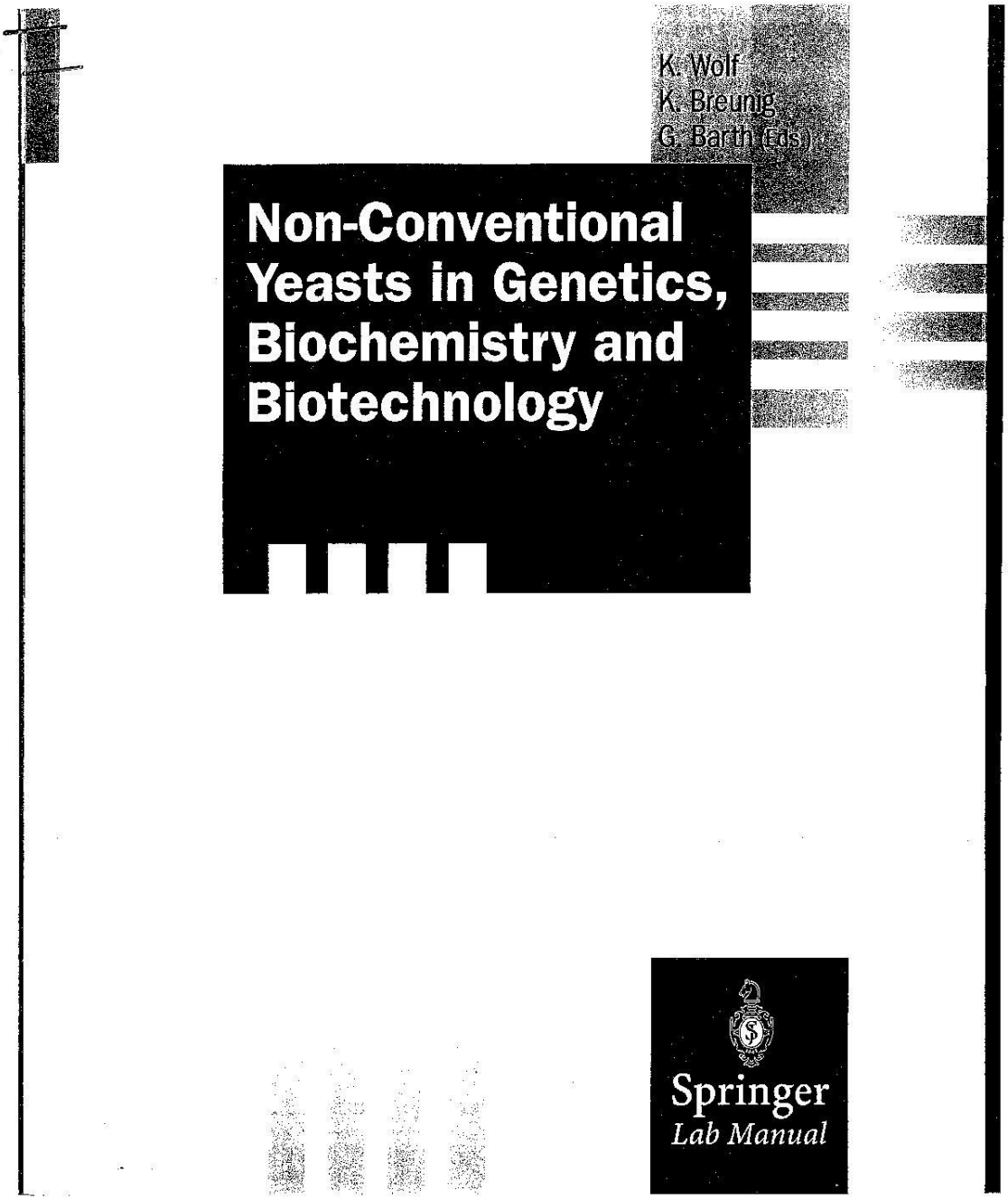

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Chapter 60

Lipase Assay in *Yarrowia lipolytica*

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Aim

To analyze lipase production in any *Yarrowia lipolytica* strain.

Introduction

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are key enzymes in fat metabolism produced by microorganisms, plants, and animals that catalyze the breakdown of triacylglycerols to free fatty acids and glycerol. In *Y. lipolytica* several genes encoding lipases have been isolated, *LIP2* coding for a secreted lipase (Pignede et al. 2000) and *LIP1* and *LIP3* encoding two lipase genes of the carboxylesterase family (Choupina et al. 1999). Lipase expression was repressed by glucose and induced by fatty acids and olive oil.

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Non-Conventional Yeasts in Genetics, Biochemistry and Biotechnology

Materials

Strains Any *Yarrowia lipolytica* strain

Media

- YED medium: 1 % yeast extract (Difco), 1 % glucose
- MM medium: 0.67 % yeast nitrogen base w/o amino acids (Difco); 1 % glucose
- YNB/olive oil: 0.67 % yeast nitrogen base w/o amino acids (Difco), 0.3 % glucose (w/v), 0.7 % olive oil; 200 mM Tris-HCl buffer, pH 7.5
- Agar/tributyrin: 0.67 % yeast nitrogen base w/o amino acids (Difco); 0.3 % glucose; 0.2 % tributyrin; 200 mM Tris-HCl buffer, pH 7.5; 2 % agar
- Agar/tributyrin/glucose: 1 % yeast extract (Difco); 0.2 % tributyrin; 0.3 % glucose; 200 mM Tris-HCl buffer, pH 7.5; 2 % agar
- Tributyrin should be sterilized alone by filtration. Solutions are mixed at 55 °C and shaken to homogenize for 5 min.

Reagents

- Tributyrin (Sigma)
- Sigma lipase substrate (SLS), 62314 (Peled and Krenz 1981)
- *p*-Nitrophenyl butyrate (Sigma)
- Olive oil (Sigma)
- Acetone
- Emulsifying mixture
  300 mM NaCl; 3 mM K_2PO_4; 0.54 % glycerol; 0.6 % gum arabic
- *p*-Nitrophenyl butyrate (*p*-NPB)
  0.42 mM *p*-NPB; 0.05 M phosphate buffer, pH 7.3; 4 % acetone

Lipase activity assays

Solid media

Cells collected from cultures under the desired conditions are washed and resuspended in sterile water at a concentration of 10^9 cells/mL. 10 µL of this solution are placed on agar/tributyrin/glucose and/or agar/tributyrin plates and halo diameters are measured 48 h post-incubation at 28 °C (Fig. 1). Lipase activity is quantified as the ratio between the squared diameter of

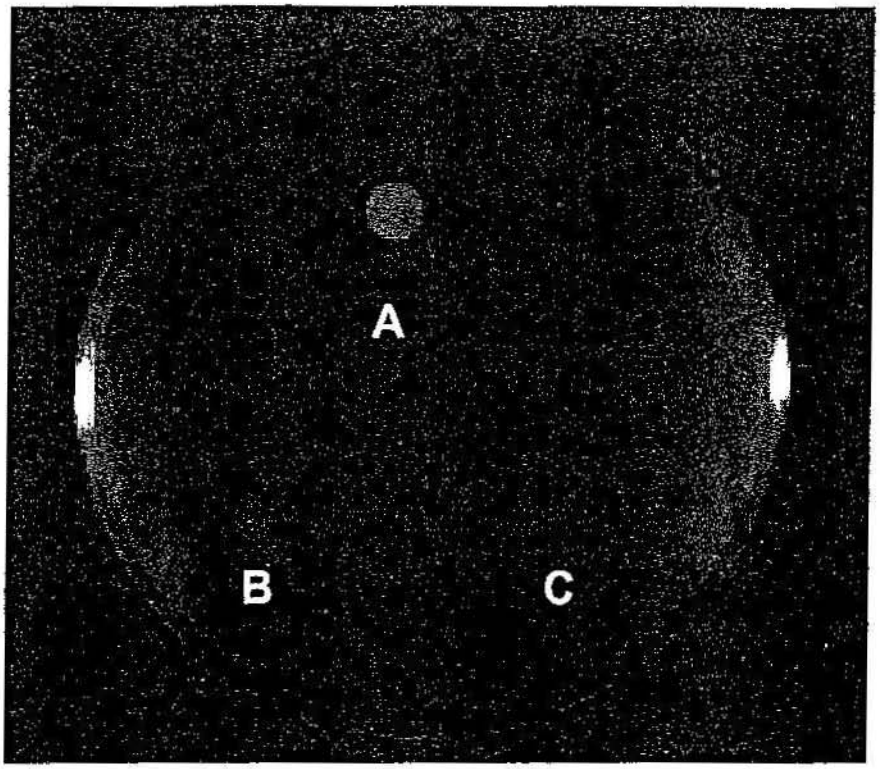

Fig. 1. Lipase production of different yeast species in solid medium (agar/tributyrin). A *Yarrowia lipolytica* (the halo is clearly visible), B *Saccharomyces cerevisiae* S288 c, and C *Kluyveromyces lactis* CBS2359

the halo and the squared diameter of the cell colony (the squared values come from comparing the surface of two circles).

Titration assays (potentiometric or pH-Stat method)

This method involves the mixing of a glyceride substrate in emulsion form with the enzyme at the optimum pH and temperature of the enzyme. During incubation, free fatty acids are released by hydrolysis, which lowers the pH of the system. Automatic titration with NaOH then gives a direct measurement of the lipase activity. Three substrates are suitable for this assay: Sigma lipase substrate (SLS), tributyrin and olive oil. SLS is an emulsified substrate (commercially emulsified olive oil) but pure tributyrin and olive oil must be emulsified before the assay. The emulsifying agent is a solution prepared as following: 17.9 g NaCl, 0.41 g KH_2PO_4, 400 mL water and 540 mL

glycerol are mixed strongly; 6 g of gum arabic and water up to 1000 ml are added and the mixture shaken until complete dissolution. The emulsified substrates are prepared as a mixture of 15 mL of substrate (tributyrin or olive oil), 50 mL of the above emulsifying agent and 235 mL water. The mixture is shaken to homogeneity by hand and re-shaken before each assay.

The release of acid is continuously titrated at pH 7.2 with the aid of a pH-Stat, the RTS 822 recording titration system from Radiometer (Copenhagen, Denmark). The general pH-Stat procedure is as follows: The reaction vessel containing 4.8 mL of the emulsified substrate is thermostatted at 25 °C for 5 min, after which pH is adjusted by the autoburet of the equipment to a fixed constant value of 7.2. Finally, 0.1 mL of supernatant of the growth cultures or 0.1 mL of the appropriate cell solutions (to leave 10^7 cells/mL in the reaction mixture) is injected into the vessel and the kinetic run is begun. Lipase activity is measured by recording the amount of titrating NaOH (10–25 mM) added to maintain the pH at the fixed value against the reaction time (10–30 min in total). Activity is expressed as the initial rate and calculated as:

$$v\ (\mu mol/L/min) = (\Delta x/\Delta y)\ M\ f$$

where $\Delta x/\Delta y$ is the initial slope of the kinetic curve determined graphically, M is the NaOH concentration in mol/L, and f is a scaling factor that takes into account the rate of the recorder, the volume of the vessel, and the conversion factors for units.

Spectophotometric assay

This involves the use of a chromogenic substrate, such as *p*-nitrophenyl butyrate (*p*-NPB) in solution, which upon hydrolysis yields colored *p*-nitrophenol as the reaction product. The substrate at 0.42 mM is prepared as a mixture of 3.6 µL of pure *p*-nitrophenyl butyrate, 48 mL of 0.05 M phosphate buffer, pH 7.3, and 2 mL of acetone as dispersing agent. The reaction mixture contains 2.4 mL of substrate and 0.1 mL of supernatant of the growth cultures or 0.1 mL of the appropriate cell solutions (to leave 10^7 cells/mL in the reaction mixture). The reaction is followed by recording absorbance at 400 nm with time (10 min) using a Beckman DU-7 spectrophotometer equipped with software to yield a least-squares fitting of the initial straight line of the kinetic run. The reaction mixture is stirred by means of a Cuv-O-Stir 333 magnetic microstirrer. A Selecta 389 thermostat with external circulation is used to maintain a constant temperature (25.0 °C)

Activity is expressed as initial rate, calculated as:

$v = 1/\varepsilon\ (dA_{410}/dt)$

where ε is the extinction coefficient of *p*-NP at pH 7.3 ($1.01 \times 10^4\ cm^{-1}\ M^{-1}$) and dA_{410}/dt is the initial slope of the absorbance-time curve.

Experimental Procedure

Grow a culture of 300 mL of one *Y. lipolytica* strain in MM at 28 °C with shaking (250 rpm) overnight. **Day 1**

Lipase induction in liquid medium. Cells in the exponential phase are collected, washed and resuspended in YNB/olive oil medium at 10^6 cells/mL. Incubation is carried out at 28 °C with shaking (250 rpm). At the desired times aliquots are taken and activity determined either in whole cells or in supernatants. Usually maximal activity is reached after 32 h of incubation. Special attention must be paid to the correct buffering of the induction media since acidification of the medium will inactivate the lipase. **Day 2**

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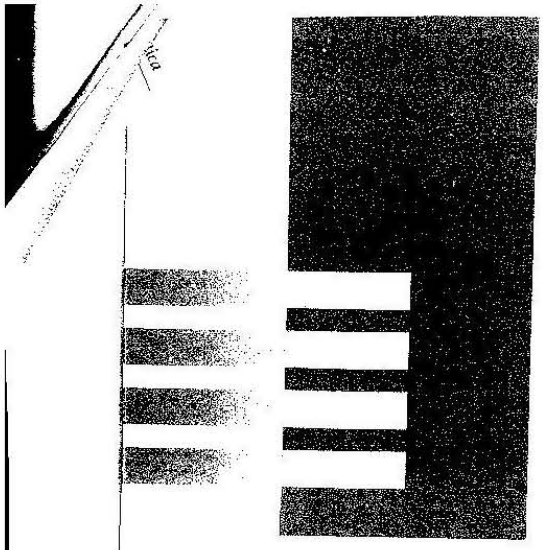

Non-Conventional Yeasts in Genetics, Biochemistry and Biotechnology

Most information on yeasts derives from experiments with the conventional yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the complete nuclear and mitochondrial genome of which has also been sequenced. For all other non-conventional yeasts, investigations are in progress and the rapid development of molecular techniques has allowed an insight also into a variety of non-conventional yeasts.

In this bench manual, over 70 practical protocols using 15 different non-conventional yeast species and in addition several protocols of general use are described in detail. All of these experiments on the genetics, biochemistry and biotechnology of yeasts have been contributed by renowned laboratories and have been reproduced many times. The reliable protocols are thus ideally suited also for undergraduate and graduate practical courses.

ISBN 3-540-44215-4

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