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Effect of Nitrogen Sources in Batch and Continuous Cultures to Lipase Production by *Candida rugosa*

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ABSTRACT

The interaction between medium components, microorganisms and the operational mode to produce lipase by *Candida rugosa* was studied. It has been observed as an emulsion formation between oleic acid and urea during continuous culture. Because of this emulsion, the nitrogen source cannot be assimilated by the cells. The lack of nitrogen source provoked a decrease in protein synthesis, being lipase synthesis suppressed. The change of the nitrogen source from urea to ammonium sulfate avoided the emulsion formation, allowing to reach biomass and lipase steady states in continuous cultures. In batch cultures, no effect of nitrogen source on lipase production and biomass was observed. The specific productivity in continuous culture was slightly higher than in batch cultures.

Index Entries: Lipase production; *Candida rugosa*; oleic acid; batch and continuous fermentation; nitrogen sources; urea; ammonium sulfate.

INTRODUCTION

Lipases (glycerol ester hydrolases EC 3.1.1.3.) comprise a group of enzymes which catalyze the hydrolysis of triacylglycerols to give free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. This hydrolysis

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reaction is reversible and, at reduced water levels often in the presence of organic solvents, lipases catalyze the reversible reaction of synthesis (*1*).

The most thoroughly studied lipase was pancreatic, but more attention is currently being paid to lipases produced by bacteria, yeast, and fungi (*2,3*). Commercial lipases are produced by a limited number of microorganisms and, among these, *Candida rugosa* is a well-known lipase producer. Its extracellular lipase has been reported to be nonspecific with respect to the glycerol position (*1*).

Although the economic importance of lipases in the enzyme industry has been lower than proteases, the use of lipases in the formulation of the detergents, in the oils and fats industry, and in the synthesis of novel compounds, will lead to their greater use in new areas, and an increase of the industrial applications (*4*).

Lipase production by *C. rugosa* in fermentation processes is commonly characterized by a mixed carbon source, usually a sugar (sucrose, glucose) and a lipid or related insoluble substances (vegetable oils, fatty acids, and so on) to grow the microorganism and to induce the production of the enzyme (*3,5,6*).

Previous studies, using a mixed carbon source (glucose and olive oil), have shown that glucose has an inhibitory effect on lipase production by *C. rugosa* (*7*). When olive oil is used as the sole carbon source, the microorganism uses sequentially the glycerol and fatty acids components of the olive oil. First, the olive oil is hydrolyzed by a low level of lipase coming from the inoculum. Second, the microorganism consumes the glycerol without lipase production. Finally, the free fatty acids are consumed simultaneously with the formation of a significant amount of lipases (*8*). After testing several free fatty acids, oleic acid was proposed as the best substrate to grow the microorganism and induce lipase production (*9*). It has been reported recently that the presence of oleic acid induces lipase production in *Geortrichum candidum*. It is suggested that long-chain fatty acids participate in the expression of the *G. candidum* lipase genes (*10*).

It also has been observed that a shortage of oxygen in the fermentation broth restricts lipase production by *C. rugosa* (*11*), in accordance with the results obtained by Tsujisaka with *G. candidum* (*12*).

The common nitrogen sources used for microbial growth are corn steep liquor, soy flour, peptone, urea, nitrate, and ammonium salts. Different effects in lipase production have been reported depending on the nitrogen source and the microorganism (*3*). *Yarrowia lipolytica* showed a significantly higher lipase activity when organic N-sources such as peptone, urea, and soy flour were used (*5*). In *Aspergillus niger*, ammonium nitrate supported the best growth as well as lipase production (*13*). In *Acremonium strictum*, organic nitrogen sources, with the exception of urea, gave higher levels of growth and lipase production than the inorganic ones (*14*). In *G. candidum*, maximum growth and lipase synthesis was achieved with peptone, while ammonium nitrate and sulphate provided

minimum lipase yield (*15*). The selection of the most suitable nitrogen source for lipase production depends on the microorganism used.

The complexity of the medium, a four-phase system, provokes additional operational problems. Different approaches in the medium composition were assayed to achieve steady states in continuous cultures. From the microscopical observation, elemental chemical formulae, and the evolution of biomass, intracellular, and extracellular lipase, a problem with urea used as nitrogen source was identified. Lipase and biomass steady states in continuous cultures were reached changing the nitrogen source to ammonium sulfate.

MATERIALS AND METHODS

Microorganism and Medium

Candida rugosa (ATCC 14830) was maintained on peptone malt extract agar plates at 4°C. The basal mineral solution, prepared using tap water, contained: KH_2PO_4, 6 g/L, $MgSO_4 \cdot 7H_2O$, 1 g/L, micronutrients: $FeCl_3 \cdot 6H_2O$, 10 mg/L, inositol, 0.004 mg/L, biotin, 0.008 mg/L, thiamine·HCl, 0.2 mg/L. The concentrations of oleic acid and nitrogen source are specified in each experiment. When ammonium sulfate was used as nitrogen source, a buffered medium was prepared using KH_2PO_4 15 g/L and Na_2HPO_4 5.5 g/L (pH 6.3).

Inocula were grown in 1-L flasks containing 250 mL of the same medium as the initial fermentation one. The loop inoculated flasks were incubated at 30°C for 24 h mixed by a magnetic stirrer.

Fermentation Conditions

Fermentation experiments were carried out in a 6-L Braun fermenter Biostat E (5 L of working volume) coupled with on-line analysis equipments (*16*). The medium was steam-sterilized in the fermenter, except the vitamins, which were microfiltered, and the nitrogen source, which was sterilized separately and added before inoculation. Standard operation conditions were: stirring rate 500 rpm (3 disc turbines, 7 cm diameter, with 6 flat blades), temperature 30°C, and airflow rate between 0 and 5 L/min to ensure a dissolved oxygen level not lower than 20% of air saturation. When ammonium sulfate was used as the nitrogen source, pH was controlled at 6.3 by adding 2*M* NH_4OH. When urea was the nitrogen source, the pH throughout fermentation was practically constant (6.3 ± 0.1) without pH control owing to the urea buffering effect and to the low solubility of oleic acid.

The difficulty in obtaining a good mixing of oleic acid with the other components of the feeding medium, in continuous culture, was solved by feeding the oleic acid separately by means of an automatic microburet

(Crison 2031) commanded by a computer through a RS232C link. The addition rate of oleic acid was fixed according to the characteristics of the fermentation.

Biomass

Samples were filtered (0.45 μm), washed with a mixture of dioxane-propionic acid (1:1), and then washed with distilled water. Finally, the filters were dried at 85°C to constant weight.

Elemental Composition

In order to determine biomass composition, cells were withdrawn from the reactor at different times during continuous fermentation growing on oleic acid. The sample was centrifuged and washed twice with 10 m*M* Tris-HCl buffer (pH 8.0). The cells were dried at 85°C to constant weight and analyzed for composition of C, H, N, and O in an elemental analyzer 240B (Perkin Elmer). It has been considered the other possible components negligible related to C molar basis.

Off-line Turbidimetric Extracellular Lipase Analysis

This analysis was based on modified Monotest Lipase (Boehringer Kit 159697). Reagent solution (1.5 mL) was mixed with sample (0.5 mL) in a thermostatically controlled cuvet (45°C) and the decrease in absorbance at 340 nm (UV) was followed for 6 min with a Perkin Elmer Lambda-5 spectrophotometer. The absorbance decrease per second was calculated from the slope of the curve. The first 2 min of analysis were not used in slope determination. If the slope was higher than 0.001 Absorbance U/s, the sample had to be diluted in order to be included in the linear range of analysis. This method was correlated with the titrimetric method (*7*). One unit of extracellular lipase activity was defined as the amount of lipase necessary to hydrolyze 1 μmol of ester bond per minute under assay conditions. For the determination of extracellular lipase, samples were filtered through a 0.45-μm filter.

Off-line Intracellular Lipase Analysis

Cells were harvested by centrifugation, washed in 10 m*M* Tris-HCI buffer (pH 8.0) and suspended in four times their wet weight of the same buffer. The suspension was sonically disrupted in a BRAUN Labsonic 2000 sonicator set at an amplitude of 170–176 for periods of 60 s/0.1 mL in a tube chilled to 0°C. The disrupted cells were centrifuged at 4000*g* for 10 min at 4°C. The resulting supernatant was used as the cell extract for the determination of intracellular lipolytic activity using the turbidimetric lipase analysis described earlier. The concentration of cell extract protein was determined by the method of Lowry (*17*) with bovine serum albumin as standard. One unit of intracellular lipase activity was defined as the

amount of lipase necessary to hydrolyze 1 μmol of ester bound per minute under assay conditions. In order to represent the intracellular concentration, the units are given per milligram of intracellular protein (or dry biomass).

Protease Analysis

Protease activity was determined by the colorimetric method of Scheuning (*18*), with azocasein as target protein.

Urea Analysis

The concentration of urea was determined using a Sigma diagnostics enzyme kit (urea nitrogen, procedure 640).

Exhaust Gas Analysis

The airflow rate was controlled and measured by a mass flow-meter/controller (Brooks 5850 TR). The oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) values were obtained by measuring the concentration of O_2 and CO_2 of the exhaust gases using a quadrupolar mass spectrometer (Spectramass PC2000). These results were used to estimate the specific growth rate (μ) (*16*).

RESULTS AND DISCUSSION

Preliminary experiments showed that free fatty acids, mainly oleic acid, resulting from olive oil hydrolysis were the inducers of lipase production. Comparing cost and productivity, it seems that, from the process development point of view, oleic acid would be a better substrate than olive oil (*8*).

The low levels of the total extracellular protein (around 100 μg/mL), obtained in batch cultures, and the absence of a detectable amount of proteases in the broth facilitates a cheap protein purification operation and a high enzyme stability (*19*). Considering that the lipase production is associated with the microorganism growth, using oleic acid as carbon source, a continuous operation mode could improve the yield and productivity obtained in batch cultures.

In all the experiments, an excess of nitrogen source related to carbon was used (microorganism elemental composition). The aim was to supply enough nitrogen to protein metabolism. Thus, a lack of nitrogen could be discarded when metabolism problems were detected. It was observed that an excess of nitrogen, at least 3–4-fold the minimum, was necessary so not to affect the biomass and lipase production.

Figure 1 shows the behavior of biomass, intracellular, and extracellular lipase in the continuous culture using oleic acid 8 g/L and urea 8 g/L.

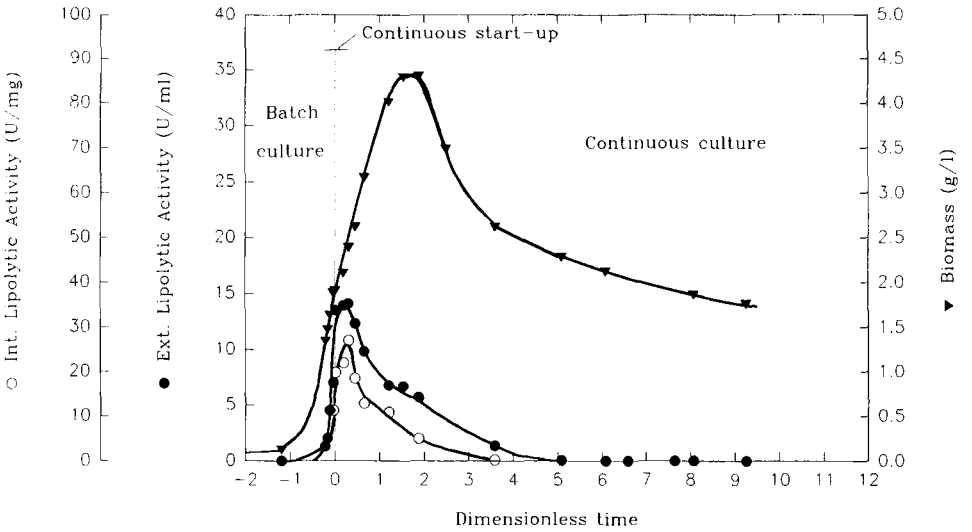

Fig. 1. Continuous fermentation by *Candida rugosa* using oleic acid (8 g/L) as a carbon source ○, intracellular lipolytic activity (U/mg of intracellular protein); ●, extracellular lipolytic activity (U/mL of culture); ▼, biomass concentration (g/L; dimensionless time (time/residence time).

Initially, a batch culture was started using the basal medium containing 2 g/L of oleic acid and 4 g/L of urea. Figure 2 displays the carbon dioxide evolution rate (CER), oxygen uptake rate (OUR) and specific growth rate (μ) evolution (*20*). During the final batch stage, the specific growth rate decreased to a value close to the planned dilution rate for the continuous operation. Then, the continuous operation was forced to start-up. This assures a good start-up, minimizing the transient phenomen involved (*21*) when changing operational mode.

A value of $\mu_{max} = 0.16\ h^{-1}$ was obtained in batch culture experiments using oleic acid (*19*). Hence, the dilution rate for the continuous culture was fixed at $D = 0.06\ h^{-1}$. It was considered a conservative value to conduct a continuous fermention, just considering it was quite far from washout dilution rate.

The level of biomass reached at two residence times was close to the expected one calculated from yield coefficient obtained on batch cultures. Thereafter, the biomass concentration started to decrease, showing that the microorganism grew with a specific growth rate below the dilution rate. This was supported with the information supplied by the mass spectrometer (μ).

The behavior of intracellular and extracellular lipase was similar. The maximum value of both lipolytic activities was reached during the transient phase. After two residence times, the lipolytic activities were reduced to undetectable levels. The decrease rate for the intracellular and extracellular lipase concentrations is according to a situation of no synthesis of lipase based on mass balance. Moreover, the lipolytic activity reduction

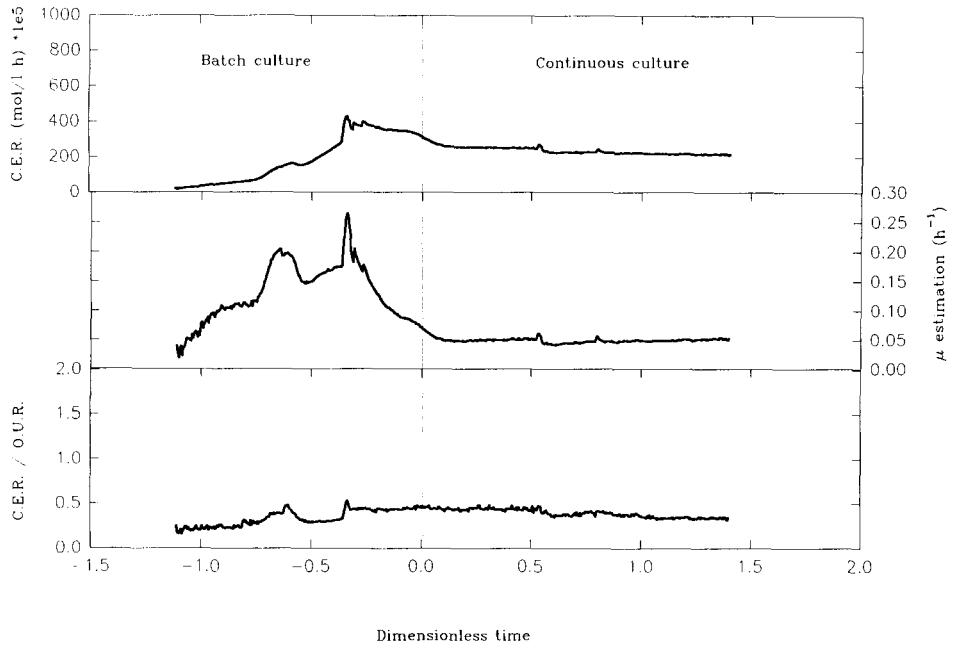

Fig. 2. CER, OUR, and μ monitoring during a batch fermentation followed by a continuous fermentation. Dimensionless time (time/residence time).

owing to excretion limitation could be rejected, as no intracellular lipase accumulation is detected.

Phase contrast microscopical observation showed that the cell morphology changed along the fermentation. During batch and at the beginning of continuous operation, the microorganism grew in aggregates trapping droplets of oleic acid. After three or four residence times, the pattern of growth was changed. It could be observed that there were more cells growing isolated than in aggregates. Around nine or ten residence times, cells appeared smaller and all of them were isolated with intracellular lipidic accumulations.

In order to evaluate if other medium components, different to the carbon source, were growth-rate limiting, new continuous cultures were carried out. In the first one, the medium was supplemented with 0.2 g/L of Calcium panthotenate, a vitamin necessary for the assimilation of fatty acids by yeasts. It was not observed any change from the general behavior showed in Fig. 1. The same was observed when the continuous medium was supplemented with 0.5 g/L of yeast extract.

When a certain level of commercial lipase (AY) Amano 30, 5 U/mL in the fermenter) was added to the feed stream, the biomass achieved a steady state without lipase production. It has been noticed that the uptake of oleic acid by C. *rugosa* is favored by the presence of extracellular lipases (22). This fact was also observed in *Pseudomonas fluorescens* (23). After six residence times, microscopic observation as well as direct visual observation of the broth in the bioreactor showed that an emulsion was

formed. When the culture broth, free of biomass, was collected at the end of the continuous experiment, it was observed a stable emulsion which remained after one month at 20°C without agitation. This suggests that the emulsion may be responsible for the wash-out phenomen observed in continuous experiments.

This set of experiments showed that the enzyme synthesis suppression is not owing to the lack of any component in the fermentation medium.

Additional experiments mixing different components of the mineral medium, without microorganisms, were made. These experiments demonstrated that urea (8 g/L) and oleic acid (8 g/L) with an agitation rate of 500 rpm make a stable emulsion after 20 h. With lower concentrations of urea and oleic acid, both at 2 g/L, and the same agitation rate, the emulsion was also formed after 72 h. This urea/oleic acid emulsion makes difficult the accessibility of urea to the microorganism. Analysis of urea in the medium showed slight differences between inlet and outlet urea concentrations, which confirms the low consumption of the nitrogen source, and hence that the microorganism grows in the absence of an effective nitrogen source.

To avoid the emulsion formation, a continuous fermentation with lower concentrations of oleic acid (2 g/L) and urea (2 g/L) was performed. In this experiment, the operation conditions were the same as the continuous with 8 g/L of oleic acid.

The behavior of intracellular and extracellular lipase, shown in Fig. 3, was similar. Both activities increased at the beginning of continuous operation, kept quite constant during two residence times, and decreased to undetectable levels. The biomass was constant until lipase became undetectable.

With reference to microscopical observation, the cells showed the same aspect as the previous continuous culture. In this experiment we also observed an emulsion, after approx 3 residence times. However, it was weaker than obtained in continuous mode with 8 g/L of oleic acid and 8 g/L of urea.

An elemental chemical formulae of cell biomass (Table 1) was determined at different times during a continuous fermentation of 8 g/L oleic acid. The microorganism formulae remains constant throughout the batch phase and the initial period of the continuous phase. In parallel with the decrease in the biomass concentration, the percentage of nitrogen in the microorganism composition decreased to one-third of its initial value.

From the urea analysis, the nitrogen source in the medium was in excess according to the microorganism composition observed in batch experiments; therefore, a lack of total nitrogen source could be discarded. It has been described that oleaginous yeasts accumulate lipids when there is a deficiency of a particular nutrient, normally nitrogen, and an excess of carbon sources. Thus, when the limiting nutrient is exhausted, cell

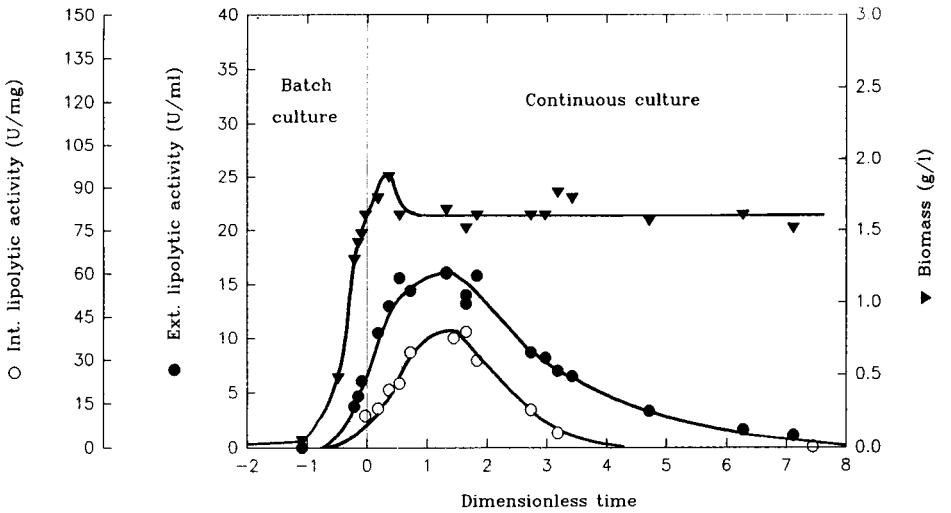

Fig. 3. Continuous fermentation by *Candida rugosa* using oleic acid (2 g/L) as carbon source and urea (2 g/L) as nitrogen source ○, intracellular lipolytic activity (U/mg of intracellular protein); ●, extracellular lipoytic activity (U/mL of culture; ▼ biomass concentration (g/L). Dimensionless time (time/residence time).

Table 1
Microorganism Elemental Composition During a Continuous Fermentation Using Urea as Nitrogen Source

Dimensionless time (time/residence time)	Microorganism elemental composition
0.0	$CH_{1.9}\ O_{0.62}\ N_{0.14}$
1.5	$CH_{1.92}\ O_{0.69}\ N_{0.13}$
3.5	$CH_{1.95}\ O_{0.58}\ N_{0.08}$
6.0	$CH_{1.94}\ O_{0.51}\ N_{0.06}$
9.0	$CH_{1.96}\ O_{0.45}\ N_{0.05}$

proliferation ceases. The excess of carbon source continues to be assimilated by the cell and channelled into lipids (24). This could explain the lipidic accumulations observed when the nitrogen percentage decreased in the microorganism.

It is therefore assumed that there is an additional problem such as accessibility or nitrogen transport into the cell. The lack of available nitrogen provokes a decrease in protein synthesis, being lipase synthesis suppressed. In order to avoid the emulsion formation, the nitrogen source was switched to an inorganic one such as ammonium sulfate.

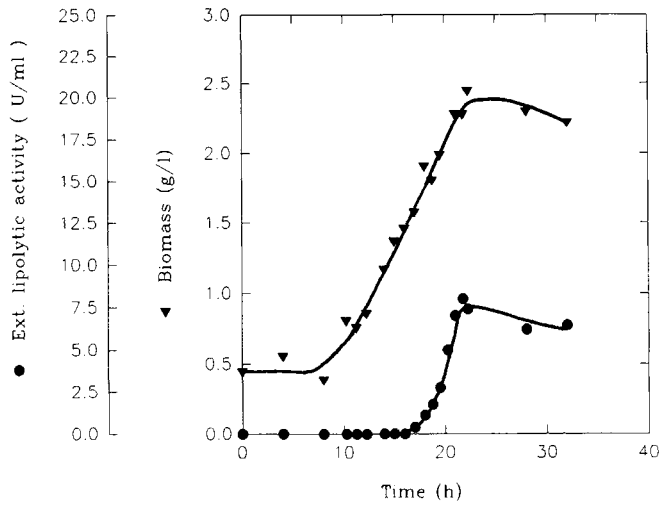

Fig. 4. Growth and lipolytic activity using oleic acid 2 g/L and ammonium sulfate 4 g/L as nitrogen source in batch culture. ●, extracellular lipolytic activity (U/mL of culture) and ▼ biomass concentration (g/L).

Experiments with Ammonium Sulfate as a Sole Nitrogen Source

Batch Experiments

First of all, the mineral medium containing oleic acid and ammonium sulfate both a 8 g/L, without microorganism, was incubated at 30°C with an agitation rate of 500 rpm during 4 d. No emulsion was observed.

In order to determine whether the production of lipase was affected by the change of the nitrogen source, batch fermentations at different concentrations of oleic acid and ammonium sulfate were performed.

Figure 4 shows *C. rugosa* batch growth using oleic acid at 2 g/L and 4 g/L of ammonium sulfate. The general behavior was the same as when urea was employed as nitogen source. The biomass yield was slightly higher ($Y_{x/s}$ = 0.85) than with urea, and similar lipase production was obtained.

Continuous Fermentations

A continuous fermentation using ammonium sulfate as nitrogen source was performed, where the operation conditions were the same as for the previous continuous cultures with urea, except that the early phase was a batch culture containing 2 g/L of oleic acid and 4 g/L of ammonium sulfate. The basal medium plus ammonium sulfate (4 g/L) was added at a dilution rate of D = 0.06 h^{-1}. Oleic acid was added separately to achieve a concentration of 2 g/L in the inlet stream.

As can be seen in Fig. 5, intracellular and extracellular lipase increased at the beginning of the continuous operation, reaching a maximum between one and two residence times. After three residence times, the activity

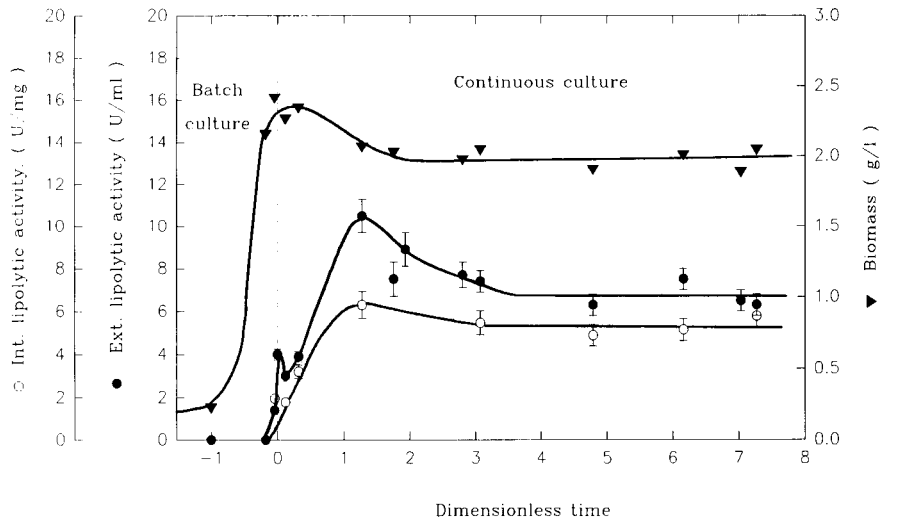

Fig. 5. Continuous fermentation by *Candida rugosa* using oleic acid (2 g/L) as carbon source and ammonium sulfate (4 g/L) as nitrogen source. ○ intracellular lipolytic activity with its standard deviation (U/mg of dry biomass); ●, extracellular lipolytic activity with its standard deviation (U/mL of culture); ▼, biomass concentration (g/L). Dimensionless time (time/residence time).

decreased to the level obtained in the batch culture and during four residence times, steady states were maintained. After one residence time the biomass reached the expected value and remained constant until the end of the experiment.

With reference to the percentage of nitrogen in the chemical formulae of *C. rugosa*, we should point out that there was no change. Microscopical observation by phase contrast showed that the microorganism grew in aggregates throughout the experiment and no intracellular lipidic accumulations were observed. Moreover, no emulsion was observed.

These results confirm that the inaccessibility of nitrogen to the microorganism was owing to the emulsion formed. It led to the decrease in lipase production, hence the wash-out in the continuous cultures was also a consequence of the emulsion. Thus, by changing the nitrogen source, we have solved the problems of working in continuous operation. Although we are unable to explain the biochemical and genetic aspects of the regulation of lipase synthesis, we suspect that the increase in extracellular lipase at the beginning of the continuous operation is a transient phenomenon that cannot be explained up to now.

CONCLUSIONS

This four-phase system caused negative interactions between medium components, microorganism, and operational mode, and also they made on-line measurements difficult.

In batch experiments, oleic acid was a better inducer than olive oil. It suggests that the lipase might be playing a different role to its catalytic function. Presumably, in the utilization of this insoluble substrate, lipase facilitates association of the cells with the insoluble substrate and permits rapid growth on fatty acids.

When urea was used as nitrogen source, continuous culture did not reach a steady state in lipase production and biomass. The behavior of the intracellular and extracellular lipase activity suggests that the wash-out phenomenon observed is owing to protein synthesis suppression and not to difficulties in excretion of the enzyme.

Additional experiments confirmed that the wash-out phenomenon was originated by an emulsion formed between oleic acid and urea. Analysis of urea in the medium fermentation showed that the nitrogen source was practically not assimilated by the microorganism, and so it was the cause of the morphological and elemental chemical formulae changes in the microorganism. These facts may explain the protein synthesis suppression and the different growth of the microorganism observed in continuous culture. Although the emulsion effect was retarded at lower oleic acid concentration, it was not eliminated.

The strategy used to avoid the emulsion effect was to change the nitrogen source. Ammonium sulfate and oleic acid did not make an emulsion. In batch experiments, similar behavior related to biomass and lipase production was observed with urea and ammonium sulfate as nitrogen source. Continuous culture with ammonium sulfate reached steady states of biomass and lipase without changes in microorganism morphology. The specific lipase productivity was (210 U/g h), slightly higher than in batch culture (180 U/g h).

The selection of culture medium and an adequate operation mode enabled us to increase 12-fold the original lipase productivity (*11,25*). The optimization of lipase production by *C. rugosa* would imply the control of oleic acid addition rate and the oleic acid concentration in the reactor. The most suitable operation strategy could therefore be a continuous culture.

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