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LIPASE PRODUCTION BY Candida rugosa : FERMENTATION BEHAVIOUR

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SUMMARY.- Extracellular lipase production by Candida rugosa growth has been studied. The main growth parameters, and the lipase activity in the culture broth were determined in order to identify the maximum of enzyme activity.
The effect of lipidic material and size and growth phase of the inoculum on enzymatic production have been studied. Maximum extracellular lipase activity was associated with an increase in enzyme production when the number of viable cells started to decrease.

INTRODUCTION

Interest in lipase enzymes has been greatly developed in the last years, due to their potential application in fat splitting as well as in synthesis of glycerides. The advantages of the enzymatic hydrolysis over the chemical process are less energy requirements and higher quality of the obtained product. (Posorske,1984).
Lipase activities have been found in different molds, yeasts and bacteria (Ibrahim et al. 1987). Although numerous papers have been published on selection of lipase producers, there is less available information on the fermentation process. (Suzuki et al.,1988; Iwai et al.,1975). This kind of information is important in order to identify optimal operation conditions for enzyme production.
Candida rugosa produces extracellular lipases in the presence of lipid material, specially when a fat and a steroid are present simultaneously (Ota et al., 1982). The behaviour of the main growth parameters, and the lipase activity changes in the culture broth are studied in order to identify the maximum of enzyme activity.

MATERIALS AND METHODS

Microorganism and medium. Candida rugosa (ATCC 14830), was maintained on malt extract, peptone and agar plates. The defined growth medium had the following composition (Ota et al., 1968): glucose 20 g/L, KH_2PO_4 6 g/L, urea 1g/L, $MgSO_4.7H2O$ 1 g/L;micronutrients: $FeCl_3.6H_2O$ 10 mg/L, inositol 0.004 mg/L, biotin 0.008 mg/L, thiamine.HCl 0.2 mg/L. Olive oil (10g/L), and cholesterol (1g/L), were fed as inducers.

Growth conditions. The yeasts were grown in a 6L Braun fermenter BIOSTAT E. The medium was steam sterilized in the fermentor, except vitamins which were microfiltered, and olive oil which was sterilized separately and added with the inoculum. Standard operation conditions were: stirring rate 500 rpm, temperature 30° C, non-controlled pH, and an air flow rate between 0-15 L/min. to ensure a dissolved oxygen value inside the culture broth not lower than 20 % saturation.

Fermenter parameters. Samples were taken periodically, and extracellular lipase activity and other growth parameters were analyzed. Total viable cells number, expressed as CFU, was counted on synthetic medium plates. Glucose and ethanol were analyzed by HPLC, using a Bio Rad Aminex HPX 87-H column and water as eluent at 0.6 mL/min flow rate. Extracellular protein was determined by the method of Lowry (Lowry et al., 1951). Intracellular lipase activity was determined taken samples and recovering yeasts cells by centrifugation at 8000 g for 10 minutes. Lipase activity tests were done with the aqueous suspension obtained after cell disruption by ultrasonication.

Lipase activity assay. Lipase assay was performed using olive oil as substrate by a modification of reported procedures (Ota et al.,1966): 5 mL sample were mixed with 5 mL of 0.03 M calcium chloride, 10 mL of 0.2 M tris acid maleate-0.2 M sodium hydroxyde buffer (pH 8.2), and 5 mL of olive oil. After 50 minutes incubation at 37°C, two phases were separated in a boiling water bath. The amount of liberated fatty acids was titrated with 0.05 N organic KOH (ethanol-trichloroethylene 1:1). Activity was expressed as units per mL of broth. One unit of lipase activity was defined as the amount necessary to hydrolyze 1 μmole of ester bond per minute under the assay conditions.

Oil hydrolysis. Batch olive oil hydrolysis were carried out in a stirred flask submerged in a constant temperature bath (40° C), by mixing 462 g of olive oil with 537 g of culture broth (previously buffered at pH 7 with 0.5 M KH_2PO_4 and 0.5 M Na_2HPO_4). Degree of olive oil hydrolysis was measured by titrating samples after separation of organic and aqueous phases as described in the previous section and calculated as:

% hydrolysis = acidity value/saponification value.

Acidity value is defined as mg of KOH necessary to neutralize the released fatty acids by 1 mg of hydrolyzed olive oil. Saponification value is defined as the mg of KOH necessary to saponify and neutralize 1 g of sample. For olive oil this value is 189.7.

The individual concentration of each fatty acid produced by the hydrolysis was determined by gas chromatography using a Chromosorb column with nitrogen as carrier and FID detector.

RESULTS AND DISCUSSION

Fermentation behaviour

Figure 1 shows the evolution of Candida rugosa batch growth, using the defined medium. Glucose (20 g/l) was used as main carbon source, and 1% olive oil plus 0.1% of cholesterol as inducers. In this experiment 1% by volume of an inoculum at the end of the logarithmic phase (42 h old) was inoculated to the 6 L fermentor.

Lipase activity.- The lipase activity evolution shows two steps. In the first part of the fermentation (until 160 h) an almost constant value of 1 unit/ml is reached, after which an increase of activity is seen when the viable cells number starts to decrease. After a maximum of 4.0 units/ml a significant decay is observed. Extracellular protein is well correlated with lipase activity when it is constant and in the maximum, showing that it may be a good indicator of its appearance.

Substrates and biomass.- At the beginning of the growth, glucose consumption is associated with oxygen depletion from the initial saturation value to the 20% (established as a minimum permitted value). When glucose has dissapeared from the culture medium, the ethanol produced (with a maximum value of 5.8 g/l) is consumed, promoting a second slower growth of biomass, that remains almost constant until its decrease at the end of fermentation. Oil is utilized by the yeast during the late phase of growth.

pH evolution.- The evolution of pH is also shown in figure 1. There is an important decrease from the initial value of 6.1 to reach a minimum of 3.2, recovering higher values during the process evolution (after glucose and ethanol consumption) and finally returning to its initial value.

Lipase excretion.- The maximum in extracellular lipase activity may be due to: a) an overall increase in the rate of total lipase synthesis or b) cell lysis releasing intracellular enzyme activities. It is important to discriminate between these two possibilities from the production point of view, because the strategy in process design to obtain the maximum yield will be different. In case a) it will be necessary to carry out batch fermentation until appearance of maximum activity, meanwhile in case b) biomass may be collected after attaining maximum growth and the cells disrupted to liberate the desired product.

To distinguish between the two mechanisms, intracellular lipase activity was evaluated as described in Materials and Methods section. The results showed no significant intracellular activity in any of the samples. Thus, extracellular lipase production by Candida rugosa under the conditions studied, is not limited by excretion.

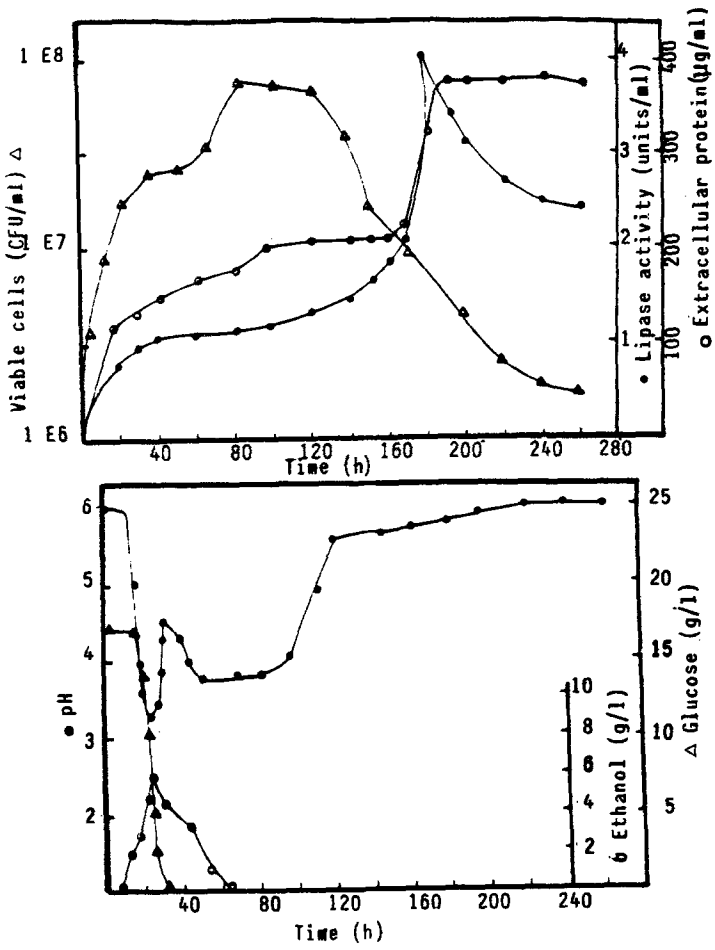

Figure 1.
Evolution of fermentation parameters.

Effect of size and growth phase of inoculum

Two additional experiments were carried out to evaluate the effect of size and growth phase of the inoculum on the moment and level of maximum lipase activity.
In a first experiment the size of inoculum was increased to 10% (v/v). The system presented the same overall evolution but the maximum lipase activity attained was lower (1.9 units/ml.) and considerably retarded (375 h.). In the second experiment, the size of inoculum was maintained at 1 % using an inoculum taken at the beginning of the logarithmic phase (24 h old). In this case the maximum of lipase activity appeared earlier (at 73 h.), but the lipase titre (3.0 units/m) was lower than when an older inoculum was employed.

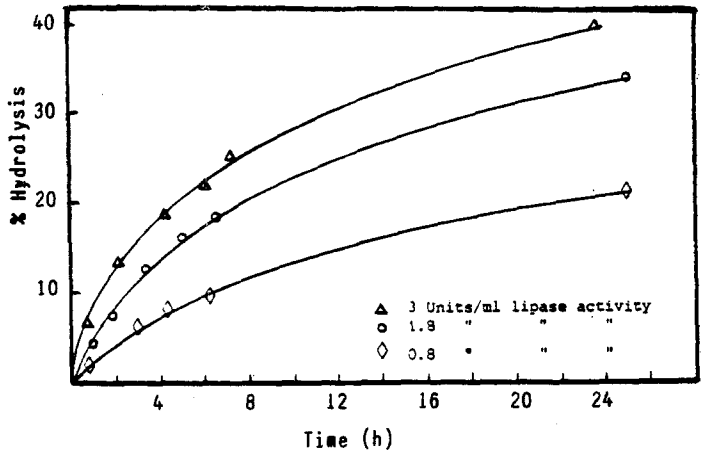

Figure 2.
Batch olive oil hydrolysis by different lipase activities.

Oil hydrolysis

The hydrolytic ability of the lipase containing culture broths was tested carrying out olive oil hydrolysis as is described in Materials and Methods. The analysis of the hydrolyzate samples showed the presence of oleic acid (79%), linoleic acid (14%), palmitic acid (7%) and stearic acid (1.5%). As can be seen in figure 2, the degree of hydrolysis obtained after 24 h was 40% for the broth with higher activity.

Influence of inducers

To test the separate effect of both inducers used (olive oil and cholesterol), a fed-batch culture was carried out. Initially, culture medium contained only 1% olive oil as inducer. The absence of cholesterol did not change the behaviour of the process (see figure 3) although the maximum lipase activity (around 2 units/ml) was lower than when cholesterol was present. When the viable cells number and the extracellular lipase activity decreased, medium containing cholesterol was added to the fermentor to obtain a 0.1% cholesterol in the culture broth. After 15 h. lipase activity increased again reaching a lipase maximum slighty lower than before, but maintaining this activity for a longer period. During this period there were no changes in pH nor oxygen consumption.

When the level of lipases droped 1.6 units/mL, medium containing olive oil was fed to restore 1% concentration in the fermentor. This addition produced a decrease in pH and the maximum lipase activity was recovered at its initial value of 2 units/mL. The presence of lipidic material has been confirmed to be necessary, although olive oil and cholesterol have different effects on the process. The presence of olive oil promoted yeasts growth and pH decrease while these effects were not observed after cholesterol addition.

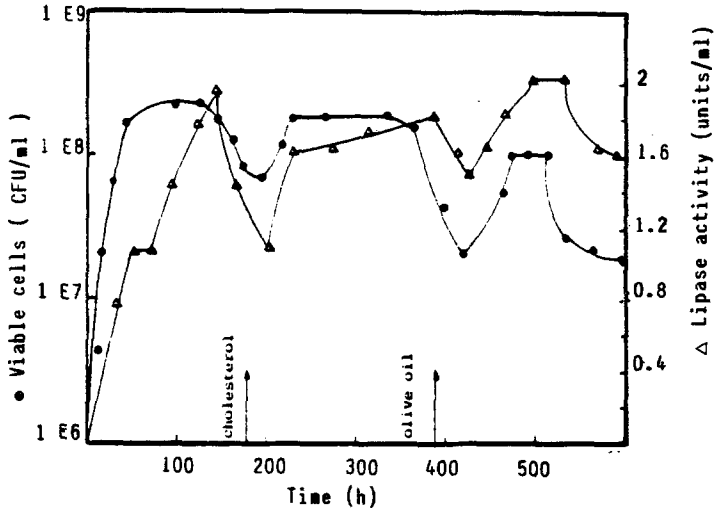

Figure 3. Activity and biomass evolution in fed-batch culture.

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REFERENCES

Ibrahim Ch. O., Hayashi M., Nagai S. (1987). Agric.Biol.Chem. 51,37-45.
Iwai M., Okumura S., Tsujisaka Y. (1975). Agric.Biol.Chem. 39,1063-1070.
Lowry O.H., RosebroughN.J., Farr A.L., Randall R.J. (1951) J.Biol.Chem. 193,265-275.
Ota Y., Miyairi S., Yamada K. (1968). Agric. Biol. Chem. 32, 1476-1478.
Ota Y., Gomi K., Kato S., Sugiura T., Minoda Y. (1982) Agric.Biol.Chem.46, 2885-2893.
Posorske L.H. (1984). JAOCS 61, 1758-1760.
Suzuki T., Mushiga Y., Yamane T., Shimizu S. (1988). Appl. Microbiol. Biotechnol. 27, 417-422.