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Utilization of Methyloleate in Production of Microbial Lipase

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Abstract

In this article, we report the development and optimization of an industrial culture medium for the production of extracellular lipase in the yeast *Yarrowia lipolytica*. Until now olive oil in combination with glucose was used as the carbon source and inducer for the production of lipase. Our results demonstrate that methyloleate, a cheap hydrophobic compound, could efficiently substitute olive oil as the inducer and carbon source for lipase production. A new process of lipase production was developed yielding a twofold increase in the level of production compared with the levels in previous reports.

Index Entries: *Yarrowia lipolytica*; lipase; bioreactor; methyloleate; olive oil; oleic acid.

Introduction

Lipases (EC 3.1.1.3) constitute a group of enzymes having the ability to hydrolyze triglycerides at the lipid-water interfaces *(1)*. In higher eukaryotes, lipases are physiologically important for the hydrolysis of oils and fats to free fatty acids and partial acylglycerols, which are essential for metabolic processes such as fatty acid transport, oxidation, and synthesis of acylglycerols and phospholipids *(2)*. During the past two decades, lipases of microbial origins have received increasing attention because of their wide variety of properties and because of the relatively simple methods of production in bioreactors and recovery from

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culture media *(3)*. However, few of them are commercially available, and they are mainly produced by filamentous fungi such as *Aspergillus oryzae, Aspergillus niger, Rhizopus arrhizus, Rhizomucor miehi,* and *Humicola lanuginosa.*

The catalytic properties of lipases have enabled the development of numerous high-tech productions of fine chemical and pharmaceutical products (for a review, see ref. *4*). In the medical and therapeutic fields, they are particularly important as substitutes for pancreatic lipases *(5)*. The fact that lipases remain active in various organic solvents has allowed their use in the catalysis of inter- and transesterification reactions of oils and fats *(6)*. Other areas of interest for their use are in the biocatalytic resolution of pharmaceutical racemic mixtures, in the development of specific flavors in food-processing industries, as an ingredient in detergent formulations, and in the treatment of wastes *(7–9)*.

The nonconventional *Yarrowia lipolytica* yeast readily grows on hydrophobic substrates such as alkanes, fatty acids, and oils. This physiologic property has been ascribed to an efficient synthesis and excretion of proteolytic and lipid-degrading enzymes (for a review, see ref. *10*). Extracellular lipase production by *Y. lipolytica* was first reported in 1948 by Peters and Nelson *(11)*, who described a single type of glucose-repressible activity. More recently, Pignede and colleagues isolated the *LIP2* gene responsible for most of the extracellular lipase activity in *Y. lipolytica* W29 strain. The gene encodes a glycosylated triacylglycerol hydrolase with a molecular mass of 38 kDa and optimum catalytic activities at pH 7.0 and 37ºC *(12,13)*.

In our laboratory, *Y. lipolytica* strain CBS6303 has been the topic of a number of investigations for the development of a large-scale bioreactor process to produce lipolytic enzyme. As a first approach, lipase production was carried out in a 100-L bioreactor on an industrial medium containing malt extract, soybean meal, and olive oil. A maximum activity of 58 U/mL was obtained after 8 h of culture *(14)*. To improve the enzymatic productivity, CBS6303 strain underwent chemical mutagenesis using *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine *(12)*. About 10 mutants of first generation were selected for their increased capacity of lipase production. Indeed, their lipase activity in the culture broth reached about 500–700 U/mL. The best of these mutants, named LgX64, was selected for a second round of mutagenesis. Among the 136 mutants of second generation, mutant LgX64.81 presented the highest capacity of lipase production (1165 U/mL). Culture of this mutant in a 500-L bioreactor on a modified medium containing whey powder, corn steep liquor (CSL), glucose, and olive oil yielded a maximum lipase production of 1150 U/mL.

The aim of the present work was to optimize further the lipase production process by increasing the yield of lipase production and by lowering the production cost.

Materials and Methods

Chemicals

Casein peptone and yeast extract were purchased from Organotechnie (LaCourneuve, France). Oleic acid was purchased from Across (Geel, Belgium) and olive oil was from Bertolli (Inveruno, Italy). Methyloleate, ethyloleate, and methyl caprylate-caproate were purchased from Cognis (Saint Forgean Ponthierry, France). Whey powder was from BHA (Homburg, Belgium), glucose was from Roquette (Lestrem, France), and $(NH_4)_2SO_4$ was from VWR (Leuven, Belgium). Antifoam Tego KS911 was purchased from Goldschmidt (Essen, Germany). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Strains, Media, and Culture Conditions

The culture medium contained 30 g/L of whey powder, 5 g/L of CSL, 8 g/L of $(NH_4)_2SO_4$, 10 g/L of glucose, and 0.5% (v/v) of inducer as stipu-lated in the text. Experiments in liquid cultures were performed as follows: Cells were inoculated in 50 mL of the same medium as for the culture and grown at 29°C to the mid–exponential phase. Yeast cells were then centrifuged for 10 min at 10,000*g* and resuspended in 5 mL of 50 m*M* sodium potassium phosphate buffer, pH 6.8. The culture media (100 mL) were then inoculated with an adequate volume of the corresponding cell suspension to give a final cell concentration of 1×10^5 cells/mL. Cultures were done in 500-mL conical flasks and grown at 29°C on a rotary shaker (150 rpm). Cell growth was determined on YPD plates as previously described *(10)*.

Cultivation Under Controlled Conditions

All fermentations were performed at 29°C in a Biolafitte bioreactor (Monze-sur-le-Mignon, France) of 2-, 20-, and 500-L capacity with an effective volume of 1.5, 15, and 450 L, respectively. Dissolved oxygen (DO) was continuously monitored with an oxygen probe (Ingold, Urdorf, Switzerland). Stirring velocity ranged from 150 to 600 rpm to ensure a minimal DO content of 30% of saturation. Airflow was set to 0.5 vvm (volume of air per volume of medium per minute), and pH was automatically maintained at 6.5 ± 0.1 by the addition of NaOH or H_3PO_4. To avoid excessive foam formation, a level probe was placed 10 cm from the top of the vessel that activated the addition of Tego KS911 antifoam.

Thin-Layer Chromatography Analysis of Culture Broth

Fatty acid esters were analyzed by thin-layer chromatography (TLC). Culture supernatants (2 μL) were spotted on silica gel TLC plates (Polygram sil G/UV; Macherey-Nagel, Duren, Germany) and developed with a chloroform:methanol:water mixture (65:15:2 [v/v/v]). Plates were

then covered with a mixture of acetic acid:sulfuric acid:anisaldehyde (100:2:1, [v/v/v]) and dried during 10 min at 150°C. Triglycerides and fatty acids appeared as black spots on the TLC plates.

Analysis of Carbon Source

Glucose concentration in the culture broth was determined using a BioMerieux kit PAP7500 (BioMerieux, Marcy Letoile, France) according to the manufacturer's recommendations. Qualitative analysis of fatty acid esters was carried out by microscopic examination of culture broth samples at × 600 magnification. At this condition, methyloleate appeared as small refractive droplets. Oleic acid concentration was assayed by high-performance liquid chromatography (HPLC) as previously described *(15)*.

Enzymatic Activity Assay

Lipolytic activity was determined by the titrimetric method described by Nagaoka et al. *(16)*. Briefly, 1 mL of pure or diluted culture supernatant was added to the reaction flasks containing 10 mL of neutralized olive oil (5%) and polyvinylic alcohol (2%) emulsion in 50 m*M* phosphate buffer, pH 7.0. Enzymatic reaction was allowed to occur during 15 min at 37°C on a rotary shaker (150 rpm). It was stopped by the addition of 20 mL of acetone-ethanol mixture (1:1 [v/v]). The free fatty acids produced were titrated with 0.05 NaOH in the presence of phenolphtaleine. One unit of lipolytic activity was defined as the amount of enzyme that released 1 μmol of fatty acid/min under the assay conditions.

Results and Discussion

Optimization of Culture Medium

We previously reported that oleic acid is the most suitable inducer for the production of lipase in *Y. lipolytica* strain LgX64.81 *(15)*. However, pure oleic acid is not convenient for the development of an industrial process owing to its excessive cost. Until now, olive oil was used as the inducer and carbon source for the production of lipase in a large-scale bioreactor *(12)*. To develop an industrial medium with low-cost raw materials, olive oil was replaced with a fatty acid ester in the fermentation medium. Different esters with various chain lengths were tested as inducers for the production of lipase. The productivities obtained with these esters were compared with those of oleic acid and olive oil. These inducers included methyloleate, ethyloleate, and methyl caprylate-caproate. Shake-flask cultures of LgX64.81 in the industrial medium containing 0.5% (v/v) inducer were realized. Samples were withdrawn at various time points for 5 d; cell growth and lipase activity were then determined. No significant difference could be observed in cell growth for all the inducers tested. Biomasses obtained on the different media ranged from 2.1 to 2.4 × 10^7 cells/mL

Table 1
Determination of Lipolytic Activity During Growth of LgX64.81 Mutant in Industrial Medium Containing Glucose (10 g/L) Using Different arbon Sources as Inducer[a]

Time (d)	Oleic acid (U/mL)	Olive oil (U/mL)	Methyloleate (U/mL)	Ethyloleate (U/mL)	Methyl caprylate-caproate (U/mL)
3	335	540	755	52	220
4	855	1080	1065	82	435
5	1045	1130	1190	195	660

[a]Values are the means of two experiments. Standard deviations are <15%.

(data not shown). These results indicate that none of the inducers seems to be toxic for cell growth.

By contrast, significant differences were observed in the level of lipase production (Table 1). Ethyloleate led to the lowest lipase production, with a value of 195 U/mL. This could be owing to the consumption of the released ethanol by *Y. lipolytica* diverting the yeast metabolism from the production of lipase. Methyl caprylate-caproate, composed of caprylate (C_8) and caproate (C_{10}) residues, led also to low values (660 U/mL). This is not surprising because *Y. lipolytica* is known to display a lipase activity acting preferentially on oleyl residues of triglycerides *(10)*. The best results were obtained with methyloleate, which led to comparable values to those obtained with oleic acid and olive oil. The lipase activities obtained after 5 d were 1045, 1130, and 1190 U/mL when oleic acid, olive oil, and methyloleate were used as inducer, respectively. These observations indicate that methyloleate could efficiently substitute olive oil as inducer for lipase production.

To optimize further the fermentation medium, the ability of methyloleate to support both cell growth and lipase production was investigated. Therefore, cell growth and lipase activities were determined after 5 d of culture of LgX64.81 growing in the industrial medium lacking glucose and supplemented with various methyloleate concentrations. The results in Table 2 show that in the presence of 0.5% methyloleate, the yield in both cell growth and lipase production was equivalent to that obtained in the glucose-supplemented medium. Biomass and lipase activity were 3.5×10^7 cells/mL and 1310 U/mL on methyloleate compared with 2.8×10^7 cells/mL and 1295 U/mL on olive oil, respectively (Table 1). These results seem to indicate that the glucose in the medium was not consumed during growth. This was confirmed by measuring the glucose concentration in the culture medium. Indeed, <10% of glucose was consumed in these conditions.

An increase in methyloleate concentration led to higher lipase production, giving a maximal value for 2.5−3% methyloleate. Surprisingly, a further increase in methyloleate concentration led to lower lipase production.

Table 2
Determination of Cell Growth and Lipolytic Activity After 3 d of Growth of LgX64.81 Mutant in Industrial Medium Lacking Glucose and Containing Different Amounts of Methyloleate[a]

Carbon source	Cell growth (10^7 cells/mL^1)	Lipolytic activity (U/mL)
0.5% Olive oil	2.8	1295
0.5% Methyloleate	3.5	1310
1.5% Methyloleate	2.6	1213
2.5% Methyloleate	4.8	2810
3.0% Methyloleate	3.8	3250
4.0% Methyloleate	2.7	1540
5.0% Methyloleate	3.0	1040

[a]Values are the means of two experiments. Standard deviations are <15%.

These lower values could be owing to the production of cell-bound lipase instead of extracellular lipase, as was previously reported. In *Candida deformans*, a yeast closely related to *Y. lipolytica*, lipase is preferentially cell bound in the presence of high concentrations of oleic acid, whereas in the presence of low concentrations of oleic acid, the lipases are located in the culture broth (17).

To confirm that methyloleate is the carbon source of choice for lipase production, shake-flask cultures of LgX64.81 on the industrial medium supplemented with 3% oleic acid, olive oil, and methyloleate were performed. Cell growth and lipase activity were measured for 8 d and lipolytic productivity was determined. As shown in Table 3, the highest lipolytic productivity (1068 U/mL and per 10^7 cells) was obtained on the medium containing methyloleate as the sole carbon source, indicating that this compound is the carbon source of choice for the production of lipase in a large-scale bioreactor.

Production of Extracellular Lipase in Bioreactor

To assess the lipase production under controlled conditions, LgX64.81 was cultivated in a 2-L bioreactor containing 1.5-L of industrial medium containing 3% methyloleate. Cell growth and lipase accumulation were monitored as a function of time until lipase production reached its maximum value. After 72 h of growth, lipase production was 3025 U/mL for a cell growth of 2.3×10^7 cells/mL (Fig. 1A). Moreover, owing to the controlled and optimized culture conditions in the bioreactor, this yield of lipase production was obtained in 72 h as compared with 120 h in shake-flask cultures.

To scale up lipase production, culture of LgX64.81 was performed in a 20-L and a 500-L bioreactor. As shown in Fig. 1B, no difference in the level of lipase production could be observed during cultures in the 20-L

Table 3
Determination of Lipase Productivity (expressed as U/mL and per 10^7 cells) During Growth of LgX64.81 in Industrial Medium Containing 3% Oleic Acid, Olive Oil, or Methyloleate[a]

Time (d)	Oleic acid	Olive oil	Methyloleate
1	0	0	0
2	70	123	157
3	125	139	253
4	152	254	361
5	388	380	473
6	418	540	591
7	625	613	800
8	609	576	1068

[a]Values are means of two experiments. Standard deviations are less than 15 %.

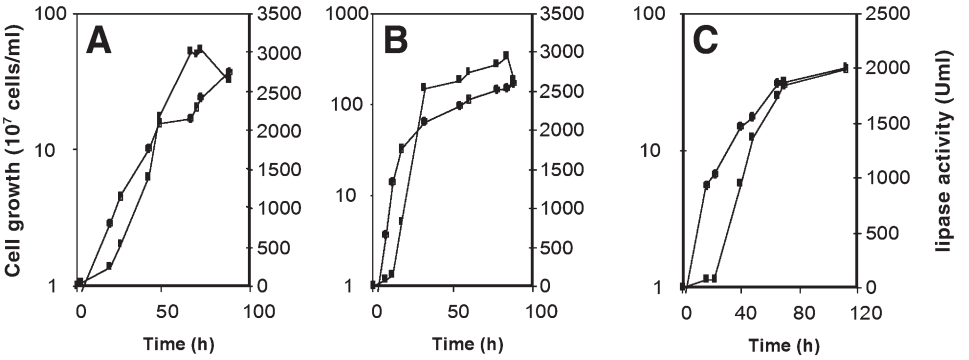

Fig. 1. Cell growth (■) and lipase activity (●) measured during culture of LgX64.81 strain in **(A)** 2-L; **(B)** 20-L, and **(C)** 500-L bioreactors on methyloleate (3%) media.

bio-reactor as compared with the 2-L culture, because the lipase activity was 2940 and 3025 U/mL, respectively. By contrast, the cell growth showed a 3.4-fold increase compared to that in the 2-L culture, indicating that the lipase productivity was lower during the 20-L culture. In addition, the morphology of the yeast cells was also different, with an average of filamentous form much higher during the culture in the 2-L bioreactor (Fig. 2). The 500-L culture led to a lipase production somewhat lower (2010 U/mL) than in the 2- and 20-L bioreactors. However, similar cell growth and cell morphology were observed as compared to those of the 2-L cultures (Fig. 2). These data suggest that the hyphal form of LgX64.81 could be a key factor for the production of lipase. These observations are in accordance with the results of Novotny et al. *(18)*, who demonstrated that the level of lipase production was modulated by the cell morphology in *Y. lipolytica*. However, no clear relationship could be established between the morphologic state and lipase production.

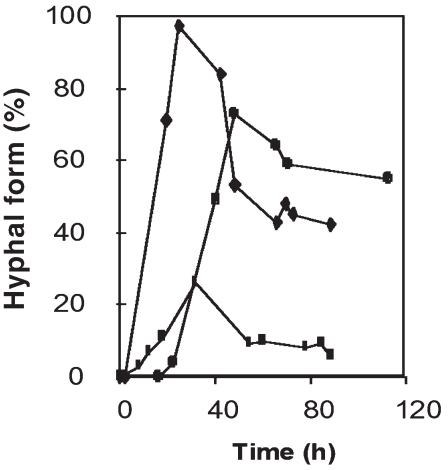

Fig. 2. Determination of hyphal form of yeast cells during culture of LgX64.81 strain in (◆) 2-L; (■) 20-L, and (●) 500-L bioreactors on methyloleate (3%) media.

Assimilation of Methyloleate

To understand better the assimilation of methyloleate, TLC analyses of culture broth samples containing methyloleate were performed (data not shown). The results suggest that the ester bonds of this carbon source were rapidly hydrolyzed during culture of LgX64.81, owing to the lipase activity, suggesting that only the oleyl residue could enter the cells. Microscopic observations of yeast cells during those cultures showed that the fatty acid accumulates in the cells to form oil bodies. The presence of oleic acid in the yeast cells was confirmed by HPLC analysis of cell extracts (data not shown). Moreover, measurements of lipase activity highlight that the maximum value of lipase activity was correlated with the disappearance of those oil bodies in the cells. These results are in agreement with a previous report on the production of lipase by *Y. lipolytica* strain IMUFRJ50682 *(19)*.

Conclusion

A suitable raw carbon source for the production of extracellular lipase in the yeast *Y. lipolytica* was found. Utilization of methyloleate led to a lipase production of 2000 U/mL in a 500-L bioreactor, which represents a twofold increase over that found by Destain et al. *(12)*. This carbon source will be now used for the industrial production of lipase with this yeast.

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