerty (1979), the enhanced solubility of hydrocarbon in the growth medium of hexadecane grown *Acinetobacter*

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Sp. has been related to the accumulation of an extracellular vesicular component, whose composition is similar to the outer membrane of *Acinetobacter* and who binds alkane in a form of microemulsion to the membrane.

Käppeli *et al.* (1980) suggested that the transport of *n*-alkane is a mediated transport. In a first step, the hexadecane partitioned into a particulate fraction which is derived from the outer membrane. These particles interact with the bacterial cell surface transporting the hexadecane to the cells. Some of our experiments demonstrated that the relation between the surface tension of the medium broth and the hydrocarbon pseudo-solubility, so called because it is impossible to measure the true solubility, which must consider only the monomolecular hydrocarbon solubilized and not the aggregate change (Goma *et al.* 1973, Alani *et al.* 1980). It is not possible to neglect neither the role of soluble hydrocarbon nor the *n*-alkane solubilization process.

Since the microbial biosurfactants are the main factors in the transfer of *n*-alkane, it could be considered that the solubilizing agents, like alcohols or surfacting agents, are the determining factors in increasing (or decreasing) the transfer potentiality. Such studies were previously carried out by number of authors (Kobayashi *et al.* 1967, Tanaka and Fukui 1971, Whitworth *et al.* 1973, Nakahara *et al.* 1981).

The aim of this work was to study the effect of three solubilizing agents, namely, methanol, ethanol and sorbitol oleate, chosen for their solubilizing properties, on the growth rate of *Candida lipolytica*.

Material and Methods

Organism

The yeast was *Candida lipolytica* M12 cultured in shake flask, stored on nutritive gelose with 0.5% *n*-tetradecane at 4°C.

Culture Medium

The medium used for the growth studies has the following composition (g per *l*), KCl: 1.15; KH_2PO_4. H_2O: 1.5; NH_4Cl: 1.5; $MgSO_4$. $7H_2O$: 0.65; Zn $(NO_3)_2$. $6H_2O$: 0.1; $FeSO_4$. $7H_2O$: 0.068; $MnSO_4$. $2H_2O$: 0.015; yeast extract Bio-mérieux: 0.1 and 1% v/v *n*-tetradecane concentration and various solubilizing agent concentrations in water. pH was brought to 3.7 by the addition of 1 N orthophosphoric acid.

Culture Conditions

The experiments were carried out in a 3 l. fermentor with agitation provided at 1200 rpm and aeration of 0.8 vvm.

Methods of Analysis

Cell concentration was estimated by gravimetrical measurement of dry cell weight on Sartorius membranes (porosity: 0.45 nm). The cells and the membrane were washed

with tap water and 10 ml of hexane; the dehydration is carried out under 200 mm pressure and 50°C temperature. These conditions were previously described by Alani *et al.* (1980).

Results

Influence of Additives

The initial concentration of hydrocarbon was 1% v/v. The concentration of sorbitol oleate (Corexit: Esso) varied between 0 and 0.5% v/v. The curve (a) of Fig. 1 indicated the change of the ratio $E = \mu_{mO.S}/\mu_{mT}$ versus the initial concentration of the solubilizing agent. (The authors consider only the maximal growth rate during exponential growth; $\mu_{mO.S}$ the maximal growth rate in presence of sorbitol oleate: O.S, and μ_{mT} as reference without O.S).

The authors observed that this ratio was greater than 1, and then the growth was enhanced. At a critical concentration, that they called P_{crit}, 0.041% v/v, they obtained the maximal value of E (E = 1.5). When the sorbitol oleate concentration increased,

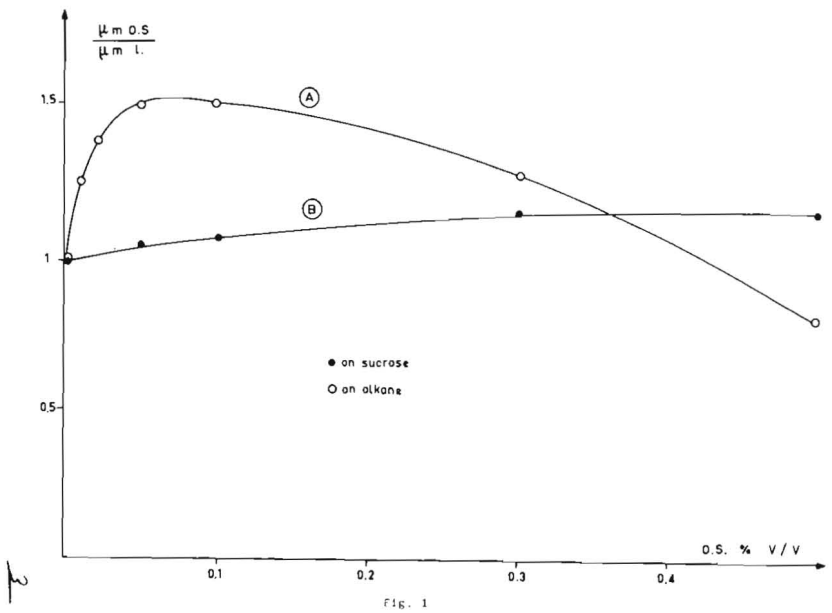

Fig. 1

Fig. 1. Effect of the sorbitol oleate concentration on the E factor when the substrate is the n-tetradecane (curve a) and sucrose (curve b)

in reference to the maximal enhancement at P_{crit}, they observed a lower activation of the growth, and sorbitol oleate exerted an inhibitory effect.

Plotting, Log E = f (P), (Fig. 2), during the activation phase, the authors got a straight line. To know the effect of the additive on the cell growth using insoluble substrate, the authors performed similar experiments with a soluble substrate: the saccharose (hydrophilic). With saccharose as carbon source, in all the range of concentrations studied, they observed an enhancement effect on the maximal growth rate (Figure 1 curve b). Two kinds of mechanisms explain the surfactant effect:

— first, emulsion of insoluble substrate,

— second, modification of the nature of all interfaces by change of the hydrophobicity both of the cell and hydrocarbon droplets by hydrophobic-hydrophobic interactions. Curve b (Fig. 1) could be explained by the increase of the hydrophobic character of the cells, on increasing the surfactant concentrations, and its adhesion to cells.

Results obtained with methanol and ethanol as additives are described in figure 2. They indicate the positive effect of the solubilizing aspects.

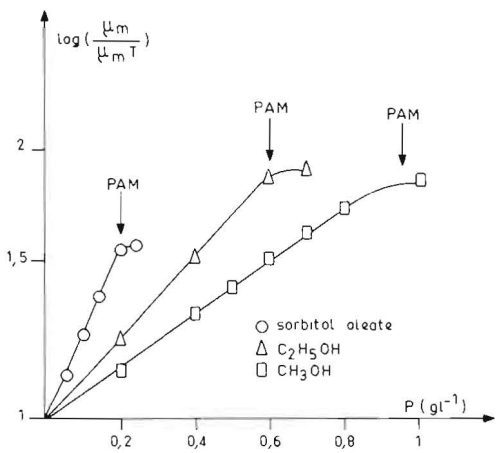

Fig.2. Evolution of $E = \mu_m/\mu_{mT}$ with addition of sorbitol oleate (—o—), ethanol (—△—), methanol (—□—), only for concentrations in the range of activator effect.

Discussion and Modelisation

In any case of *Candida lipolytica* cultured on *n*-tetradecane (10 gl^{-1}), the effect of solubilizing agents (methanol and ethanol) or surfactant (sorbitol oleate) is variable depending upon the concentration used. When the additive concentration is low, the activating effect increases until a critical concentration, P.A.M. Beyond this concentration, one can find a competition between the inhibitory and the enhancement effects.

During the strict enhancement phase, the maximum growth rate increases exponentially with the additive concentration as indicated in Fig. 2, where one finds the influence of the concentration of the product with solubilizing effect on the E factor, P concentration is lower than P_{crit}. The main effect of the additive product is to increase the hydrocarbon solubility. This shows that the solubilized hydrocarbons are involved in mass transfer, and confirms that the most probable pathway of liquid hydrocarbon uptake, is the dissolved *n*-alkane in aqueous phase and this opposes the theory of several authors (Erickson *et al.* 1969, Aiba *et al.* 1969). The effects of methanol and ethanol enhance this last mechanism. The sorbitol oleate effects are compatible with both pathways considering uptake from dissolved alkane or from submicron oil droplets (accomodated hydrocarbons according to the Aiba's concept).

If we consider the theoretical results of Goma (1975) on the cell growth kinetics limited by substrate solubilization, the activator effect could be understood and modelised. The results of Stephen and Stephen (1964) used by Chakravarty *et al.* (1975) indicate that the solubility of the dispersed phase increases exponentially with the amount (P) of the solubilizing factor added to the medium:

$$S_d = S_{d0}\, e^{\beta(P)} \tag{1}$$

(S_d alkane solubility, when the concentration of solubilizing factor is P and S_{d0} alkane solubility when P = 0).

Goma (1975) and Goma and Ribot (1978) theoretically demonstrated that when the cell growth kinetics are limited by the substrate solubilization before their assimilation, the cell growth kinetics are described by a relation like Contoi's law:

$$\mu = \mu_m \cdot \frac{1}{A\,\frac{X}{S} + 1} \tag{2}$$

in which

$$\mu_m = Z \cdot k_\mu \cdot \bar{a}_\mu \cdot S_d \tag{3}$$

(k_μ: mass transfer coefficient, $\bar{a}_\mu$ cell area, Z constant) and (A) is dependent on power input for agitation and aeration and on rheological and surface tension properties of the fermentation broth.

Considering only the exponential growth one can write, according to Stephen's relation:

$$\mu_{mP} = Z \cdot k_\mu \cdot \bar{a}_\mu \cdot S_{d0}\, e^{\beta(P)} \tag{4}$$

The activation factor $E = \frac{\mu_{mP}}{\mu_{mT}}$ have for value:

$$E = \frac{\mu_{mP}}{\mu_{mT}} = e^{\beta(P)} \tag{5}$$

The last equation (5) is in agreement with the experimental results described in Fig. 2, only in the activation step, where one observes, a linear relation between log E and P. This relation is also observed for growth activated by ethanol and sorbitol oleate. The ß value which characterizes each of the three products are summarized in Table 1.

The critical concentration P_{crit}, is the maximal concentration in solubilizing agent that is necessary to use.

The values of the three parameters of the products that have been used are summarized in Table 1.

Table 1. Values of the parameters describing the effect of the solubilizing agents on hydrocarbon assimilation by *C. lipolytica*.

Paremeters / Agents	ß l/g	$P_{crit.}$ $g l^{-1}$
Sorbitol oleate	2.17	0.18
Ethanol	1.035	0.631
Methanol	0.69	0.91

If one can understand and describe the real solubilizing effects of methanol and ethanol, the surfactant effect is more difficult to be explained. Many authors intensively studied this problem (*Kobayashi et al.* 1967, Tanaka and Fukui 1971, Whitworth *et al.* 1973). According to these authors, the stimulating effect is obtained especially from surfactant with H.L.B. (hydrophilic, lipophilic balance) of a value between eleven and fifteen. The role of sorbitol oleate may be described in many ways; it occurs:

— on *n*-alkane emulsification by decreasing the interfacial tension between hydrocarbon and water, and so decreasing the droplet diameters of dispersed phase, and consequently increasing the real solubility of *n*-alkane.

— by adsorbing to interface *n*-alkanes-water and so by rigidifying the droplets in emulsion. The frequency of coalition between droplets decreases and the mass transfer is unfavorable, which is in agreement with the observations of Blanch and Fiechter (1974).

— by adsorbing to cell walls and modifying the hydrophobic character of the cell, so their affinity decreased for *n*-alkanes because the hydrophobic character of the cell is necessary for the assimilation of *n*-alkane. Nakahara *et al.* (1977) and Miura (1978) reported that it is necessary to consider the cell surface properties to well understand the mechanism of hydrocarbon uptake by cells. The effects of tensioactive adsorption by cells is accredited by the stimulated effect on the *Candida lipolytica*

growth on saccharose in presence of large amount of surfactant. Sorbitol oleate adsorption renders cell more hydrophilic and explains: i) the growth enhancement on hydrophilic substrate, ii) the inhibition at high concentration on hydrophobic substrate.

Conclusion

The solubilizing agents, as methanol and ethanol, have an activating effect on hydrocarbon solubilization at low concentrations, but have an inhibiting effect at high concentrations in the medium

The tensioactive agents have, at low concentrations, an emulsifying and solubilizing effects which stimulate the growth. Beyond a critical concentration, however, their adsorption to cell walls and the interface hydrocarbon-water has an inhibiting effect due to the increasing hydrophilic character of the cell walls and the hydrocarbon droplets rigidification.

The biosurfactant secreted during the cell growth in the medium probably has the same effects of sorbitol oleate, and this observation can explain some growth limitations due to hydrocarbon uptake, in particular linear growth. These results can find their application in the treatment of water spoiled by hydrocarbons, with some limitations discussed by Atlas (1981) who indicates from the point of view of microbial hydrocarbon degradation, dissolution and emulsification of hydrocarbons appear to have a positive effect on degradation rates. If there are no adverse toxic effects, dispersion of oil should accelerate microbial hydrocarbon degradation. This is an important consideration when determining whether dispersants should be added to oil spills. Increased toxicity must remain, however, a major concern when considering the use of such chemical dispersants.

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تأثير العوامل المذيبة على خميرة الكانديدا ليپوليتيكا النامية على مادة التفاعل: ن ـ ألكان

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درس تأثير إضافة تركيزات مختلفة لكل من: الميثانول والإيثانول وأوليات السوربيتول على تكاثر خميرة الكانديدا ليپوليتيكا *Candida lipolytica* بوجود الألكانات المشبعة (هيدروكربونات) كمادة تفاعل.

وجد أن إضافة تركيزات منخفضة من المواد المضافة تزيد من تكاثر الخميرة إلى درجة يصل فيها النمو إلى معدله الأقصى (Maximum growth rate).

عند زيادة تركيزات المواد المضافة عن التركيزات الحرجة، تمارس هذه المواد تأثيراً مثبطا للتكاثر.

عند إضافة التركيز الأمثل للتكاثر من الميثانول يصبح معدل النمو الأقصى مضاعفاً بعامل قدره (١,٩٥) في وجود الميثانول، وبعامل قدره (١,٩) بوجود الإيثانول، وبعامل قدره (١,٥) بوجود أوليات السوربيتول.

قد يُعزى التأثير المنشط لهذه المواد المضافة إلى زيادة ذوبان مادة التفاعل (الهيدروكربونات).

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تم إخضاع زيادة معدل التكاثر (E) وعلاقته بتركيز المادة المضافة المنشطة للتفاعل (P) لمعادلة رياضية.