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# Effect of Fermentation Conditions in the Enzymatic Activity and Stereoselectivity of Crude Lipase from *Candida rugosa*

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## Abstract

Different fed-batch cultures of *Candida rugosa* were carried out using oleic acid as the only carbon source. The crude lipases obtained under several operational conditions and downstream processes showed different catalytic activity and isoenzymes ratio. This fact implied that the performance of the lipase produced could be modulated by using different operational fermentation conditions. These powders were compared with commercial lipase from Sigma (St. Louis, MO) in hydrolysis and synthesis reactions. Especially interesting was the fact that the enantioselectivity of a crude lipase was higher than that observed with commercial lipase in the resolution of racemic Ketoprofen. In addition, response of both lipases in the presence of water was different.

**Index Entries:** *Candida rugosa* lipase; downstream; isoenzymes; lipolytic activity; esterification; Ketoprofen.

## Introduction

Microbial lipases (EC 3.1.1.3) have found numerous applications in the synthesis of pharmaceutical compounds, especially when synthesis involves an intermediate with a chiral center *(1,2)*. *Candida rugosa* is a

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well-known industrial lipase producer. It needs the presence of an inducer (lipid or related substance) to produce the enzyme. The production can be modulated by the nature of the inducer *(3)*. Oleic acid was one of the best inducers tested *(4)*. The operation mode strategy selected also influences the final production; fed-batch culture maintained at a constant specific growth rate was the best strategy tested, better than batch and continuous fermentations at different conditions *(5)*. Finally, an intelligent system and mathematical model of the process has been developed to obtain a reproducible and predictable product *(6,7)*.

Commercial *C. rugosa* lipase results in a mixture of different isoenzymes *(8)*. Genetic studies have identified up to seven genes coding for different lipases in *C. rugosa (9)*. This could demonstrate that the different isoforms are not owing to posttranslation modifications *(10)* but to the presence of different genes, whose expression could be modulated by culture conditions *(11)*.

Preparations from the same microorganism, but from different suppliers, batches, or even storage age, may show different enzymatic activity and selectivity. Thus, it is difficult to understand the synthetic properties of these mixtures in biotransformations *(12)*.

Cloning and expression of one of the lipase genes selected should be the most suitable approach for the production and characterization of pure *C. rugosa* lipase. Nevertheless, *C. rugosa* obeys a noncanonical codon usage *(13)*, and, consequently, the heterologous expression of the gene selected in classical host microorganisms such as *Saccharomyces cerevisiae* has resulted in an inactive lipase *(14)*. To avoid this problem, two different strategies in order to work in catalytic reactions under controlled enzyme conditions can be followed: (1) using the pure isoenzymes, after a relative complex downstream process, which could also be affected by posttranslational modifications, or (2) working with a powder enzyme produced under controlled fermentation to ensure an effective, predictable, and reproducible biocatalyst.

In this article, we show that several lipases produced in different controlled fermentation conditions present different enzymatic activities in hydrolysis reactions using triolein and tributyrin as substrates, and in the synthesis in organic media of Ketoprofen compared with *C. rugosa* lipase from Sigma.

## Materials and Methods

### *Fermentation*

#### Fermentors

Braun Biostat E (5 L) and Braun Biostat UD (50 L) Braun (Melsungen, Germany) fermentors were used.

#### Microorganism and Medium

*C. rugosa* (ATCC 14830) was maintained on peptone malt extract agar plates at 4°C. The basal mineral solution, prepared using tap water con-

tained 2 g/L oleic acid; 15 g/L $KH_2PO_4$; 5.5 g/L $K_2HPO_4$; 4 g/L $(NH_4)_2SO_4$; 1 g/L $MgSO_4 \cdot 7H_2O$; 10 mg/L $FeCl_3 \cdot 6H_2O$; 0.004 mg/L inositol; 0.008 mg/L biotin; 0.2 mg/L thiamine-HCl; and 50 µL/L Antifoam Braun Biotech DF 7960.

### Fermentation Standard Operation Conditions

Temperature was 30°C, and pH was controlled at 6.3 by adding $NH_4OH$ (2 *M*). Other conditions are specified in Table 1.

### Lipase Production

A constant volume fed batch with a constant oleic acid feeding rate was carried out. Different feeding rates are given in Table 1.

### 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 105°C to constant weight.

### Analysis of Off-Line Turbidimetric Extracellular Lipolytic Activity

Analysis was based on modified Monotest Lipase Boehringer (Mannheim, Germany) kit 1596972. Reagent was suspended with 200 m*M* Tris-HCl buffer (pH 7.4). Reagent solution (750 µL) was mixed with sample (250 µL) in a thermostatically controlled cuvet (42°C), and the decrease in absorbance at 340 nm (UV) was followed for 7 min with a UV-VIS Varian Cary 3 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 the linear range of analysis. This method was correlated with the titrimetric method *(15)*. 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.

## *Downstream and Purification*

### Obtention of a Concentrated Liquid Lipase

The culture broth was first centrifuged at 3000*g* and the supernatant microfiltered through a 0.45-µm filter to eliminate the biomass. At this point, 0.02% of sodium azide was added to prevent possible contamination. The lipase was then concentrated by ultrafiltration (10,000 Dalton cutoff was used) with a Minitan® Millipore system equipped with four plaques of polysulfone membrane (PTGC OMP 04®) and dialyzed afterward with Tris-HCl buffer (20 m*M*, pH 7.4) at the same cutoff.

### Ethanol Precipitation (UAB-1000)

Ethanol was slowly added to the sample to get the desired rate of 2 vol of ethanol/vol of water with low stirring rate and working at 0°C. Finally, when all the ethanol was completely added, the sample was maintained under low stirring for 1 h. Precipitated protein was separated by centrifugation at 3000*g*, and the pellet was dried at room temperature *(8)*.

#### Lyophilization (UAB)

Concentrated liquid lipase was frozen in the presence of lactose (10 g/L) within less than 10 min using liquid nitrogen. Afterward, the frozen product was introduced in a lyophilizer (Virtis Sentry 5L, Virtis Company Gardiner, NY) for 24 h.

#### Chromatography Characterization

A solution of 20 m*M* Tris-HCl, pH 7.4, was used throughout the purification. A fast protein liquid chromatography system (FPLC) from Pharmacia (Uppsala, Sweden), equipped with a column Memsep 1000 DEAE from Millipore (Ibénica, Spain), was used for protein purification. The crude enzyme preparation (10 mg of powder/mL) was applied to the column equilibrated with the buffer. The column was first washed with the buffer containing 0.3 *M* NaCl in order to elute proteins without lipolytic activity. Then, two major peaks with lipolytic activity were eluted at NaCl concentrations of 0.45 *M* and 1 *M*, respectively *(16)*.

#### *C. rugosa* Commercial Lipase

Powder was obtained from Sigma (type VII lipase).

### *Synthesis Reactions*

#### Esterification Reaction

The reaction mixture was composed of *R* or *S* Ketoprofen (2[3-benzoyl]-phenyl propionic acid) (66 m*M*), 1-propanol (66 m*M*), and 10 mL of isooctane used as solvent. The reaction was carried out at 30°C with magnetic stirring (500 rpm) in the presence of an initial amount of water of 200 µL/mL of solvent.

#### Analysis of Samples

Samples of 0.1 mL were withdrawn at different times and diluted with the solvent until 1.4 mL. These samples were analyzed in a Shimadzu (Kyoto, Japan) GC-14A gas chromatograph with a flame ionization detector and SPB-1 column (15 m × 0.32 mm). Injector temperature was 300°C. For (*R*,*S*)-Ketoprofen, nitrogen flux was 30 mL/min and the column temperature was 165°C.

## Results and Discussion

### *Fermentation*

Two crude lipase powders named UAB and UAB-1000 obtained in different bioreactors under different oleic acid constant feeding rate and downstream strategies were used. Table 1 presents a summary of these conditions with the extracellular lypolytic activity/biomass yield ($Y_{L.A./X}$) values at the end of the fed-batch fermentation, previous to downstream processing. The fermentations in Table 1 were performed to obtain a similar final biomass between 8 and 10 g/L.

Table 1
Fermentation, Downstream Conditions, and Values of the Extracellular Lipolytic Activity and $Y_{L.A./X}$ at the End of the Fed Batch

| Parameter | UAB | UAB-1000 |
|---|---|---|
| Fermenter | Braun Biostat E | Braun Biostat UD |
| Culture volume (L) | 5 | 50 |
| CFR[a] | 0.2 | 0.4 |
| Agitation (rpm) | 500 | 500 |
| Airflow (vvm[b]) | 0.1-1 | 0.1-1 |
| Lipolytic activity (U/mL) | 40 | 8.8 |
| $Y_{L.A./X}$ (U/mL · g biomass) | 4.7 | 0.9 |
| Downstream | Lyophilization | Ethanol precipitation |

[a]CFR, constant feeding rate (g of oleic acid/h · L of bioreactor).
[b]vvm, air volume/medium volume.

Previous studies have reported that in a constant feeding rate fed-batch strategy, the maximum lipolytic activity per unit of biomass is obtained at low substrate feeding rates and that it decreases as the feeding rate increases *(5)*. This unique effect cannot justify the extremely low yield reached at the highest feeding rate fed-batch fermentation (UAB-1000). Recently, it has been demonstrated that the size of oleic acid drops influences drastically the extracellular production of lipase *(17)*. Although stirring and airflow rate conditions were similar, the different configuration of the bioreactors made in the lipase production has proven that the size of the oleic acid drop was quite different. This fact justifies the low levels of lipolytic activity obtained under these conditions in the Braun Biostat UD.

Nevertheless, these two powders were used to demonstrate that the fermentation conditions could affect the catalytic performance of the enzymes obtained. Moreover, a comparison with one of the most common commercial lipases from *C. rugosa*, lipase Sigma type VII named CRCL, was carried out.

## *Downstream and Characterization*

The main difference in the downstream process was the protocol used to obtain the final powder. UAB-1000 was precipitated by adding ethanol with a final lipolytic activity yield of about 30%, quite different compared with data using commercial lipases *(8)*. On the other hand, lipase UAB was lyophilized in the presence of lactose with a final lipolytic activity yield of about 90%. It has been previously found that this treatment improves the ester synthesis activity *(18)*.

These lipase powders obtained under controlled selected fermentation and downstream conditions are quite different compared with commercial lipases (Sigma) from the point of view of percentage of isoenzymes. Specifically, when both fermentation powders and commercial lipases were applied to an anion-exchange chromatography, different profiles were obtained. Figure 1A–C shows profiles for UAB, UAB-1000, and Sigma

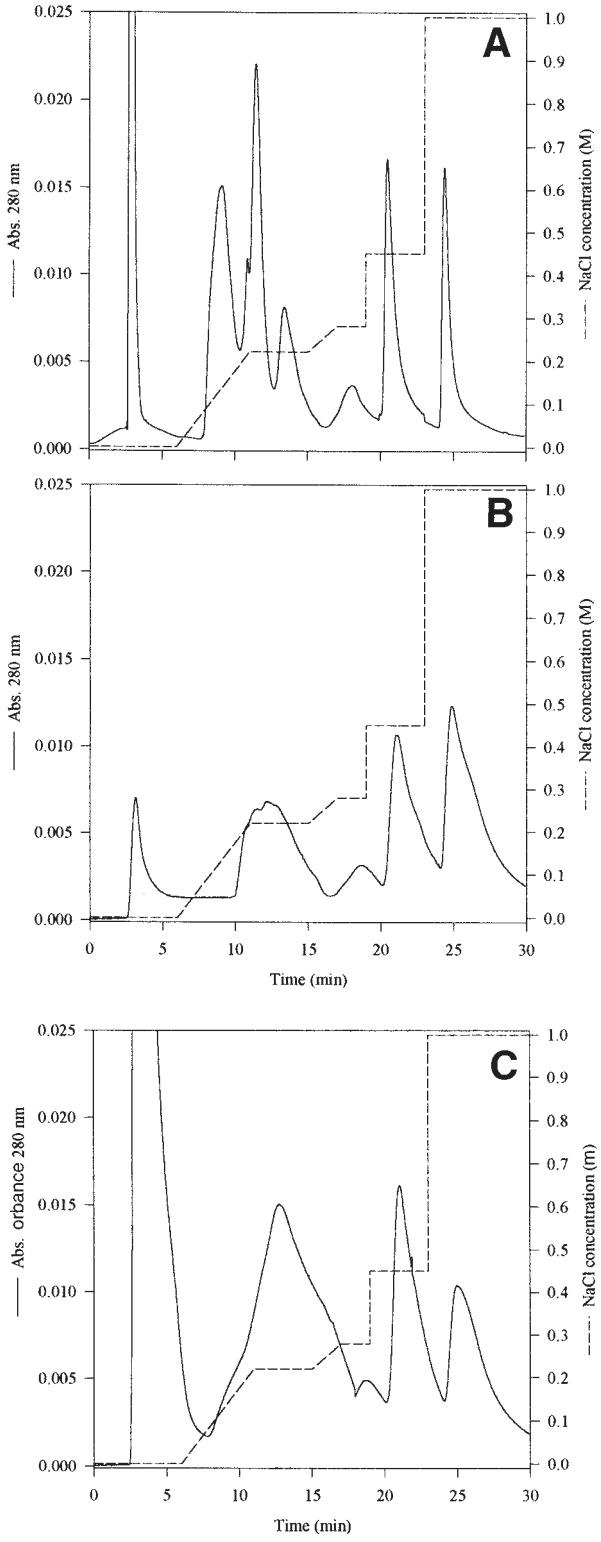


Fig. 1. FPLC chromatograms obtained with a Millipore Memsep DEAE 1000 anion exchange column: (a) UAB lipase; (b)UAB-1000 lipase; (c) Sigma lipase.

Table 2
Lipolytic Activity with Two Substrates
for UAB, UAB-1000, and Sigma Lipase

| | Substrate | |
|---|---|---|
| Lipase | Triolein (U/mg powder) | Tributyrin (U/mg powder) |
| UAB | 20.0 | 0.41 |
| UAB-1000 | 1.0 | 0.044 |
| CRCL (Sigma) | 4.9 | 0.24 |

lipase, respectively. In all chromatograms the presence of two final fractions (F1 and F2) with lipolytic activity was observed (retention times: 21 [F1] and 25 [F2] min). Although sodium dodecyl sulfate-polyacrylamide gel electrophoresis of these fractions showed slight contamination (data not shown), it was evident that the relative ratio of these fractions was completely different. In fact, UAB lipase showed a similar proportion of both fractions, as well as UAB-1000 lipase, in which the F2 fraction was slightly predominant. On the contrary, the major fraction in Sigma lipase was F1, almost twice that of the second fraction. Moreover, a larger amount of proteins without lipolytic activity (at the beginning of the chromatogram) appeared in Sigma lipase. This fact was confirmed by the value of specific lipolytic activity per milligram of protein, which was lower than the UAB lipase (ratio UAB/Sigma = 2). Thus, the ratio of the isoenzymes obtained by fermentation was quite different compared with Sigma lipase.

Identification of the type of isoenzymes (LIP1–LIP5) is in progress. Recently, it has been demonstrated that the secretion of the isoenzymes can be modulated by modifying the feeding rate strategies in fed-batch cultures *(5)*.

### *Lipolytic Activity*

The three lipase powders, obtained after downstream, were characterized using different substrates and assays, and the results compared with commercial lipase are presented in Table 2. The enzymatic activity was expressed as micromoles of acid released per minute and milligrams of powder.

The relative enzymatic activity for both long (triolein) and short (tributyrin) chain triglyceride was UAB > CRCL > UAB-1000. UAB powder showed a much higher activity with triolein than CRCL and UAB-1000, with a rate of 20:4.9:1 respectively, but these differences were lower in the case of tributyrin, in which the rate was 9.3:5.5:1. This fact indicates that the UAB lipase shows a higher specific activity with long-chain triglycerides than CRCL. On the other hand, the behavior of UAB-1000 lipase seems to be closer to that of Sigma lipase with the substrates tested.

### *Esterification Reaction*

The powder lipases obtained under controlled fermentations and selected downstream is quite different compared with commercial lipase

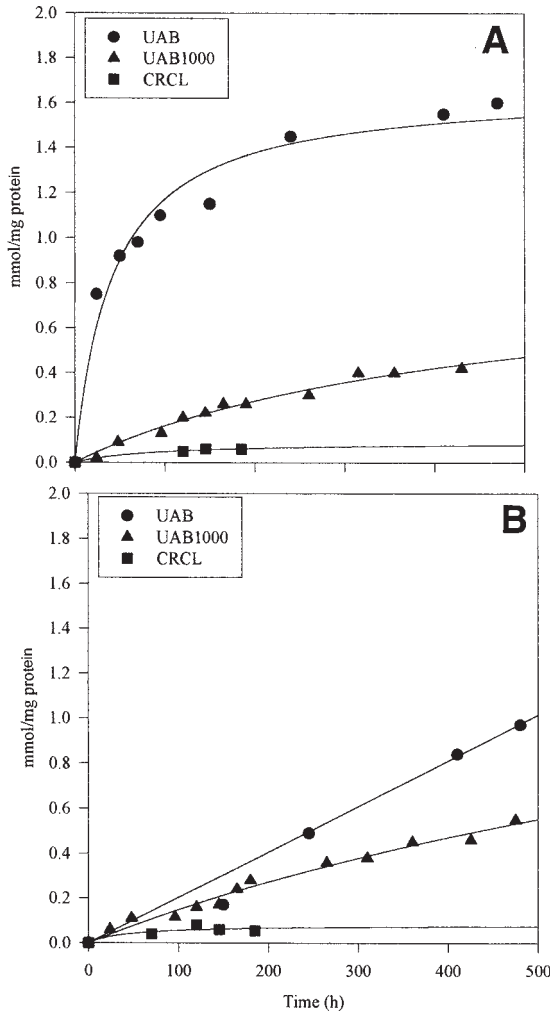


Fig. 2. Esterification of (*S*)-Ketoprofen **(A)** and (*R*)-Ketoprofen **(B)** without addition of water to the reaction medium.

from the point of view of percentages of isoenzymes and lipolytic activity in front of different substrates (triolein, tributyrin). Thus, these powders were tested in the esterification of chiral compounds.

CRCL (from Sigma) and pure isoenzymes Lipase A and Lipase B are stereoselective to *S*(+)-2-arylpropionic acids *(19)*. Nevertheless, Ketoprofen is referred to as a bad substrate when lipase from *C. rugosa* is used as biocatalyst because poor yields and enantiomeric excess were achieved *(20,21)*. Figure 2 gives the results obtained in the synthesis of Ketoprofen in the absence of water in the medium. In this case, the relative enzymatic activity is UAB > UAB-1000 > CRCL. Moreover, enantioselectivity was very high in the case of UAB lipase, in which a ratio $v_S/v_R$ of 11 was reached. Although UAB-1000 is more active than CRCL, similar enantioselectivity ($v_S/v_R$ <1)

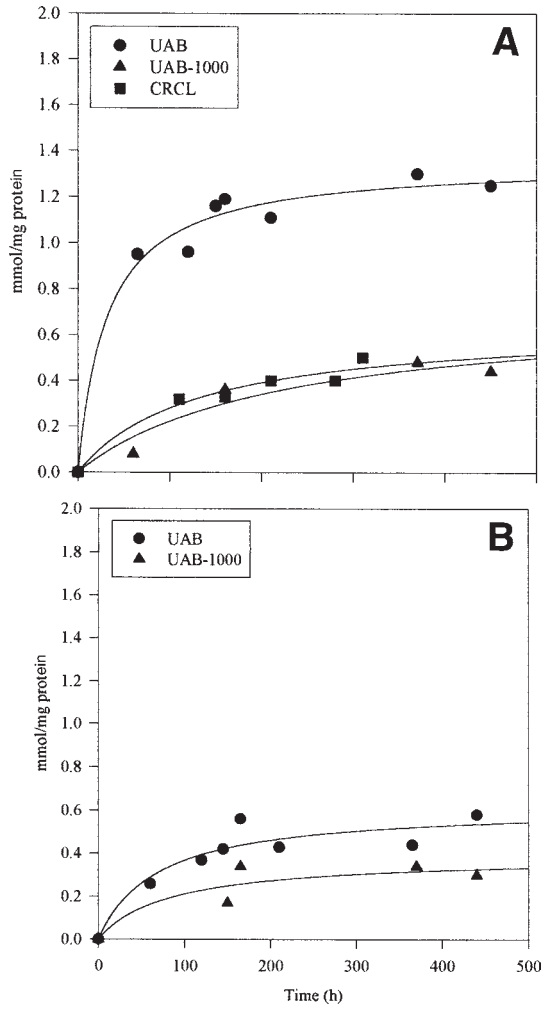


Fig. 3. Esterification of (*S*)-Ketoprofen **(A)** and (*R*)-Ketoprofen with 200 µL water/mL medium.

was observed. This difference in behavior between the powders may be related to the different proportion of isoenzymes observed (Fig. 1) as well as to the different activity shown.

### *Influence in the Esterification Yield of the Water Added to the Medium*

The addition of water to the reaction mixture increased the yield and stereoselectivity in the esterification of (*R,S*)-2(4-isobutylphenyl) propionic acid (Ibuprofen) *(22,23)*. To explore this topic and to homogenize the initial water content in the different powders in the case of the esterification of (*S*) or (*R*)-Ketoprofen, reactions with 200 µL of $H_2O$/mL of media were carried out (Fig. 3). Again, in the presence of water, UAB lipase is the most active enzyme.

The influence of water is different depending on the lipase powder used. The addition of water increases the yield of CRCL in the esterification of (*S*)-Ketoprofen (Fig. 3); however, synthesis of (*R*)-Ketoprofen practically was not detected. Thus, increase in the enantioselectivity ($v_S/v_R$) toward the (*S*)-enantiomer was observed; a ratio value of 4.2 was reached. On the contrary, no influence of water in the synthesis rate of (*S*)-enantiomer was observed using UAB powder and a slight increase using UAB-1000. Although the esterification of the (*R*)-enantiomer was very low, a slight increase in the synthesis rate was observed in both powders.

The effect of water in the reaction rate and enantioselectivity could be related not only to the difference in percentage of isoenzymes but also to the different downstream used and other components present in the commercial powder, or produced jointly with the enzyme in the fermentation process. The lyophilization in the presence of lactose (UAB lipase) results in the best biocatalyst in all the reactions proved. The lack of effect of the water in this enzyme may be related to the hydration of the lipase, because lactose acts like a water reservoir, giving enough water to the lipase to be active. When lactose is not present (UAB-1000 lipase), the addition of water is used to hydrate the external surface of the protein, which slightly increases the esterification reaction rate of both enantiomers, which keeps the enantioselectivity but increases the yield. In general, we can conclude that the presence of water in the reaction medium changes the rate of esterification of these lipases, and that the downstream process can regulate this effect.

## Conclusion

The control of the fermentation conditions of the yeast *C. rugosa* using oleic acid as the only carbon source produces crude lipases with different relative proportions of isoenzymes compared with Sigma type VII lipase. As a consequence, these crude lipases show different enzymatic activity both in hydrolysis and in esterification reactions. UAB lipase produced under these conditions is a suitable biocatalyst to carry out a high stereoselective esterification of racemic Ketoprofen, in which case no good resolution had been obtained using commercial lipase.

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