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Screening of yeast and fungal strains for lipolytic potential and determination of some biochemical properties of microbial lipases

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Abstract

For the screening of yeast and fungal strains with lipolytic potential from Chem. Pharm. Res. Institute Culture Collection, we employed a sensitive agar plate method, using a suitable medium supplemented with $CaCl_2$ and Tween 80. By determination of the opacity areas developed around the colonies of lipolytic microorganisms consisting of calcium soaps, the good lipase producing strains were selected. *Candida lipolytica* CPhI-50 and *Hansenula anomala* CPhI-23 from the yeast strains and *Rhizopus nigricans* CPhI-Rn-8, *Aspergillus oryzae* CPhI-20-9 and *Aspergillus niger CPhI-8-N*-9 from the fungal strains were good lipase producers. When these strains were submerged cultivated on modified GYP medium, *Candida lipolytica* CPhI-50 and *Aspergillus oryzae* CPhI-20-9 had the highest yield of lipase in the medium at the end of the bioprocess. Also, we determined the pH dependence of lipase activities from the good producing strains, and we found that these enzymes exhibited various pH sensitivities, dividing them into three groups: I. lipase active at pH 5.0–6.5 produced by *Candida lipolytica* CPhI-50. II. lipase active at pH 6.5–7.5 produced by *Hansenula anomala* CPhI-23 and *Rhizopus nigricans* CPhI-Rn-8. III. lipase active at pH 7.5–8.5 produced by *Aspergillus oryzae* CPhI-20-9 and *Aspergillus niger CPhI-8-N*-9. By screening at 50°C and pH 8.5 we identified a thermostable semialkali-tolerant lipase produced by *Aspergillus niger CPhI-8-N*-9.

Keywords: *Candida lipolytica*; *Hansenula anomala*; *Rhizopus nigricans*; *Aspergillus oryzae Aspergillus niger*; Lipase; Screening

1. Introduction

Lipases (glycerol–ester–hydrolases), E.C.3.1.1.3. are carboxyl esterases that hydrolyze glycerides present as aqueous emulsions. Certain microorganisms are the sources of choice for lipase production, because these are able to utilize lipids of plant and animal origin as the source of carbon and energy for growth [1]. Practical use of microbial lipases determined a great interest in biotechnological research, concerning the improvement of both, the producing strains and the biochemical properties of the lipolytic enzymes. New applications of the microbial lipases like: hydrolysis of fats, transesterification, stereospecific hydrolysis of racemic esters, organic synthesis, and the use of lipases in detergents have been developed in the last years [2]. For the isolation, identification and screening of lipase producing microorganisms, microbiological, biochemical and genetic methods are utilized [3–5]. Yeast and fungal strains can produce lipase cell-bound or ex-

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creted into the medium, by submerged cultivation under optimum conditions [6,7]. The presence of triglycerides, surfactants or vegetable oils in the culture medium has in most cases an inducible effect on lipase synthesis by yeast and fungal strains [6–9].

This paper deals with the screening of yeast and fungal strains for lipolytic potential, as well as the determination of pH dependence and thermostability of lipase activities from the good producing strains.

2. Materials and methods

2.1. Microorganisms

See Table 1 . *A. oryzae* CPhI-20-9, *A. oryzae* CPhI-20-9-71 and *A. oryzae* CPhI-20-9-118 strains are protease producing mutants which were obtained from *A. oryzae* CPhI-20 strain by ethyl–methane–sulphonate treatment. *A. niger CPhI-8-N*-2, *A. niger CPhI-8-N*-9, *A. niger CPhI-8-N*-19 and *A. niger CPhI-8-N*-27 strains are cellulase producing mutants which were obtained from *A niger* CPhI-8 by UV irradiation.

2.2. Media

(1) G.Y.P.–agar medium with the following composition: (%w/v) glucose 2.0, yeast extract – 0.5, peptone – 0.5, agar–agar – 2.0, supplemented with (%w/v) $CaCl_2$ – 0.1 and Tween 80 – 1.0.

(2) A modified GYP medium containing: (%w/v) glucose 2.0, yeast extract – 1.0, peptone – 2.0, and olive oil 2.0.

The pH of the media was 4.5 for the yeast strains and 6.0 for the fungal strains.

2.3. Cultivation conditions

The solid cultures were grown on agar plates containing volumes of 20 ml medium, at 30°C, for 72 h (yeast strains) and 120 h (fungal strains).

The submerged cultures were grown in 500 ml Erlenmeyer flasks containing volumes of 100 ml medium, at 30°C, for 24 h (yeast strains) and 48 h (fungal strains), by using a reciprocal shaker (240 rpm).

2.4. Determination of cell number

The cell number was measured by the viable plate count method. The GYP–agar medium was spread with decimal diluted samples and the number of colonies was counted after incubation at 30°C (48 h for yeast strains, and 72 h for fungal strains).

2.5. Screening methods

The screening of some yeast and fungal strains for lipase production consisted of the following stages:

(1) The determination of the opacity areas

Table 1
Microorganisms

Yeast strains	Fungal strains
Candida lipolytica CPhI-50	*Rhizopus nigricans* CPhI-Rn-8
Candida rugosa CPhI-15	*Rhizopus stolonifer* CPhI-Rs-5
Candida utilis CPhI-10	*Aspergillus oryzae* CPhI-20
Candida tropicalis CPhI-29	*Aspergillus oryzae* CPhI-20-9
Candida boidinii CPhI-44	*Aspergillus oryzae* CPhI-20-9-71
Hansenula anomala CPhI-23	*Aspergillus oryzae* CPhI-20-9-118
Hansenula polymorpha CPhI-3-5	*Aspergillus niger CPhI-8-N*-2
Saccharomyces cerevisiae CPhI-34	*Aspergillus niger CPhI-8-N*-9
Saccharomyces carlsbergensis CPhI-38	*Aspergillus niger CPhI-8-N*-19
Rhodotorula rubra CPhI-63	*Aspergillus niger CPhI-8-N*-27
	Absidia repens CPhI-A-3

developed around the colonies of microorganisms cultivated on GYP–agar medium initial supplemented with $CaCl_2$ and Tween 80, and the determination of the R/r values.

(2) The determination of the enzyme activities in the medium at the end of the bioprocess with good lipase producing strains selected in the first stage and cultivated in modified GYP medium [7].

We have extended our screening method to focus on the pH dependence and thermostability of lipase activities from the good producing strains.

2.6. Enzyme assay

The enzyme activities were estimated by R/r values, where R is the radius of the opacity areas in cm, developed during incubation time and consisting of calcium soaps, and r is the colony radius in cm. The lipase activity in the medium was assayed by Willstater modified method on olive oil as substrate, as will be explained below.

A lipase unit is the mass of the enzyme which, in standard conditions, hydrolyzes the vegetable oil, so it causes the release of 1 μ equivalent of carboxyl groups in 1 min. The biomass was separated by centrifugation at 4000 rpm for 20 min. The supernatant (crude enzyme) was then assayed for lipase activity.

The substrate emulsion was composed of: (v/v) olive oil 3, sodium deoxycholate (soln. 2%, w/v) 1 (emulsifier) and ovalbumin (soln. 0.05%, w/v) 0.5 (stabilizer).

For each determination, a sample of crude enzyme (1–5 ml) and a control sample (1–5 ml) were assayed; in the second one the enzymatic reaction was stopped at point zero with 20 ml methanol/ethyl ether (9/1 v/v) mixture. The incubation was made at 30°C for 30 min. The carboxyl groups released were determined by titration with 0.1 N NaOH, in the presence of thymol blue, until the blue color was stable for 1 min. To find out the pH dependence of enzymatic activity for the selected microorganisms, the pH of the culture supernatant (crude enzyme) was adjusted at various values, and incu-

Table 2
Screening of some yeast and fungal strains for lipase production

Microorganism	Viability (cell No./ml)	R/r	Classification
Candida lipolytica CPhI-50	$0.82 \cdot 10^8$	$R/r > 2.0$	good
Candida rugosa CPhI-15	$3.80 \cdot 10^8$	$1.0 < R/r < 2.0$	moderate
Candida utilis CPhI-10	$1.50 \cdot 10^8$	$R/r < 1.0$	weak
Candida tropicalis CPhI-29	$2.20 \cdot 10^8$	$R/r < 1.0$	weak
Candida boidinii CPhI-44	$4.30 \cdot 10^8$	0	—
Hansenula anomala CPhI-23	$2.40 \cdot 10^8$	$R/r > 2.0$	good
Hansenula polymorpha CPhI-3-5	$3.70 \cdot 10^8$	0	—
Saccharomyces cerevisiae CPhI-34	$3.90 \cdot 10^8$	0	—
Saccharomyces carlsbergensis CPhI-38	$2.50 \cdot 10^8$	$R/r < 1.0$	weak
Rhodotorula rubra CPhI-63	$2.60 \cdot 10^8$	$R/r < 1.0$	weak
Rhizopus nigricans CPhI-Rn-8	$2.0 \cdot 10^6$	$R/r > 2.0$	good
Rhizopus stolonifer CPhI-Rs-5	$1.2 \cdot 10^6$	$1.0 < R/r < 2.0$	moderate
Aspergillus oryzae CPhI-20	$4.70 \cdot 10^7$	$R/r < 1.0$	weak
Aspergillus oryzae CPhI-20-9	$3.80 \cdot 10^7$	$R/r > 2.0$	good
Aspergillus oryzae CPhI-20-9-71	$2.90 \cdot 10^7$	$1.0 < R/r < 2.0$	moderate
Aspergillus oryzae CPhI-20-9-118	$2.50 \cdot 10^6$	$1.0 < R/r < 2.0$	moderate
Aspergillus niger CPhI-8-N-2	$0.70 \cdot 10^7$	0	—
Aspergillus niger CPhI-8-N-9	$1.80 \cdot 10^8$	$R/r > 2.0$	good
Aspergillus niger CPhI-8-N-19	$0.90 \cdot 10^7$	0	—
Aspergillus niger CPhI-8-N-27	$1.20 \cdot 10^7$	0	—
Absidia repens CPhI-A-3	$0.85 \cdot 10^6$	$R/r < 1.0$	weak

Table 3
The lipolytic enzyme production in submerged cultures of some yeast and fungal strains

Microorganism	Enzyme activity (UE/ml)
Candida lipolytica CPhI-50	12.0
Hansenula anomala CPhI-23	5.8
Rhizopus nigricans CPhI-Rn-8	6.2
Aspergillus oryzae CPhI-20-9	9.5
Aspergillus niger CPhI-8-N-9	7.6

bated at 30°C for 30 min. In order to determine the thermal stability of enzymatic activity, the culture supernatant (crude enzyme) was incubated at various temperatures for 30 min, cooled and assayed for activity.

3. Results

About 75% of the cultures tested in the first stage were lipase positive. The strains were classified by R/r value as good ($R/r > 2$), moderate ($1.0 < R/r < 2.0$) or weak ($R/r < 1.0$) enzyme producers (Table 2).

C. lipolytica CPhI-50 and *H. anomala* CPhI-23 from the yeast strains and *R. nigricans* CPhI-Rn-8, *A. oryzae* CPhI-20-9 and *A. niger CPhI-8-N-9* from the fungal strains were good lipase producers. From below mentioned strains, *C. lipolytica* CPhI-50 and *A. oryzae* CPhI-20-9 had the highest yield of lipase in submerged cultures (Table 3).

The crude lipase from the medium exhibited

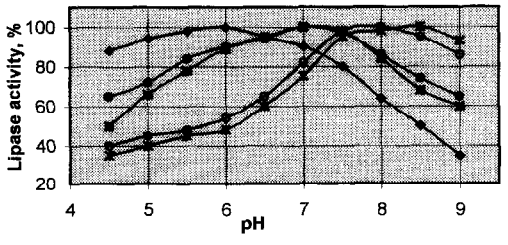

Fig. 1. Influence of pH on lipase activity. To find out the pH dependence of enzymatic activity for the selected microorganisms, the pH of the culture supernatant (crude enzyme) was adjusted at various values, and incubated at 30°C for 30 min. ◆ *C. lipolytica* CPhI-50, ■ *H. anomala* CPhI-23, ● *R. nigricans* CPhI-Rn-8, ○ *A. oryzae* CPhI-20-9, * *A. niger CPhI-8-N-9*.

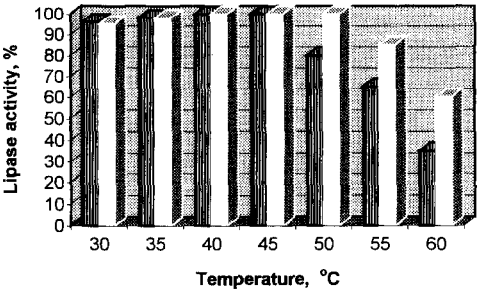

Fig. 2. Influence of temperature on lipase activity from *A. oryzae* CPhI-20-9 and *A. niger* CPhI-8-N-9. The maximum values were obtained for *A. niger* CPhI-8-N-9 and *A. oryzae* CPhI-20-9. The lipase activity was assayed by titrimetric method described at Enzyme assay, after 30 min incubation at the mentioned temperatures.

various pH sensitivities and were divided into three groups (Fig. 1):

I. lipase active at pH 5.0–6.5 produced by *C. lipolytica* CPhI-50.

II. lipase active at pH 6.5–7.5 produced by *H. anomala* CPhI-23 and *R. nigricans* CPhI-Rn-8.

III. lipase active at pH 7.5–8.5 produced by *A. oryzae* CPhI-20-9 and *A. niger CPhI-8-N-9*.

We have also tested the selected microorganisms (Table 3) for various temperatures: 30, 35, 40, 45, 50, 55, and 60°C, but only two of them showed significant values of lipase activity at high temperatures (Fig. 2). By screening at 50°C and pH 8.5 we identified a thermostable, semi-alkali-tolerant lipase produced by *A. niger* CPhI-8-N-9.

4. Discussion

Screening and isolation of microorganisms for lipase activity is most frequently carried out employing agar plates containing triglycerides or Tweens. Lipase catalyzed hydrolysis gives rise either to clearing or opacity zones developed around colonies of lipolytic organisms [10–12].

About 75% of the yeast and fungal strains which we had tested using GYP–agar medium supplemented with $CaCl_2$ and Tween 80 were lipase positive. By determination of the opacity

areas developed around the colonies of the lipolytic microorganisms consisting of calcium soaps, *C. lipolytica* CPhI-50 and *H. anomala* CPhI-23 from the yeast strains and *R. nigricans* CPhI-Rn-8, *A. oryzae* CPhI-20-9 and *A. niger CPhI-8-N-9* from the fungal strains were selected as good lipase producers ($R/r > 2.0$).

Certain characteristics of microbial lipases as, for example, pH dependence or a high stability and activity at elevated temperatures were used for screening of microorganisms with lipolytic potential.

Thus, by screening at 60°C and pH 9.0 [4] identified some bacteria and yeasts — from tested cultures (including bacterium, yeast, actinomycete and fungal strains) that produce thermostable alkali-tolerant lipases.

By screening at 50°C and pH 8.5 we found a fungal strain, *A. niger* CPhI-8-N-9 which produced a thermostable semi-alkali tolerant lipase.

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