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Strategies in Lipase Production by Immobilized *Candida rugosa* Cells

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ABSTRACT

Growing cells of *Candida rugosa* immobilized in polymethacrylamide-hydrazide and polyurethane foam were employed in fluidized and packed bed reactors, for discontinuous and continuous fermentations to obtain extracellular lipase. In spite of hydrodynamic problems, fermentation cultures using polyurethane foam showed higher lipolytic activity than cultures employing polymethacrylamide-hydrazide beads, which was probably owing to the high immobilized biomass concentration in polyurethane observed by direct microscopy enumeration. Different oleic acid concentrations were assayed. The maximum level of lipase was achieved at 4 g/L of oleic acid. These results reaffirm that lipase production is a direct function of cell-substrate contact and that the organic substrate dispersion is important in this system.

Index Entries: Lipase production; *Candida rugosa*; immobilized cells; fluidized reactor; packed bed reactor; polymethacrylamide-hydrazide; polyurethane.

INTRODUCTION

Lipases (EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of triacylglycerols and they are of increasing importance in industrial processes (*1*). The use of immobilized cells in fermentation provides known advantages: simplification of the reaction processes, reduction of the

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pollution problems, and allowance for continuous operation, working at dilution rates higher than with free cell cultures. Immobilization of whole cells is a technique that can be used in several processes in order to increase the reactor productivity. Two successful approaches to cell immobilization are entrapment within natural or synthetic polymers and immobilization by adsorption onto solid surfaces and within porous supports. One method, using a synthetic polymer (polymethacrylamide) to entrap the cells and a porous support (polyurethane) to adsorb the cells, has been selected.

Studies of extracellular lipase production by immobilized *Candida rugosa* cells previously have been reported (2). Using Ca-alginate and a mixed matrix of polyurethane foam/Ca-alginate beads as an immobilization support, it has been concluded that cell-substrate contact is essential for substrate uptake by microorganisms and lipase production, with these supports substrate dispersion and contact with cells are limited.

In order to improve this contact, we have studied different supports for cell immobilization, types of bioreactors, operating strategies, and carbon source substrates. Two immobilization techniques were considered; adsorption to polyurethane foam and entrapment in polymethacrylamide-hydrazide gels. Two kinds of bioreactors were used: a packed bed and a fluidized bed reactor. Both bioreactors were operated in batch and continuous culture.

MATERIALS AND METHODS

Microorganism and Medium

Candida rugosa (ATCC 14830) was maintained on malt extract agar plates at 4°C. The basal mineral solution contained: 15 g/L KH_2PO_4 (Merck), 5.5 g/L K_2HPO_4 (Merck), 1 g/L $MgSO_4 \cdot 7H_2O$ (Merck), 4 g/L $(NH_4)_2SO_4$ (Merck), micronutrients: 10 mg/L $FeCl_3 \cdot 6H_2O$ (Merck), 0.004 mg/L inositol (Fluka), 0.008 mg/L biotin (Fluka), and 0.2 mg/L thiamine·HCl (Fluka). The carbon sources used were oleic acid (Merck), caprylic acid (Merck), and glucose (Merck). The concentrations are specified in each experiment.

Chemicals

Polyurethane foam was supplied by Bayer (Spain). This polyurethane is a 50:50 mixture of two components: Desmodur 44 V 20 and polyol 15/14 B/1-35. It has a density of 0.7 g/L. It has a very hydrophobic nature. Copolymer acrylamide-methacrylamide-hydrazide was prepared following the procedure described by Dror et al. (3). A hydrophobic analog was obtained by chemical modification of polyacrylamide, using a copolymerization with 30% of methacrylamide. We are interested in a hydrophobic support to increase the diffusion of organic substrate (oleic acid).

Biomass Determination

Free cell mass was counted by direct microscopy enumeration. To determine the immobilized cells, a method measuring protein content was used. Protein was determined by the method of Freeman et al. (*4,5*), according to the procedure of Bradford (*6*).

Immobilization

C. rugosa cells were immobilized in polyurethane cubes of 8 mm^3 by recycling. A fermentation broth containing cells was circulated through the fermenter at a constant rate using a peristaltic pump.

Immobilization in polymethacrylamide gel was carried out by suspending cells in a 5 or 7% (w/v) distilled water solution of prepolymer. Alginate 0.75% was added (*7*). The mixture was pumped through a hypodermic needle into a solution of 2% $CaCl_2 \cdot 2H_2O$ and glyoxal (1 g of glyoxal per 3 g of polymer). Calcium ions promote the instantaneous gelification of alginate, whereas polyacrylamide crosslinking by glyoxal takes 1 h. After this time, the beads were transferred to a phosphate buffer for 30 min and were stirred in order to remove the alginate. Then polymethacrylamide beads of 2–2.5 mm in diameter were obtained.

After immobilization, the beads were incubated in a fresh medium with glucose as a carbon source for 2 d to increase the cell concentration.

Lipase Activity in Aqueous Phase Assay: Off-Line Turbidimetric Analysis

This analysis was based on a modified Monotest Lipase (Boehringer kit 159697). 0.75 mL of reagent solution was mixed with 0.25 mL of the sample in a thermostatized cuvet at 45°C. The decrease in absorbance at 340 nm was followed for 6 min with a Varian Cary 3E Spectrophotometer. The absorbance decrease per second was calculated from the slope of the curve within the last 4 min. This method was correlated with the titrimetric lipase method (*8*). All samples were filtered through a 0.45-μm filter. Activity was expressed as U/mL of broth. One unit of lipase activity was defined as the amount of lipase necessary to hydrolyze 1 μmol of ester bond per minute under the assay conditions.

Experimental Procedure

A columnar glass reactor was used for the experiments. It was 450 mL in volume and water jacketed at 30°C. An airflow was introduced in fine bubbles from the bottom of the reactor (Fig. 1). The airflow rate used, 0.37 vvm, ensured a dissolved oxygen level not lower than 20% of saturation in fermentation broth. A shortage of oxygen in fermentation broth restricts lipase production by *C. rugosa* (*8*), this has also been noticed in *Geotrichum candidum* (*9*) and *Pseudomonas fluorescens* (*10*). The dissolved

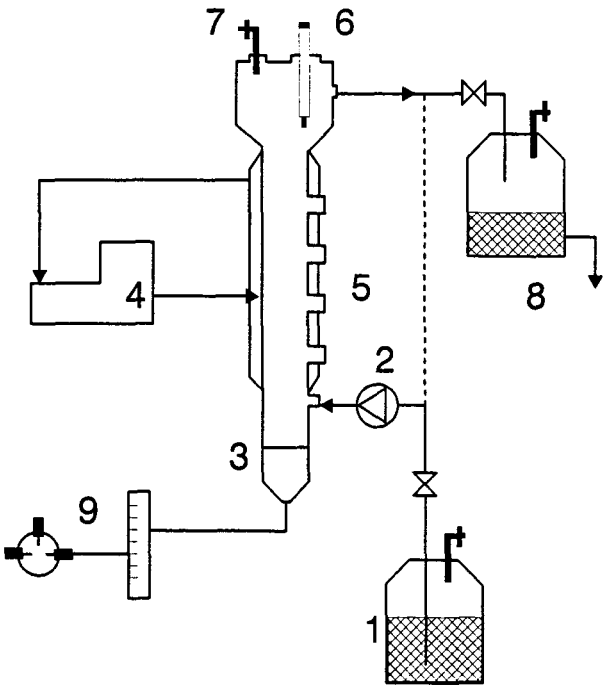

Fig. 1. Experimental set-up of the columnar glass reactor. 1, feed tank; 2, peristaltic pump; 3, air-diffuser; 4, thermostated water; 5, sample ports; 6, pO_2 electrode; 7, sterile filter; 8, effluent tank; 9, aeration units; ------, recirculation loop.

oxygen was continuously measured with a sterilizable oxygen electrode (Instruments LTD Uniprobe) connected to a Braun Biolab oxygen meter. The drops of dispersed oleic acid did not disturb the measurement. Along the fermentation, the pH value was maintained quite constant around 6.0 owing to the buffer effect of the medium without pH control. Two kinds of support were used: cube-shaped pieces of polyurethane foam for the packed bed reactor and spherical beads of polymethacrylamide-hydrazide for the fluidized bed reactor. Batch and continuous operational strategies had been tested. For batch culture experiments the tubular reactor was equipped with a closed recirculating loop. The total working volume was 750 mL. The broth was recirculated by an Eyela MP3 peristaltic pump. The recirculation rate was 145 $mL \cdot h^{-1}$.

For continuous experiments, the fresh medium was continuously pumped from a feeding system equipped with a magnetic stirrer in order to mix the organic substrate. The dilution rate for continuous experiments was 0.3 h^{-1}.

RESULTS AND DISCUSSION

Fermentation experiments were carried out using two different immobilization techniques. The first support studied was a polyurethane

foam of a very hydrophobic nature. Examination of cut slices of polyurethane after a batch fermentation (Fig. 2A,B) and after a day of incubation (Fig. 2C) revealed that it is a very good support for cell growth. The different cell morphology observed are in accordance with the growth phase of the microorganism. Stationary growth phase (Fig. 2A,B) and exponential growth phase (Fig. 2C). Batch and continuous experiments were conducted at different concentrations of oleic acid in a packed bed reactor, filled with the polyurethane particles. The first substrate used was oleic acid because it is a well-known lipase inducer in *C. rugosa* (*11*).

As can be seen in Fig. 3, appreciable differences were observed in batch culture after 20 h of fermentation. The lipolytic activity detected in the medium increased according to the amount of oleic acid fed. Concentrations of oleic acid higher than 4 g/L have not been tested because the hydrodynamic properties of these reactors cannot offer a good substrate dispersion. When the microorganisms grew on 1 g/L of oleic acid, low lipase production (0.8 U/mL) was detected. When the carbon source concentration was increased to 4 g/L, lipase production rose (1.9 U/mL). The free cell mass in each experiment, when the maximum level of lipase was achieved, was similar ($2 \cdot 10^7$ cells/mL).

These results confirm that lipase production is a direct function of substrate-cell contact, as could be observed by microscopical observation. In order to increase this contact, we decided to feed the substrate continuously with an oleic acid concentration of 1 g/L. The early phase of the continuous culture was a batch culture containing 2 g/L of oleic acid. Lipase concentration was higher (1.6 U/mL) than in batch culture using the same initial oleic acid concentration (Fig. 4).

In order to improve the reactor mass transfer characteristics, the fluidized bed offers better mixing and mass transfer coefficients, but the physical properties of the polyurethane used do not allow its fluidization.

The following step consisted of a study using another method of immobilization, thus permitting work in fluidized bed conditions. Batch experiments in a fluidized bed reactor with polymethacrylamide beads were carried out. Figure 5 shows lipase production in these conditions at the same concentrations of oleic acid as before. Although the lipolytic activity detected in the medium was also enhanced with the oleic acid concentration added, it was found to be lower than in the polyurethane batches. The free biomass levels were higher ($4 \cdot 10^7$ cells/mL) than in the polyurethane batch cultures. A common result of these experiments was the low yield lipase/substrate obtained. In previous fermentation studies with *C. rugosa* free cells, higher levels of lipase were achieved (*8,11*).

In order to improve the lipase yield, we tried a new strategy: testing the effect of a mixed feed glucose (2 g/L) and oleic acid (0.5 g/L) as a carbon source, with a feed rate that allows glucose to be completely consumed, whereas the oleic acid still remaining in the reactor induced lipolytic activity. The effect of a mixed carbon source has been explored continuously with both immobilization techniques. The early phase of the continuous

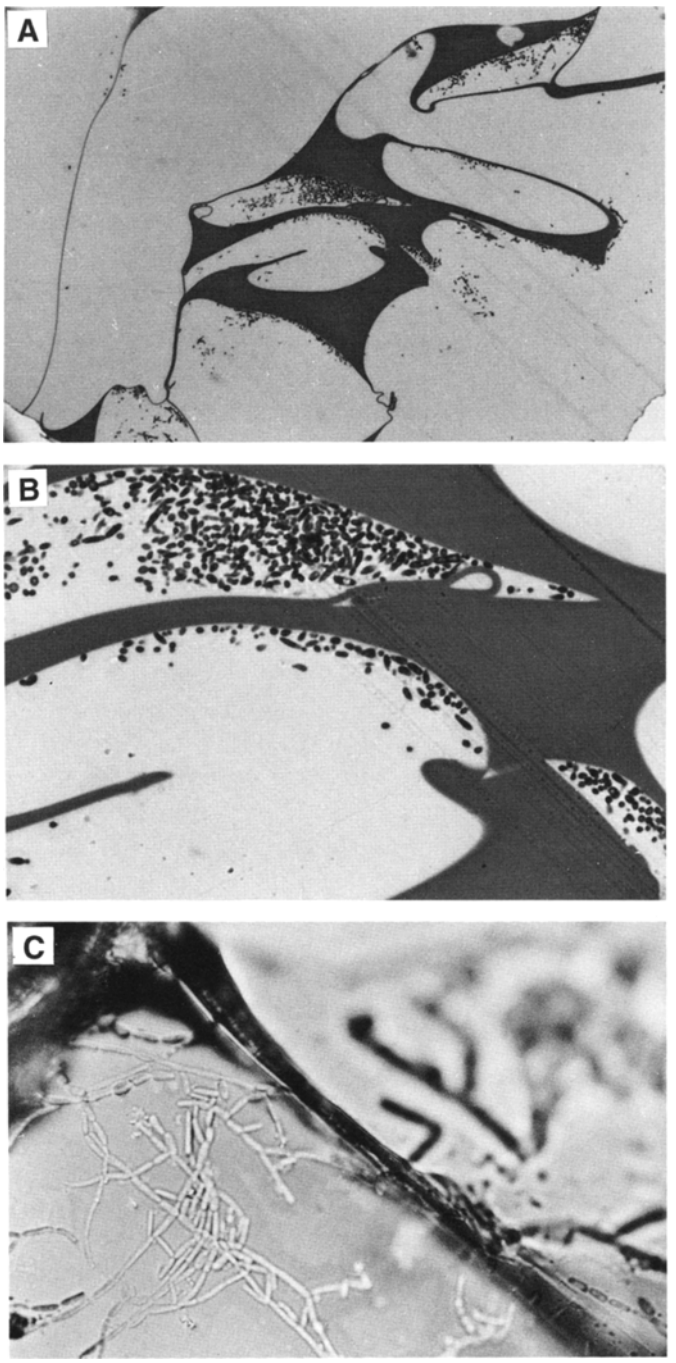

Fig. 2. Sections of polyurethane cubes showing the growth of the yeast cells inside the foam. (**A,B**) Fixed cut of polyurethane (100x and 400x respectively) after a batch fermentation. (**C**) A fresh cut of incubated polyurethane foam (400x) after a day of incubation.

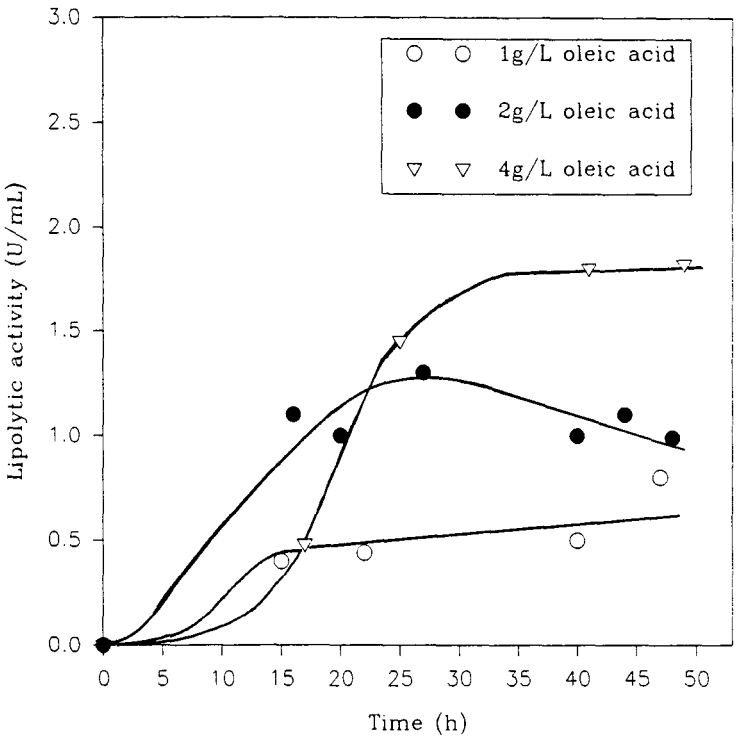

Fig. 3. Batch experiments at different initial oleic acid cencentration, using polyurethane foam.

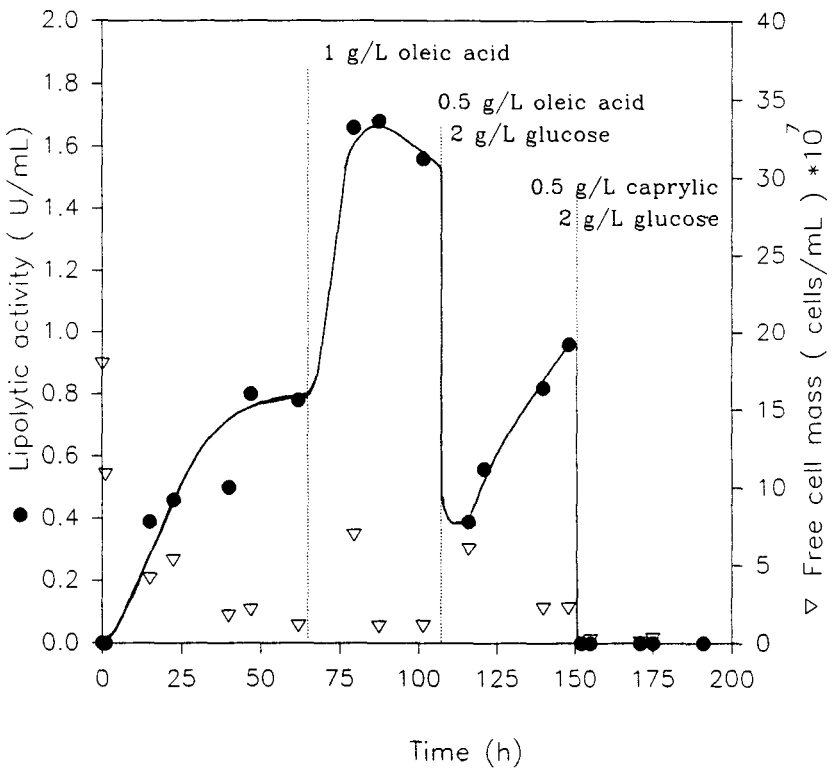

Fig. 4. Continuous fermentation cultures with polyurethane foam using different carbon sources using a dilution rate of 0.3 h^{-1}.

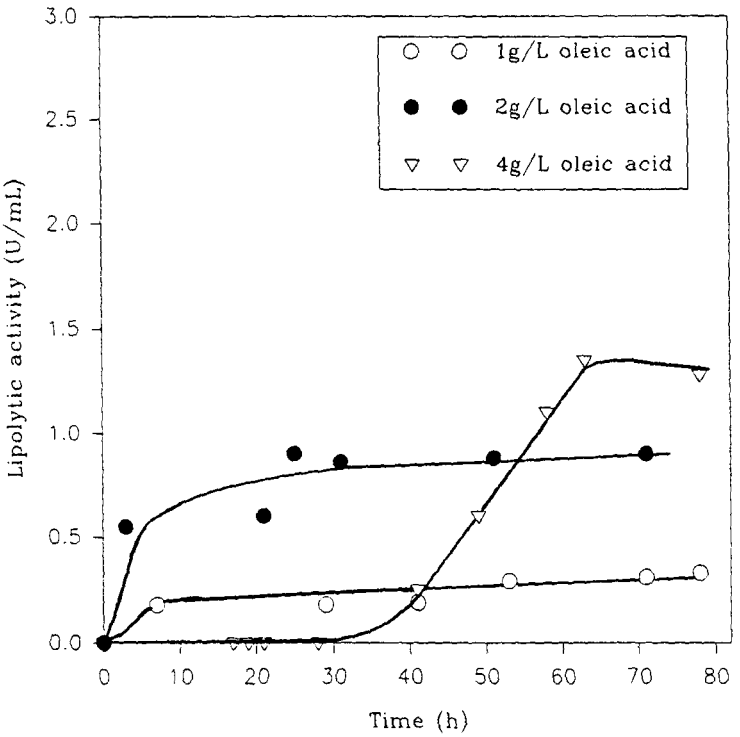

Fig. 5. Batch experiments at different initial oleic acid concentration with polymethacrylamide-hydrazide beads.

culture with polymethacrylamide-hydrazide was a batch culture containing 2 g/L of oleic acid. As shown in Fig. 6, a high lipolytic activity level has been achieved with polymethacrylamide-hydrazide. However, with polyurethane (Fig. 4) the level was lower than with 1 g/L of oleic acid.

In another series of continuous experiments, a different lipase inducer was assayed. Taking into account the observed deficient substrate-cell contact, we planned to use a soluble substrate as lipase inducer (*12*) in order to solve the substrate dispersion and to improve the contact and diffusional problems. Previous studies in our laboratory had demonstrated that caprylic acid, used as a single carbon source, was a good inducer of lipolytic activity, despite its use at low concentrations owing to its toxicity. At low concentrations, caprylic acid is water soluble. When the carbon source used in culture was caprylic acid alone, the biomass grew slowly. To achieve higher biomass concentration, caprylic acid was mixed with glucose. The results obtained (Fig. 4,6) show no lipolytic activity using polyurethane foam as support, while a negligible lipase concentration was detected with polymethacrylamide beads after 50 h of fermentation.

In continuous fermentation, the free cell mass behaves differently, depending on the support used. When the support was polymethacrylamide, the increase of lipase activity was correlated with free cell growth. When the support used was polyurethane, this fact was not observed.

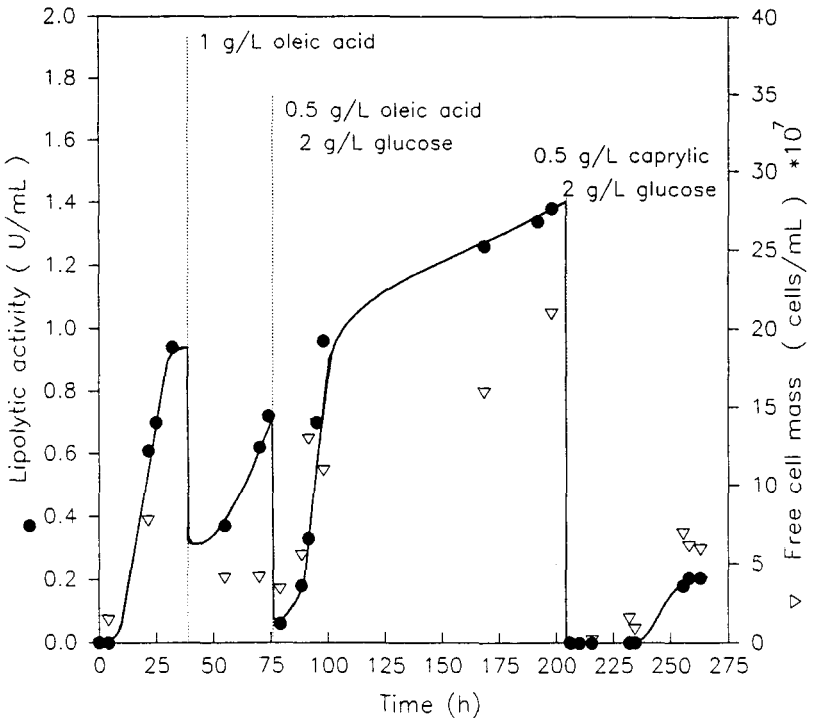

Fig. 6. Continuous fermentation cultures with polymethacrylamide-hydrazide beads using different carbon sources using a dilution rate of 0.3 h^{-1}.

CONCLUSIONS

Different strategies based on immobilized cells were employed in order to find the best scheme. The results obtained indicate the importance of support-substrate contact problems for lipase production. Working with free cells, lipase production was higher in the case of immobilized systems, probably owing to mass transfer limitations.

Although polyurethane foam is a good growth support, low lipolytic activity was obtained. However, lipolytic activity was higher than that corresponding to the use of polyacrylamide as a support. The analysis of immobilized biomass in each support showed a higher increase in immobilized cell mass in polyurethane.

In relation to the experiments conducted to find a good lipase inducer for this system, the use of caprylic acid barely induced the production of lipase. Attention should be paid to the use of a mixture of oleic acid and glucose, because it offers interesting prospects and at the same time gives high levels of biomass and lipase induction.

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