Appl Biochem Biotechnol (2010) 160:1371–1385
DOI 10.1007/s12010-009-8599-7

An Enhanced Process for the Production of a Highly Purified Extracellular Lipase in the Non-conventional Yeast *Yarrowia lipolytica*

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Received: 10 January 2009 / Accepted: 2 March 2009 /
Published online: 31 March 2009

Abstract *Yarrowia lipolytica* LgX64.81 is a non-genetically modified mutant that was previously identified as a promising microorganism for extracellular lipase production. In this work, the development of a fed-batch process for the production of this enzyme in this strain was described. A lipolytic activity of 2,145 U/mL was obtained after 32 h of batch culture in a defined medium supplemented with 10 g/L of tryptone, an enhancer of lipase expression. To maximize the volumetric productivity, two different fed-batch strategies had been investigated. In comparison to batch process, the intermittent fed-batch strategy had not improved the volumetric lipase productivity. In contrast, the stepwise feeding strategy combined with uncoupled cell growth and lipase production phases resulted in a 2-fold increase in the volumetric lipase productivity, namely, the lipase activity reached 10,000 U/mL after 80 h of culture. Furthermore, this lipase was purified to homogeneity by anion exchange chromatography on MonoQ resin followed by gel filtration on Sephacryl S-100. This process resulted in an overall yield of 72% and a 3.5-fold increase of the specific lipase activity. The developed process offers a great potential for an economic production of Lip2 at large scale in *Y. lipolytica* LgX64.81.

Keywords *Yarrowia lipolytica* · Lipase · Fed-batch culture · Bioreactor · Purification

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Introduction

Lipases (E.C.3.1.1.3) constitute a class of hydrolases that are primarily responsible for the hydrolysis of acylglycerides [1]. They are ubiquitous and widely distributed through different kinds of organisms. In addition to their biological significance, lipases hold tremendous potential for application in biotechnology [2]. Promising application fields include waste treatment [3], the resolution of racemic mixtures to produce optically active compounds [4], and the development of characteristic flavors in food processing industries [5]. In the medical and therapeutic fields, they are particularly used as substitutes for pancreatic lipases for the treatment of mucovicidosis and cystic fibrosis [6, 7].

Lipase production was reviewed by Vakhlu and Kour [2]; most of the papers describe the production of this enzyme by *Candida* species. However, an increasing interest in the extracellular lipase produced by the non-conventional yeast *Yarrowia lipolytica* (formally *Saccharomycopsis lipolytica*) can be noted over the two last decades.

Strains of this species have been most frequently isolated from lipid-containing substrates such as cheese and olive oil. This substrate preference had been attributed to an efficient production and secretion by the yeast of lipolytic enzymes [8]. Among them, great attention has been paid to the extracellular lipase Lip2. It was established that most of the extracellular lipase activity in *Y. lipolytica* results from *LIP2* gene expression. This gene has been isolated and shown to encode 334 amino acid polypeptides in which the first 33 amino acids consist of a signal peptide [9]. Cloning, sequencing, and functional analysis of two other genes *LIP7* and *LIP8* encoding for functional lipases were later described by Fickers and coworkers [10]. They suggested that, in contrast to Lip2 which was secreted in the medium, Lip7 and Lip8 could correspond to cell-bound lipases [10].

Different approaches were applied to optimize *Y. lipolytica* extracellular lipase production. Destain et al. [11] reported the selection of mutants with increased lipase secretion after chemical mutagenesis of *Y. lipolytica* CBS6303 wild-type strain. Other studies described the construction and selection of overproducing mutants using genetic engineering approaches [12, 13].

Factors affecting lipase production, mainly carbon and nitrogen sources [14, 15], or enzyme induction and catabolite repression were also studied [16, 17, 18].

Furthermore, considerable efforts dedicated to the setup of a scaleable process for lipase production by *Y. lipolytica* have been deployed. Enzyme production, carried out in a 2,000-L bioreactor using the *Y. lipolytica* LgX64.81-overproducing mutant, led to a lipase activity of approximately 1,100 U/mL after 53 h of fermentation. Under these conditions, the culture medium was composed of olive oil, glucose, whey powder, yeast extract, corn steep liquor, and ammonium sulfate [19]. A medium composed of methyloleate as an alternative to olive oil was later developed by Destain and coworkers [20]. They reported the production of a lipolytic activity of 3,025 and 2,940 U/mL within 3 days of culture in 2- and 20-L bioreactors, respectively. Unfortunately, a marked decrease in lipase productivity was observed when scaling up the production process to a 500-L bioreactor; they only reached 2,010 U/mL after 120 h of culture. Furthermore, they observed a great variability between 2- and 20-L bioreactor batch cultures regarding *Y. lipolytica* cell growth profiles. Biomass yield showed a 3.4-fold increase in 20-L culture compared to that in the 2-L culture [20]. This process inconsistency could be explained by the presence in the culture medium of complex nutrient components such as yeast extract, corn steep liquor, whey powder,

etc. Indeed, these complex nutrients are of a biological origin and therefore have a non-constant composition that could make the cultivation process less reproducible [21].

Besides medium composition, culture strategy greatly affects the effectiveness of a process. In this context, most of the lipase production processes are usually performed in batch culture. Even though this culture mode is simple and enables a short process time, it has fundamental disadvantages. Due to substrate limitation, it results in limited product yield. Furthermore, the growth rate in a batch culture can not be controlled. This parameter is therefore constantly changing during fermentation and ceases when one or more nutrients are depleted or if metabolic by-products inhibit cell growth [21].

Fed-batch culture mode emerges consequently as an attractive choice in numerous biotechnological processes due to its operational simplicity, reliability, and flexibility for implementation in multipurpose facilities [22]. It is a very promising culture mode for the production of secreted protein with regard to the maximization of volumetric productivity.

A fed-batch culture of the *Y. lipolytica* LgX64.81-overproducing mutant of lipase was optimized in this work. First, lipase production was assessed in defined medium supplemented with tryptone and compared to the production in a complex medium commonly used for yeast culture. To reach a high volumetric productivity of the target protein, various feeding strategies were then investigated. A downstream process was also developed to obtain a highly purified enzyme.

Materials and Methods

Microorganism

Y. lipolytica LgX64.81-overproducing mutant isolated by Destain and coworkers was used in this study [11].

Chemicals

Yeast extract, potassium phosphate monobasic, potassium phosphate dibasic, and Triton X-100 were purchased from USB Corporation (Cleveland, OH, USA). Methyloleate was provided by Cognis (Saint Forgean Ponthierry, France). Thiamin was obtained from Merck (Darmstadt, Germany) whereas myo-inositol was from Calbiochem (La Jolla, Canada). Antifom 204 and all other chemicals, unless stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Culture Media

The yeast strain was isolated on yeast extract, peptone, and glucose (YPG)-agar plates containing 20 g/L glucose, 10 g/L yeast extract, 10 g/L casein peptone, and 16 g/L agar. The inocula were prepared in YPG liquid medium.

The complex medium (YTA) contains 10 g/L yeast extract, 10 g/L tryptone N1, and 4 g/L ammonium sulfate.

Tryptone-defined medium (TD) has the following composition: 15 g/L KH_2PO_4, 5.5 g/L K_2HPO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 10 g/L tryptone N1, 4 g/L $(NH_4)_2SO_4$, 10 mg/L

$FeCl_3 \cdot 6H_2O$, 4 μg/L myo-inositol, 8 μg/L biotin, and 200 μg/L thiamine HCl. Methyloleate was used as a carbon source at a final concentration of 20 g/L.

Shake Flask Culture

Preparation of the inoculum was carried out in 250-mL shake flask containing 50 mL of YPG medium, and pre-cultures were incubated for 16 h at 29 °C and 140 rpm.

Shake flask cultures were carried out in 1-L-baffled shake flask containing 200 mL of growth medium. The flasks were incubated at 29 °C, 140 rpm for 3 days. Samples were taken daily for analytical assays.

Bioreactor Cultures

Cultures were performed in a 5-L-stirred bioreactor (Infors, Switzerland). Bioreactor inoculum was prepared as follows: first, 50 mL of a sterile YPG medium was inoculated by a single colony isolated on YPG-agar plates and incubated at 29 °C in an orbital shaker at 140 rpm for 24 h. This culture was then used as an inoculum for a second pre-culture carried out in 1-L-baffled shake flask containing 200 mL of the medium that will be used during bioreactor culture, except that glucose was used as carbon source. After 20 to 22 h of incubation at 29 °C and 140 rpm, the inoculum was aseptically added to the bioreactor.

Bioreactor culture conditions were as follows: working volume 2.5 L, temperature 29 °C, pH regulated at 6.5 by KOH 3 M or H_3PO_4 3 M addition. A minimal dissolved oxygen of 30% air saturation was ensured using an air flow rate of 1 vvm (air volume per volume of medium per minute) and stirring speed ranging within 600–700 rpm. To avoid excessive foaming, 5 mL of antifoam 204 was initially added to the medium before sterilization.

Kinetic parameters of cell growth were determined during batch cultures of *Y. lipolytica* LgX64.81 in a 5-L bioreactor. Glucose and methyloleate were tested separately as a unique carbon source. Cultures were carried out under similar conditions. Cell yield coefficients ($Y_{X/S}$), maximal growth rate (μ_{max}), and maintenance term (m) were equal to 0.4 g/g, 0.11 h^{-1}, and 0.09 g/g when the cells were grown on glucose and to 0.29 g/g, 0.09 h^{-1}, and 0.1 g/g when methyloleate was used as a carbon substrate.

Intermittent fed-batch process was performed by pulse addition of methyloleate at a final concentration of 2% when the lipid substrate was depleted in the culture medium.

Stepwise feeding strategy was carried out as follows: An initial cell growth phase was first carried out in batch mode using 1% glucose as a unique carbon source. Then, after consumption of the initial glucose as indicated by an increase of dissolved oxygen level, a fed-batch cell growth phase on glucose was started. Feeding was adjusted to allow an exponential growth. Hence, the substrate feeding rate was calculated from the glucose mass balance equation according to the model described by Kortz et al. [23]. The feed rate of carbon substrate is given by the following equation:

$$m_S(t) = F(t)S_F(t) = \left(\frac{\mu(t)}{Y_{X/S} + m}\right)V(t)X(t)$$

where m_S is the mass flow of substrate (grams per hour), F the volumetric feeding rate (liter per hour), S_F the concentration of the substrate in the feeding solution (grams per liter), μ the specific growth rate (per hour), $Y_{X/S}$ the biomass/substrate yield coefficient (gram per gram), m the maintenance coefficient (gram per gram per hour), X the biomass concentration (grams per liter), and V (liter) the culture volume.

After 24 h of fed-batch culture on glucose, a starvation period was established for total glucose exhaustion in the culture medium. Upon complete depletion of glucose, a fed-batch culture on methyloleate was started. Lipid substrate was added in pulse mode allowing lipase gene induction and secretion of the enzyme into the medium.

Downstream Processing Steps

After cell removal by centrifugation (4,000 rpm for 30 min), the culture supernatant was clarified using 0.2 μm Minisart filter (Sartorius Stedim Biotech, Goettingen, Germany) and desalted on Sephadex G-25 prepacked PD10 column (Amersham Biosciences, Uppsala, Sweden). The clarified and desalted fraction was then loaded on 1 mL HiTrap Q column (Amersham Biosciences, Uppsala, Sweden) equilibrated with Tris–HCl 25 mM pH 7. Elution was carried out with stepwise gradient 0.1–1 M of NaCl at a flow rate of 0.5 mL/min. Absorbance was monitored at 280 nm. Fractions displaying high lipase activity were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Lipase was then further purified on a Sephacryl S-100 HR XK 16/60 column (Amersham Biosciences, Uppsala, Sweden) using PBS pH 7 at a flow rate of 0.5 mL/min.

Analytical Methods

Microbial growth was monitored by measuring the absorbance at 600 nm (OD_{600}) of culture samples. For cells grown on methyloleate, lipids were extracted from culture samples with 2.5 volume of propanol/butanol mixture (1:1, *v*/*v*) before OD_{600} determination.

To determine biomass concentration in terms of dry cell weight (DCW), samples of culture broth (5 mL) were taken in duplicate, centrifuged at 4,500 rpm and washed twice with deionized water. They were dried at 80 °C for 60 h. One unit of OD_{600} was found to be equivalent to 0.18 g/L dry cell weight (for both substrates). Glucose concentration was determined using an enzymatic kit (01GLO2125, Eurodiag, France). Methyloleate concentration was estimated by the colorimetric method based on a sulfo-phospho-vanillin reaction described by Frings and Dunn [24].

The lipolytic activity was determined by the titrimetric method as described by Destain et al. [20] using olive oil as substrate. One lipase unit (U) was defined as the amount of enzyme required for liberating 1 μmol of fatty acid per minute at 37 °C and pH 7.0.

Protein production was analyzed by SDS-PAGE according to the method of Laemmli [25] and stained with Coomassie brilliant blue or silver nitrate.

Results

Selection of a Semi-defined Medium for Enhanced *Y. lipolytica* Extracellular Lipase Production

Most of the culture media used to grow *Y. lipolytica* LgX64.81 for extracellular lipase production contain complex and undefined nutrients such as whey powder, yeast extract, peptone, etc. Nevertheless, the use of a synthetic medium composed exclusively of chemically defined substances with a mineral nitrogen source did not result in significant lipase production [26]. Supplementation of the defined medium with 10 g/L of tryptone was necessary for a valuable production of extracellular lipase in *Y. lipolytica*. This was ascribed

to the presence of bioactive peptides in tryptone that stimulate lipase production in this strain [26].

Therefore, lipase production in shake flask and 5-L bioreactor cultures was assessed in defined medium supplemented with 10 g/L of tryptone named TD medium and compared to a complex medium (YTA) commonly used for yeast cell growth. As shown in Fig. 1, specific lipase production was higher in complex medium in comparison to the tryptone-defined medium during the first 48 h of *Y. lipolytica* mutant culture in a shake flask. Thereafter, an improvement in specific lipase production was observed in TD medium. At the end of the culture, the specific lipase production in TD medium was 1.5-fold higher than that observed in the complex medium. Comparatively, a marked improvement in specific lipase production was noted soon after the first 24 h of *Y. lipolytica* culture in a 5-L bioreactor in the TD medium. Under these conditions, the specific lipase production was around 1,000 U/mg of biomass whereas a specific production of only 600 U/mg was obtained using YTA-rich medium. Therefore, TD medium was selected as the optimal culture medium for subsequent experiments.

Setup of a Fed-Batch Process for Lipase Production

Various feeding strategies were investigated in an attempt to develop a cost-effective and efficient process for the production of extracellular lipase in *Y. lipolytica* LgX64.81 mutant.

Intermittent Fed-Batch Process

A batch culture in TD medium containing 2% methyloleate as a unique carbon source was first carried out. After the depletion of the carbon source as indicated by an increase of dissolved oxygen level, methyloleate was added to the medium to restore its concentration at 2%.

As shown in Fig. 2a, nearly 5 g/L of biomass was obtained at the end of the batch culture. At that time, the lipase activity was around 2,000 U/mL. After methyloleate addition, a 2-fold increase in biomass was observed. However, no improvement in lipase

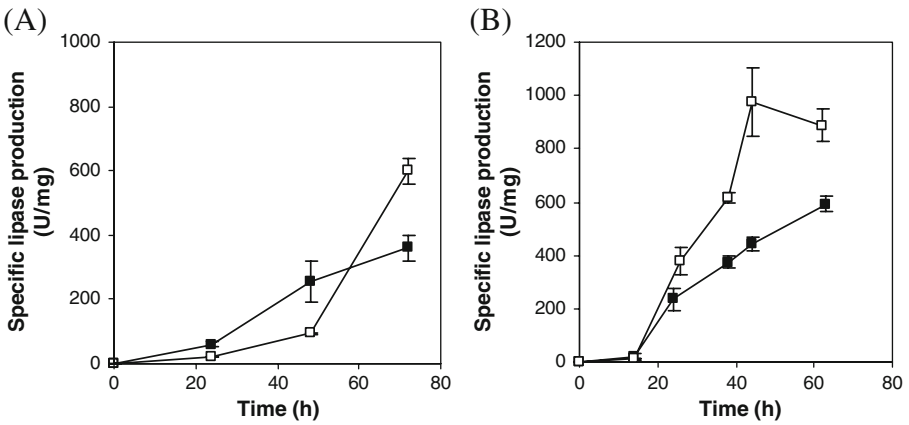

Fig. 1 Time course of specific lipase production during shake flask (**a**) and 5-L bioreactor (**b**) cultures of *Yarrowia lipolytica* LgX64.81 mutant. The yeast was grown in either tryptone-defined medium (*empty square*) or YTA-rich medium (*filled square*). Experiments were carried out in duplicate

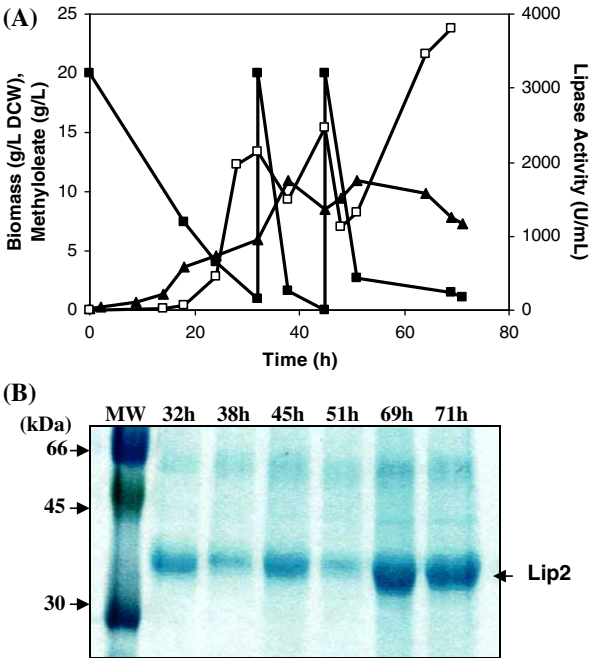

Fig. 2 Intermittent fed-batch culture of *Yarrowia lipolytica* LgX64.81 mutant in a 5-L bioreactor. **a** Time course of cell growth (*filled triangle*), lipase activity (*empty square*), and methyloleate level (*filled square*). **b** SDS-PAGE analysis of culture supernatants. *Lane 1* standard protein size marker (*MW*). Ten microliters of culture supernatant collected at 32, 38, 45, 51, 69, and 71 h was loaded per lane

production was noted; soon after the addition of the methyloleate, a decrease in lipase activity was even observed. Nevertheless, when methyloleate became limiting (lower than 0.3%), the lipase activity increased again but it did not exceed 2,500 U/mL.

After the second methyloleate pulse, cell growth had ceased, and a decrease of biomass level was observed at the end of the culture. The pattern of lipase production was similar to that observed after the first methyloleate pulse, i.e., a decrease of the lipase activity soon after lipid addition. However, a significant improvement of lipase production occurred later. At 70 h of culture, the lipase activity reached 4,000 U/mL corresponding to a production level of 0.4 g lipase/L of culture supernatant.

SDS-PAGE analysis of culture supernatants (Fig. 2b) confirmed these results. The decrease of lipase band intensity was clearly demonstrated at 38 and 51 h after methyloleate pulses. Then, the enzyme had accumulated in the medium and an intensive band of lipase was observed at the end of the culture. The overall productivity was equal to 57 U/mL/h.

Stepwise Feeding Process

Cell growth was initially carried out using glucose as a unique carbon source since this substrate resulted in a higher cell yield coefficient ($Y_{X/S}$). Lipase production was then initiated by switching to the lipidic carbon source (methyloleate).

Cell growth phase was started with a batch phase at an initial glucose concentration of 1%. Once fully consumed, glucose was exponentially fed to the medium keeping the cell growth rate at $\mu_{max}/2$ around 0.06 h^{-1}. Thereafter, a carbon starvation period was allowed to ensure full consumption of glucose before the initiation of lipase production step.

Three cultures were carried out separately to study the effect of multiple methyloleate pulses on lipase production. During the first culture, a unique pulse of methyloleate was performed whereas in the second culture two successive pulses of the lipidic carbon source were carried out, and the second pulse was performed after the complete depletion of methyloleate. During the third culture, three pulses of methyloleate were consecutively added to the medium.

As shown in Fig. 3a, nearly 4 g/L of biomass was obtained within 24 h of batch growth on glucose. At the end of the fed-batch growth phase, over a 3-fold increase in biomass level was obtained; nearly 15 g/L of biomass was reached within 48 h of culture.

An accumulation of glucose was observed at high glucose feeding rates (Fig. 3c), and glucose residual level reached 6–7 g/L over a glucose feeding rate of 4 g/L/h. However, this accumulated glucose was fully consumed during the starvation period.

In contrast to the former phase where the glucose was used as a carbon source, variability in cell growth profile was noticed, with no obvious reason when switching to methyloleate as a carbon source and an inducer of extracellular lipase production.

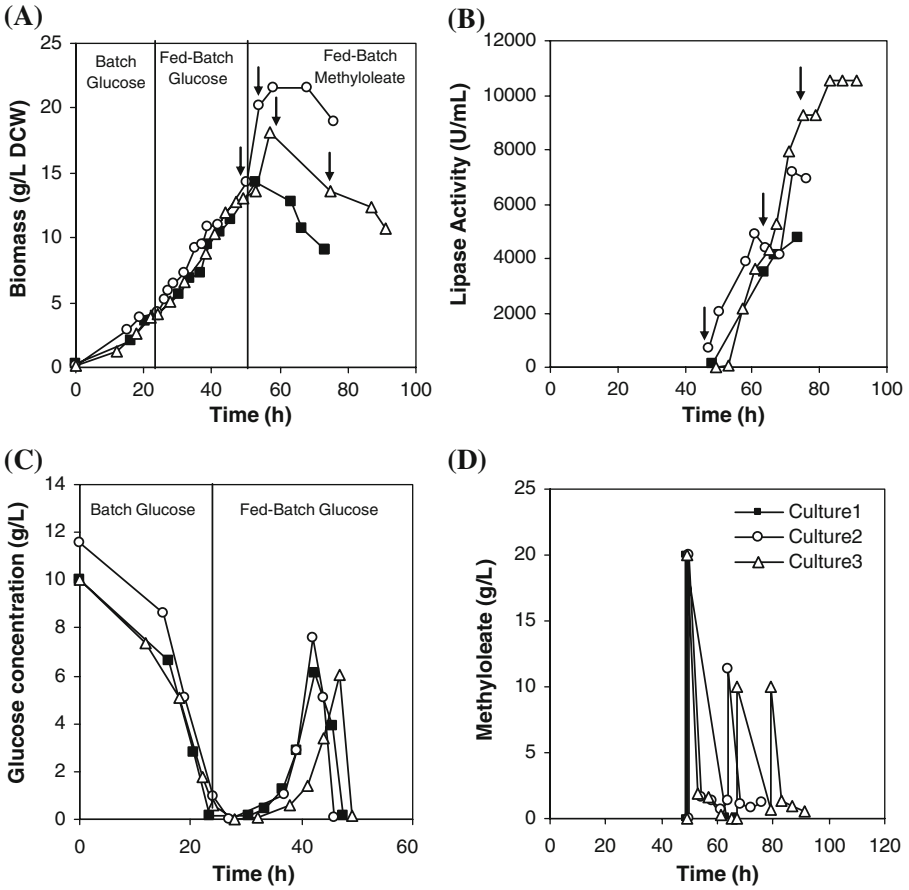

Fig. 3 Stepwise fed-batch cultures of *Yarrowia lipolytica* LgX64.81 mutant in a 5-L bioreactor. Time course of cell growth (**a**), lipase production (**b**), glucose (**c**), and methyloleate (**d**) levels during the three cultures. *Downward arrow* indicates methyloleate pulse

No further increase in biomass level was observed in culture 1. During lipase production phase, a decrease of biomass level was even observed after methyloleate pulse. Nevertheless, in cultures 2 and 3, biomass level reached nearly 22 and 18 g/L DCW, respectively, soon after the first pulse of methyloleate. Additional pulses of methyloleate resulted in a decrease of biomass level for both cultures.

Nevertheless, regardless of cell growth variability, lipase production seemed to be quite comparable. As shown in Fig. 3b, over 4,000 U/mL of lipase activity was obtained in all cultures within 14 h following the first addition of the lipidic substrate. A further one-step addition of methyloleate yielded a higher lipase activity around 7,000 U/mL (culture 2). When a third pulse of methyloleate was performed (culture 3), the highest lipase production was observed. Over 10,000 U/mL of lipase activity was obtained within 80 h of culture corresponding to nearly 1 g of lipase/L of culture supernatant.

SDS-PAGE analysis of the culture supernatants recovered at different times during the three cultures (Fig. 4) showed an accumulation of the enzyme in the medium at the end of the culture. An intensive band of approximately 38 kDa corresponding to the extracellular lipase of *Y. lipolytica* was observed in supernatants collected after the addition of the lipidic carbon source. Higher lipase production was clearly observed with increased pulses of methyloleate. In all cases, lipase appeared to be the major protein in the culture supernatants; this would facilitate its purification.

Downstream Processing of the Extracellular Lipase

Even though the lipase was shown to be the major protein secreted in the medium, further purification steps are needed to comply with the regulatory requirements regarding the use of proteins intended for therapeutic purpose [27]. Hence, ion exchange chromatography was used as a first step in the purification process. After clarification and desalting steps, cell-free culture supernatant was loaded into an anion exchange column (MonoQ). Elution profile of the lipase as well as SDS-PAGE analysis of the eluted fractions are shown in Fig. 5.

At pH 7, lipase protein was efficiently adsorbed to the negatively charged matrix and eluted at a low NaCl concentration (0.1 M). No loss of the enzyme was observed in the flow through fraction and the washing fractions (Fig. 5b). Although the lipase was purified 2-fold with a high yield recovery of 73% (Table 1), contaminants were co-eluted with the enzyme indicating that a polishing step is still needed.

Since these contaminants have a molecular weight far from lipase, size exclusion chromatography was applied to the pooled fractions eluted from MonoQ column (F1 to F8).

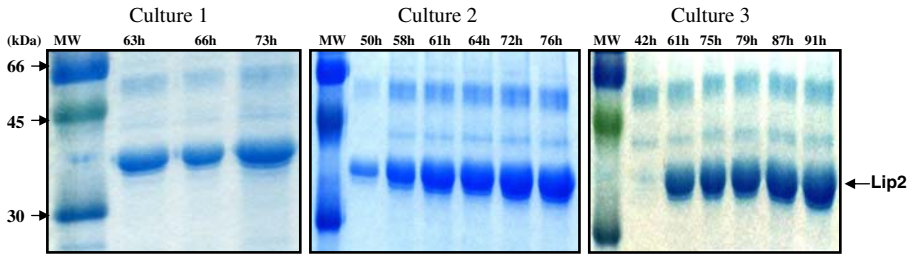

Fig. 4 SDS-PAGE analysis of culture supernatants collected at different times of cultures 1, 2, and 3 during stepwise fed-batch cultures of LgX64.81 mutant in a 5-L bioreactor. *Lane 1* standard protein size marker (*MW*). Sampling time in hours is indicated on each lane

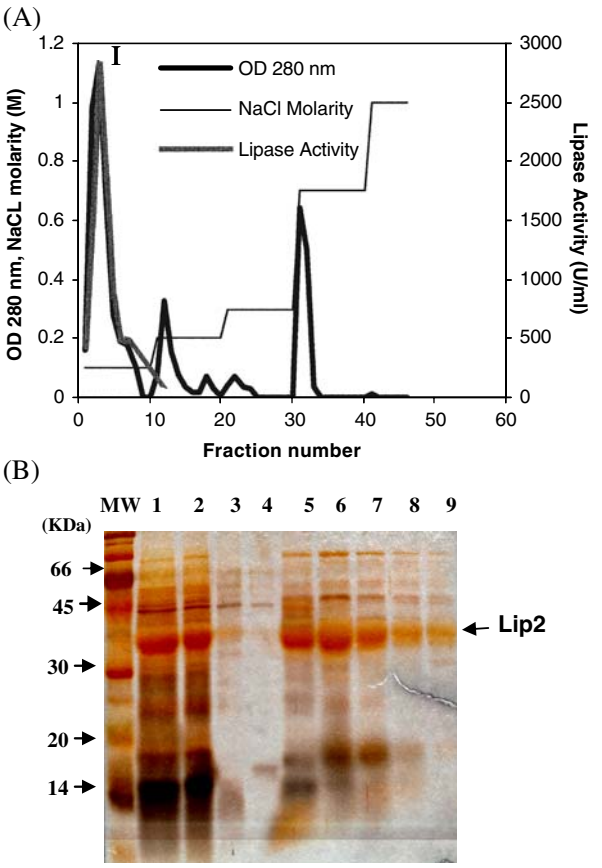

Fig. 5 Stepwise gradient elution of lipase on MonoQ (**a**) and SDS-PAGE analysis (**b**) of various fractions collected during the MonoQ purification step (12% acrylamide and silver nitrate staining gel). *MW* standard protein size marker; *lane 1* clarified culture supernatant; *lane 2* desalted fraction loaded on MonoQ; *lane 3* flow through fraction; *lane 4* washing product; *lanes 5–9* fractions eluted at 0.1 M NaCl (fractions 3, 4, 5, 6, and 7 of peak I)

Elution profile of the lipase as well as SDS-PAGE analysis of the eluted fractions containing the target protein is shown in Fig. 6. Sephacryl S-100 purification had led to the removal of all the residual contaminant proteins. The purity of the lipase was confirmed by SDS-PAGE analysis with the presence of a single band at a molecular weight of 38 kDa (Fig. 6b). In summary, this purification process resulted in a purification factor of 3.4 along with a high recovery yield of 72% (Table 1).

Table 1 Flow sheet of lipase purification procedure from *Yarrowia lipolytica* culture supernatant.

Purification step	Specific activity (U/mg)	Yield (%)	Purification factor
Culture broth	ND	100	–
Desalted supernatant filtrate	2,408	87	1
MonoQ anion exchange	5,249	73	2.2
Sephacryl S-100 gel filtration	8,173	72	3.4

ND not determined

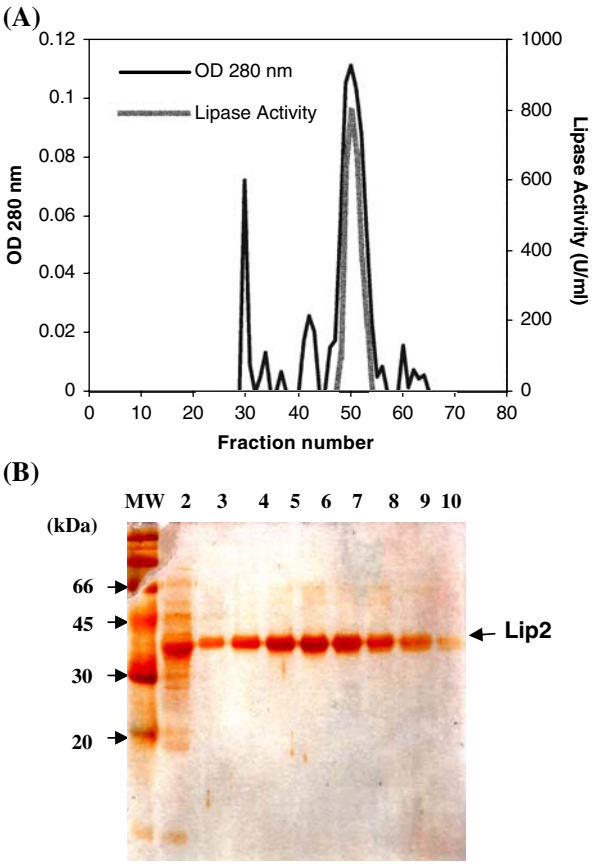

Fig. 6 Sephacryl S-100 gel filtration of lipase. Protein elution profile (**a**) and SDS-PAGE analysis (**b**) of fractions obtained through the gel filtration step (12% acrylamide and silver nitrate coloration). *Lane 1* standard protein size marker; *lane 2* pooled fractions collected during the MonoQ purification step (peak I); *lanes 3–10* fractions eluted after gel filtration (from 47 to 54)

Discussion

The aim of this work is to design a cost-effective process for the production of lipase using the non-genetically modified overproducing mutant of *Y. lipolytica* LgX64.81.

Media components have a strong impact on economics of industrial fermentation processes and can account for up to 30% of the total production cost [28]. Hence, the design of an economic and efficient growth/production medium compatible with commercial needs must be considered in the early steps of process development.

Complex substances are often used for industrial fermentations. Nevertheless, their use is not recommended for large-scale production since they have numerous drawbacks including batch-to-batch variability, uncertainty about the exact composition, difficulty to estimate stoichiometric yields, etc. [21]. There is a strong tendency nowadays to develop processes using the most defined media [29].

The performance of a tryptone-defined medium to support cell growth and lipase production was first assessed during shake flask and 5-L bioreactor batch cultures of the LgX64.81 mutant. Results were compared to those observed in a complex medium.

These data showed that the tryptone-defined medium described in the present work leads to promising results in terms of lipase productivity compared to other studies of lipase

production optimization by *Y. lipolytica* CBS6303 wild-type strain and its two derivative mutants [19, 20, 30]. Batch culture of LgX64.81 mutant in 5-L bioreactor using tryptone-defined medium yielded a lipase activity of 2,145 U/mL within 32 h of culture which is equivalent to a productivity of 67 U/mL/h. This level is 1.6-fold higher than that reported by Destain et al. [20] for *Y. lipolytica* LgX64.81 grown on a complex medium.

Aiming to enhance lipase productivity and to develop a competitive process, two fed-batch strategies were also assessed in this work. An intermittent feeding strategy based on pulse addition of methyloleate was first investigated. This process resulted in nearly 4,000 U/mL of lipase activity within 70 h of culture. However, a decrease in lipase activity was observed soon after the addition of the hydrophobic substrate.

Lower enzyme activity observed at higher lipid substrate concentration can be explained by the inhibition of lipase production by free fatty acids. This effect, however, has not been properly characterized [31]. Similarly, it was previously observed that increasing the feeding rate of lipidic substrate provoked an inhibitory effect of lipase production, and lipase levels in aqueous phase decreased markedly because of enzyme adsorption to the excess of substrate [32].

Despite the high lipase activity reached at the end of the intermittent fed-batch culture, no improvement in lipase productivity was achieved in comparison to batch operation. Hence, a stepwise fed-batch strategy based on uncoupled cell growth and lipase production phases was carried out. Cell growth was first undergone using glucose as unique carbon source. This step led to a higher biomass level (15 g/L) in comparison to batch (5 g/L) and intermittent fed-batch cultures (10 g/L). The lipase production was then induced by methyloleate addition. Three cultures were carried out to study the effect of multiple methyloleate pulses on lipase production. In contrast to cell growth phase, variability in biomass levels was observed between the three cultures. Similarly, variability in cell growth was also reported by Destain et al. [20] during batch cultures of *Y. lipolytica* LgX64.81 on methyloleate. A 3.4-fold increase in biomass level was observed in a 20-L bioreactor compared to that obtained in a 2-L bioreactor [20].

This cell growth variability could most probably be related to the presence of insoluble carbon substrate in the medium. Presence of insoluble substrate such as lipids in the medium could lead to several problems in biomass measurement due to the heterogeneity of culture broth [33]. In addition, Aguedo et al. [34] suggested that cell growth rate is linked to the adhesion between lipid droplets and cells. With lipid globules smaller or bigger than cells, cell growth rate was rapid but this rate decreased for similar sizes [34].

Regarding enzyme production, a lipase activity of 10,000 U/mL was reached after 80 h of culture after the addition of three successive pulses of methyloleate. This yield corresponds to a production level of around 1 g of lipase/L of culture supernatant and a productivity of 125 U/mL/h. This productivity is 2-fold higher than the level reached in batch and intermittent fed-batch. It is also worth noting that this strategy resulted in a 2.5- and 5-fold increase of lipase activity in comparison to intermittent fed-batch and batch strategies, respectively.

It is also the highest productivity among relevant data reported for lipase production in *Y. lipolytica* LgX64.81 strain. Lipase productivity of 48 U/mL/h was obtained by Fickers and coworkers [35] during fed-batch culture of this strain in a 20-L bioreactor. The lipase activity reached 3,044 U/mL after 63 h of culture when a combination of glucose and olive oil was added to the medium.

Fed-batch strategies were also described for *Y. lipolytica*-overproducing mutants obtained by different genetic engineering approaches. Using a 10-copy recombinant strain

of *Y. lipolytica*, lipase activity achieved 90,500 U/mL after 70 h of fed-batch cultivation [12]. However, the level of biomass (80 g/L DCW) was too much higher than that obtained in this study (15 g/L). It should be stressed that the LgX64.81 strain was previously reported to have an altered glucose metabolism. In this mutant, a trans-acting mutation had resulted in a reduced capacity to phosphorylate hexose compared to the wild-type strain [36]. This probably explains the low level of biomass obtained in this study.

An amplified multicopy *Y. lipolytica* strain named JMY 1105 that presents a far higher potential of lipase production was reported by Fickers et al. [18]. The increase in lipase productivity was estimated to range from 10- to 46-fold compared to LgX64.81 and up to 4,472-fold compared to CBS6303 parental wild type. Fed-batch culture of this strain resulted in a lipase productivity of 1,978 U/mL/h [18].

More recently, Aloulou et al. [37] reported the production of about 3 g of lipase/L after 60 h of fermentation in YPD complex medium, of a 20-copy recombinant strain of *Y. lipolytica*. A lipase activity of 12,500 U/mL was also reported by Yu et al. [38] when overexpressing the *LIP2* gene in the methylotrophic yeast *Pichia pastoris*.

Nevertheless, the choice of protein expression system is most often dictated by the ultimate use of the product and not necessarily the yield of the upstream process. Hence, for a protein intended for therapeutic use, factors considered for the evaluation of protein expression in a host include: (a) the regulatory path to approve a drug produced in a given expression platform, and (b) the overall royalties associated with the production of a protein in a given host by recombinant DNA technology [39].

Therefore, the adoption of the non-genetically engineered strain *Y. lipolytica* LgX64.81 as a host system for lipase production can be considered as a promising alternative, particularly for nutraceutical and pharmaceutical applications.

Even though most commercial applications do not require homogenous lipase, a high degree of purity is needed in applications employing the enzyme for biocatalytic production of fine chemicals, pharmaceuticals, and cosmetics.

Most purification schemes for microbial lipases are based on multistep strategies [27]. In this study, *Y. lipolytica* lipase was purified to electrophoretic homogeneity by a procedure involving anion exchange and gel filtration chromatographic steps with an overall yield of 72%. Such performance is higher than that achieved by Fickers et al. [19] who reported a yield of 60% when lipase produced by *Y. lipolytica* LgX64.81 strain was partially purified by centrifugation, filtration, and ultrafiltration steps.

Conclusion

The production of extracellular lipase in fed-batch culture of *Y. lipolytica* LgX64.81 mutant was studied. An improvement in lipase productivity was obtained using a defined mineral medium enriched with tryptone. Different strategies were carried out to optimize lipase production. A stepwise fed-batch strategy based on separate phases of cell growth and lipase production had led to the highest lipase yield. Over 10,000 U/mL of lipase activity was reached. A competitive process based on anion exchange chromatography and gel filtration was developed to obtain a highly purified lipase compatible with pharmaceutical use.

Acknowledgment The authors are grateful to the "Commissariat Général aux Relations Internationales de la Communauté Française de Belgique" for financial support.

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