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Homologous High-Level Lipase and Single-Cell Protein Production with Engineered *Yarrowia lipolytica* via Scale-Up Fermentation for Industrial Applications

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Abstract: *Yarrowia lipolytica* is a promising feed additives. Here, we aimed to produce extracellular lipases and single-cell proteins (SCPs) at high levels simultaneously through fed-batch fermentation of engineered *Y. lipolytica*. The parameters for 500 mL shake flask cultures were optimized with a single factorial design. The resultant activity of lipase reached 880.6 U/mL after 84 h of fermentation, and 32.0 g/L fermentation broth of dry SCP was obtained at 120 h. To attain high SCP and lipase productivity, the high-density fed-batch fermentation of *Y. lipolytica* was scaled up in 10 L, 30 L, and 100 L fermentors. Using glycerol as the sole carbon source, the lipase activity peaked to 8083.3 U/mL, and the final dry SCP weight was 183.1 g/L at 94.6 h in 10 L fermentors. The extracellular lipase activity and SCP weight reached 11,100.0 U/mL and 173.3 g of dry SCP/L at 136 h in 30 L fermentors, respectively. Following 136 h of fed-batch fermentation, the extracellular lipase activity and dry SCP weight reached 8532.0 U/mL and 170.3 g/L in 100 L fermentors, respectively. A balance between the lipase secretion and growth of *Y. lipolytica* recombinant strain was achieved, indicating that an efficient fermentation strategy could promote further scale-up for industrial SCP production from engineered *Y. lipolytica*.

Keywords: *Yarrowia lipolytica*; homologous lipase expression; scale up; single-cell protein; high-cell-density fermentation

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1. Introduction

Yarrowia lipolytica naturally produces several useful compounds and is generally recognized as safe by the American Food and Drug Administration (FDA) [1–3]. Owing to its safety, ability to use various substrates, post-translational modifications, and high-secretion ability [4–6], *Y. lipolytica* has been developed as an expression system to successfully generate non-native proteins since the 1990s [7–10]. *Y. lipolytica* has great utility and offers a new perspective for cell factories with high potential for biotechnological applications, including biotransformation of steroid, and the production of citric acid, aroma, and tailored chain-length fatty acids [11–14]. This oleaginous yeast shows great industrial prospects, with pharmaceutical and food-related applications [15–19].

Studies have attempted to develop *Y. lipolytica* using renewable low-cost substrates, such as plant oils, fats, and glycerol, to obtain high-value products, including single-cell proteins (SCPs) [20,21]. SCPs, the bulk of dried cells, can also be termed bioproteins, microbial proteins, or biomass. Entire cells or lipases of *Y. lipolytica* have been used in enantioselective resolution to produce single enantiomers [22–24]. S-Enantio preference towards 2-bromo-p-tolylacetic acid ethyl ester catalyzed by *Y. lipolytica* Lip2 is similar to

the best result attained with *Burkholderia cepacia* lipase [25]. It is important to obtain as many entire cells of *Y. lipolytica* as possible along with lipases at a low cost. Nonetheless, comprehensive reports on industrial production of SCP and lipase simultaneously with *Y. lipolytica* indicate the need for further development in this area.

In our previous study, an auxotrophic engineered *Y. lipolytica* strain producing lipase and SCP was first investigated for feeding purposes, and its oral feeding assessment in fish demonstrated that the best engineered yeast was an excellent feed additives [26]. Scaling up processes for the simultaneous and efficient production of lipase and cell mass is important. In this study, we investigated pilot-scale fermentation for the simultaneous production of extracellular lipase and high-yield SCP from engineered *Y. lipolytica* in 30 L and 100 L bioreactors for potential industrial applications. Raw glycerol was evaluated for cell growth and lipase expression from shaking culture to 100 L fermentation, and two-stage feeding-batch culture on glycerol was achieved and fermentation parameters were optimized for pilot-scale fermentation.

2. Materials and Methods

2.1. Strains, Plasmids, and Media

A wild-type *Y. lipolytica* strain was isolated and identified from marine-oil-contaminated sludge. An auxotrophic *Y. lipolytica* host strain was constructed and preserved in the laboratory. The multicopy integrative vector pINA1297 was constructed at the National Institute for Agronomic Research, France [26]. The expression cassette, comprising the hp4d promoter, a secretion signal peptide (*xpr2pre*), the *pro-mlip* gene, and the *xpr2* terminator, was amplified using the plasmid pINA1297 as a template. Multicopy-integrated strains were selected based on growth on a minimal YNBD medium. The YNBD selective medium was composed of a yeast nitrogen base without amino acids (YNB) (6.7 g/L) and glucose (10 g/L).

Yeast extract and peptone, obtained at low prices, were of industrial grade. Other routinely used reagentswere commercially available analytical-grade products. The seed culture medium, Yeast Extract–Peptone–Dextrose (YPD) medium, included 1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, and 2% (*w*/*v*) glucose. The culture of the *Y. lipolytica* recombinant strain was investigated on different media, including a modified YPD medium and a buffered complex glycerol medium (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) dextrose, 1.34% (*w*/*v*) yeast nitrogen base (YNB) with no amino acids, 4×10^{-5}% (*w*/*v*) biotin, 100 mM potassium buffer, and 1% (*w*/*v*) glycerol). MD medium plates (1.34% (*w*/*v*) YNB, 4×10^{-5}% (*w*/*v*) biotin, 2% (*w*/*v*) dextrose, and 2% (*w*/*v*) agar) were utilized for the selection of transformants. The YPS medium consisted of yeast extract (10 g/L), peptone (10 g/L), and sucrose (50 g/L). The YPG medium consisted of yeast extract (10 g/L), peptone (10 g/L), and crude glycerol (50 g/L). The YPO medium consisted of yeast extract (10 g/L), peptone (10 g/L), and olive oil (20 g/L). The YPL medium consisted of yeast extract (10 g/L), peptone (10 g/L), and lactic acid (20 g/L). Agar (20 g/L) was added to prepare the solid plates.

The scale fermentation medium FM22 contained 4.29% KH_2PO4, 0.5% $(NH_4)_2SO_4$, 0.079% $CaSO_4$, 1.43% K_2SO_4, 0.57% $MgSO_4$, 4% raw glycerol, and 2.5 mL/L *Pichia* trace minerals 4 (PTM4) solution. PTM4 was composed of 2.0 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.08 g L^{-1} NaI, 3.0 g L^{-1} $MnSO_4 \cdot H_2O$, 0.2 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.02 g L^{-1} H_3BO_3, 0.5 g L^{-1} $CaSO_4 \cdot 2H_2O$, 0.5 g L^{-1} $CoCl_2$, 7.0 g L^{-1} $ZnCl_2$, 22.0 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.2 g L^{-1} biotin, and 1 mL L^{-1} concentrated H_2SO_4 [27]. In addition, 1000 × vitamin included 0.05 g L^{-1} biotin, 1.0 g L^{-1} calcium pantothenate, 1.0 g L^{-1} nicotinic acid, 25.0 g L^{-1} inositol, thiamine 1.0 g L^{-1} HCl, 1.0 g L^{-1} pyridoxine HCl, and 0.2 g L^{-1} para-aminobenzoic acid. The feeding medium contained 4% raw glycerol, 0.2% PTM4, and 0.1% 1000 × vitamin.

2.2. Shake Flask Optimization of the Engineered Y. lipolytica Strain Culture

The recombinant *Y. lipolytica* strain was cultured in a 500 mL flask containing 120 mL of the YPD medium at 220 rpm for 24 h at 28 °C. To improve lipase productivity and achieve

maximal bioactivity with sufficient amounts of SCPs, various parameters, including carbon sources, initial pH (pH 4.5–6.5), initial cultivation medium volume (20–200 mL in a 500 mL shaking flask), and inoculum size (1–5%) based on the above initial culture conditions, were investigated to evaluate the effects on lipase and SCP productivity.

Each condition was assessed in triplicate, and each experiment was repeated three times. The results are shown as mean ± standard deviation (SD) from three independent experiments.

2.3. Fed-Batch Fermentation Process

Based on the optimal conditions for the 500 mL flask culture, a fermentation process for lipase and SCP production using the engineered *Y. lipolytica* strain was performed in a 10 L bioreactor (BIOTECH-10JGZ, Baoxing Co., Shanghai, China). The fermentation inoculum of the recombinant strain was prepared by culturing the cells at 28 °C for 18–20 h at 220 rpm in a 500 mL shake flask containing 120 mL of the YPD medium. After that, 10% (*v*/*v*) of the inoculum was inoculated in a 10 L fermenter. The culture parameters were as follows: the pH was adjusted to 5.0 by adding 25% ammonium hydroxide and the dissolved oxygen (DO) concentration was maintained at over 20% of air saturation by controlling the air flow rate (1.0–4.0 m^3/h) and stirring speed (200–600 rpm) at 28 °C.

In the early stages of the 10 L fed-batch fermentation, a two-stage feeding strategy was investigated using different culture and feeding media. First, the engineered *Y. lipolytica* strain was incubated with 400 mL of pre-culture of the recombinant strain in 4 L of the YPS medium for growth at 28 °C and pH 5.0, until glycerol depletion led to a sharp increase in the DO concentration. Subsequently, cell growth and lipase production were further investigated by sucrose feeding after carbon source depletion, which is indicated by the OD_{600} value(above 100).

Considering cell growth and target compound production, the culture medium was changed to a modified FM22 medium. After incubation in the FM22 medium for 12 h, the cells were further grown by 50% crude glycerol feeding until the OD_{600} value above 100 in phase I was attained. Sucrose feeding started in phase II after glycerol feeding was stopped for 30–60 min of carbon starvation, and then, the concentration of DO rapidly increased again.

To increase biomass and lipase productivity at a low cost, a two-stage feeding method was used in 10 L scale fermentation with 4 L of modified FM22. The 10 L fermentation was controlled at pH 5.5, and a temperature of 28 °C was maintained for cell growth within 12 h. The cells were allowed to grow substantially; this was followed by 50% crude glycerol (as the sole carbon source) feeding until OD_{600} exceeded 100 because of the depletion of glycerol. Additionally, the pH was adjusted to pH 5.5 ± 0.05, and the temperature was reduced to 27 °C for target protein expression. Furthermore, the antifoaming agent J647 (Shanghai Luer Chemical Trading Co., Ltd., China) was pumped to control excessive foam production if necessary. The feed frequency was adjusted once for 2–3 h, and we controlled the agitation rate and air flow rate to maintain the DO concentration at 20–50%.

OD_{600}, biomass, lipase activity, and total protein concentration of the samples were analyzed at regular intervals. Fermentation was disrupted when lipase activity decreased. Each condition was assessed in triplicate, and each experiment was repeated three times. The results are shown as the mean ± SD from three independent experiments.

Using the same 10 L fermentation strategy described above, fed-batch fermentation in 30 L and 100 L bioreactors was carried out with the initial volumes of 15 L and 45 L, respectively, and low-cost crude glycerol as the sole carbon source.

2.4. Biomass Analysis and OD Measurement

Biomass is expressed as the dry cell weight (DCW, g/L) and was measured by centrifuging 10 mL samples in a pre-weighed centrifuge tube at 8000× *g* for 10 min at 4 °C, and the supernatant was refrigerated for further analysis. The precipitates were subsequently washed twice with distilled water and measured in triplicate after treatment at 65 °C to

a constant weight. To measure the OD at 600 nm, the fermentation broth samples were diluted with distilled water.

2.5. Enzyme Assay and Total Protein Production

Extracellular lipase activity was determined using the alkali titration method with olive oil [28,29]. The reaction was carried out in the mixture containing 5 mL of 50 mM Tris-HCl (pH 8.0), 4 mL of an emulsion of olive oil (1 mL of 25% (*v*/*v*) olive oil emulsified with 3 mL of 2% (*w*/*v*) polyvinyl alcohol solution as a stabilizer), and 1 mL of an appropriately diluted enzyme solution at 40 °C for 10 min in a shaking water bath. Subsequently, the hydrolysis was terminated by adding a 15 mL mixture of acetone/ethanol (1:1, *v*/*v*). The amount of liberated fatty acids was measured using titration with 50 mM NaOH, with phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of lipase that liberated 1 µmol of fatty acids from olive oil per minute. The total protein concentration was determined using the Bradford method [30]. Each condition was measured in triplicate, and each experiment was repeated three times. The results are shown as mean ± SD of three independent experiments.

2.6. SDS-PAGE Analysis

The supernatant was harvested from the culture via centrifugation at 12,000× *g* for 12 min at 4 °C. The supernatants were mixed with 2 × sample buffer, boiled for 15 min, and then subjected to SDS-PAGEwith a 12% separating polyacrylamide gel to examine the recombinant lipase [31]. A protein ladder from Fermentas (Burlington, Canada) was used to determine the molecular weight of the separated proteins. The proteins were stained and visualized using Coomassie Brilliant Blue R-250 (Amresco, Solon, OH, USA).

3. Results

3.1. Optimization of the Best Engineered Y. lipolytica Strain in a Shaking Flask

The parameters for the 500 mL shaking flask culture were optimized with a single factorial design. As shown in Figure 1, the optimal conditions were as follows: carbon source, 5% sucrose; initial medium pH 5.5, inoculation volume, 2%; and culture medium volume, 10%. The highest specific activity of the total protein was 2.16 × 10^3 U/mg in the culture supernatant after 72 h. The resultant activity of extracellular lipase reached 880.6 U/mL at 84 h of fermentation, and 32.0 g of dry SCP per liter of fermentation broth was obtained at 120 h.

3.2. Fed-Batch Fermentation (10 L)

Y. lipolytica 10 L fermentation was performed to achieve a high yield of both SCP and lipase production at 28 °C and pH 5.0, with a stirring speed of 300–600 rpm and an air flow of 1–4 L/min to ensure a DO concentration above 30% air saturation. Under the optimal conditions for the 500 mL flask culture, the maximum lipase activity reached 2012.5 U/mL, and the final biomass (DCW) was 102.2 g/L at 101.7 h when sucrose and glycerol were used as carbon sources in a 10 L fermentation (Figure 2a). Additionally, when glycerol was used as the sole carbon source, the highest lipase activity achieved was 8083.3 U/mL, and the final dry SCP weight was 183.1 g/L at 94.6 h in 10 L fermentation (Figure 2b). Lipase and dry biomass productivity using glycerol as the sole carbon source far exceeded those of sucrose feeding. Considering the productivity of lipase and biomass, glycerol was utilized as the sole carbon source in a subsequent study of larger fed-batch fermentation.

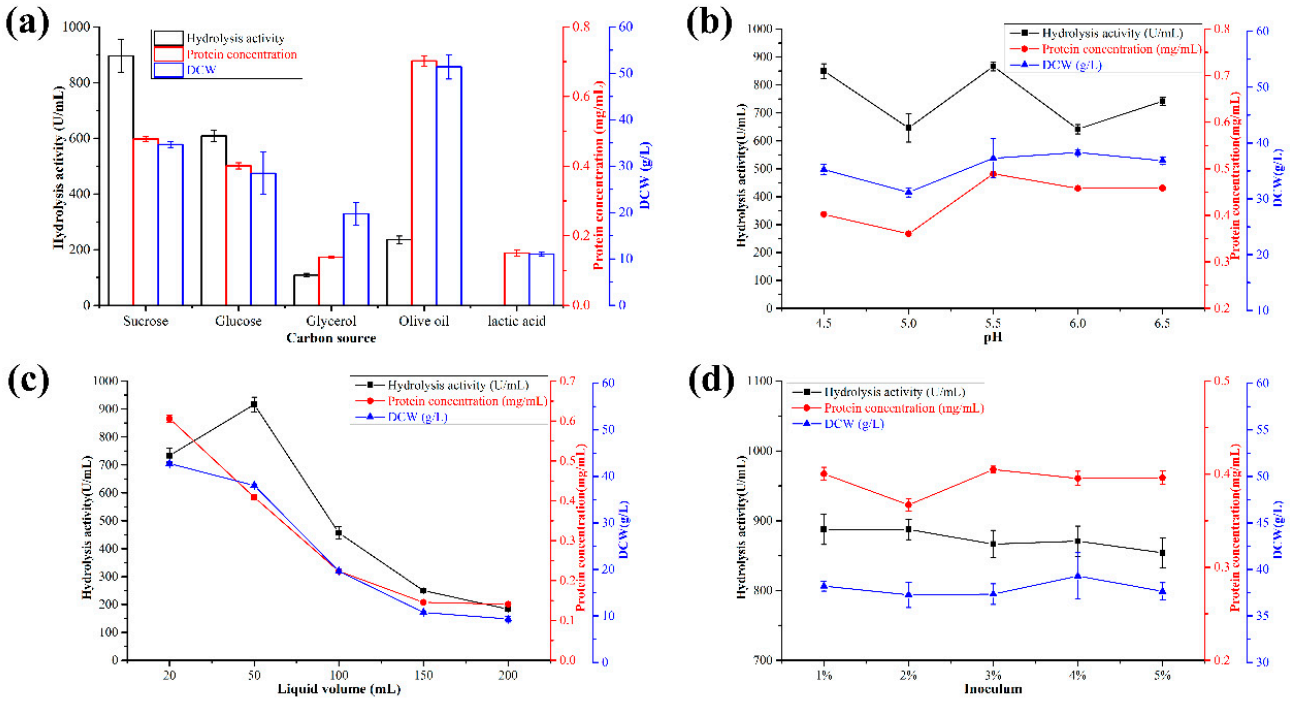

Figure 1. Optimization of the best engineered *Y. lipolytica* strain cultivation in 500 mL shake flasks. Effects of (**a**) different carbon sources in culture media on lipase hydrolysis activity, single-cell proteins (SCPs), and total protein concentration; (**b**) variation in initial pH on lipase hydrolysis activity, SCPs, and total protein concentration; (**c**) different initial liquid volumes of culture medium on lipase hydrolysis activity, SCPs, and total protein concentration; and (**d**) inoculum size on lipase hydrolysis activity, SCPs, and total protein concentration. All values are mean ± SD from three independent experiments.

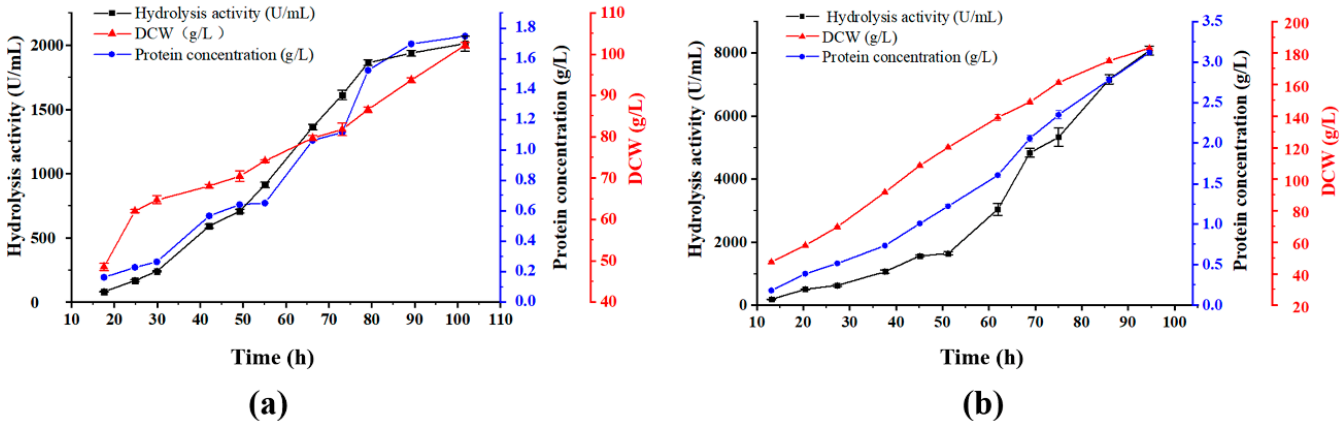

Figure 2. Time course of lipase hydrolysis activity (■ filled square), biomass (SCP) dry cell weight (DCW) (▲filled triangle), and total protein concentration (●filled circle) of the best engineered *Y. lipolytica* strain during fed-batch fermentation in a 10 L fermenter with 4 L initial modified FM22 medium. (**a**) Raw glycerol and sucrose were used as co-feeding carbon sources; (**b**) raw glycerol was used as the sole carbon source.

3.3. Fed-Batch Fermentation (30 L)

To achieve high cell biomass and lipase productivities, fed-batch studies were carried out in 30 L fermenters using the engineered *Y. lipolytica* strain. High lipase activity and biomass were achieved in 30 L pilot-scale studies using glycerol as the sole carbon source

through careful control of pH and aeration. At 136 h, the biomass, lipase activity, and protein concentration reached 173.3 g/L (DCW), 11,100.0 U/mL, and 2.1 g/L, respectively (Figure 3).

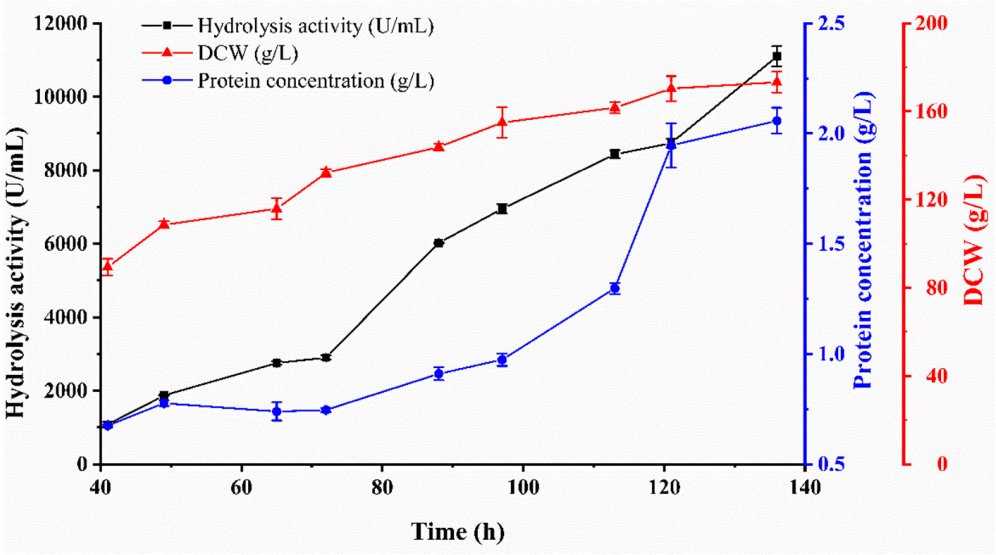

Figure 3. Time course of lipase hydrolysis activity (■ filled square), DCW of biomass (SCP) (▲filled triangle), and total protein concentration (● filled circle) during fed-batch fermentation of the best engineered *Y. lipolytica* strain in a 30 L fermenter with 15 L initial modified FM22 medium. Raw glycerol was used as the sole carbon source.

3.4. Pilot-Scale Fermentation (100 L)

Using the same two-stage shift strategy as described above, 100 L fermentation achieved a good balance between lipase expression and biomass productivity at a controlled pH of 5.0 and an aeration rate of 2.0–4.0 m^3/h for a 137 h duration. At 136 h, the biomass, lipase activity and protein concentration reached 170.3 g/L (DCW), 8532.0 U/mL, and 2.5 g/L, respectively (Figure 4).

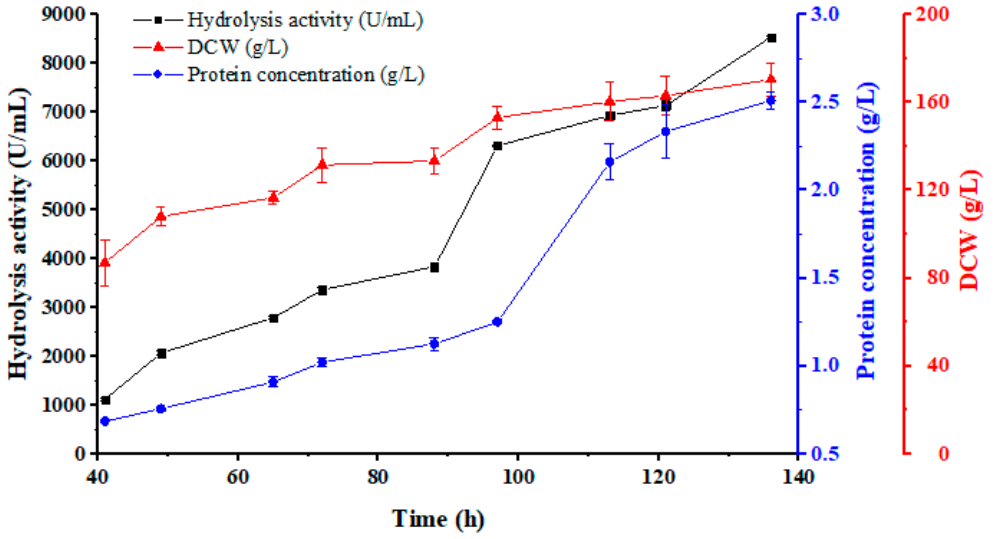

Figure 4. Time course of lipase hydrolysis activity (■ filled square), DCW of biomass (▲filled triangle), and total protein concentration (● filled circle) in a 100 L fermenter with 45 L initial modified FM22 medium of the best engineered *Y. lipolytica* strain during fed-batch fermentation. Raw glycerol was used as the sole carbon source.

3.5. SDS-PAGE Electrophoresis

Equal amounts of the supernatants from the 10 L, 30 L, and 100 L fermenters were boiled and subjected to 15% SDS-PAGE. In Figures 5 and 6, we identified a main band with a molecular mass of approximately 38 kDa in the supernatant from the fed-batch fermentation, which is the molecular mass of *Y. lipolytica* lipase (YLL) Lip2 [29]. As shown in Figure 5, the productivity of the recombinant target lipase increased with the extension of the fermentation time in a 10 L fermenter.

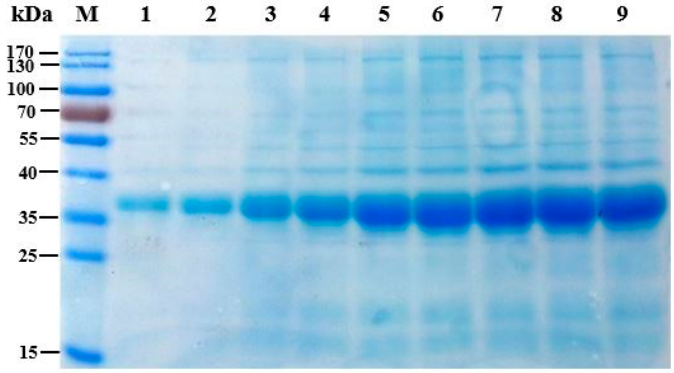

Figure 5. SDS-PAGE analysis ofsupernatant obtained during culture in a 10 L fermenter; raw glycerol fed-batch fermentations in a 10 L fermenter with a 4 L initial modified FM22 medium. Lane M, protein molecular weight marker (Fermentas, Burlington, Canada); lanes 1–9, 15 µL of culture supernatant at 27.3 h, 37.6 h, 45.1 h, 51.2 h, 61.9 h, 68.8 h, 75.0 h, 85.9 h, and 94.6 h, respectively.

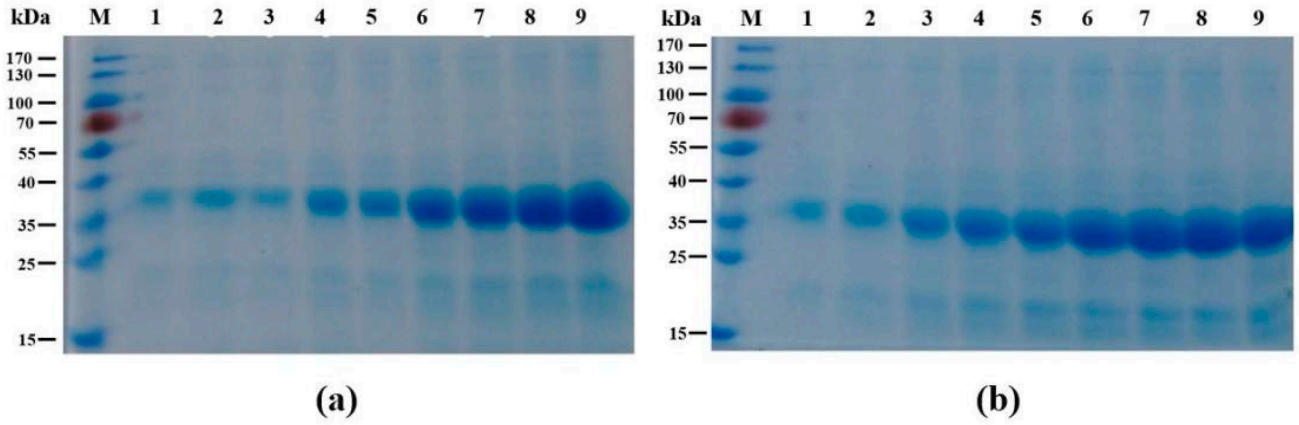

Figure 6. SDS-PAGE analysis of culture supernatant obtained during cultivation in 30 L and 100 L fermenters. (**a**) Raw glycerol fed-batch fermentations in a 30 L fermenter with an initial modified FM22 medium of 15 L. Lane M, Fermentas pre-stained protein ladder 26616 (Fermentas, Burlington, Canada); lanes 1–9, 15 µL of culture supernatant at 41 h, 49 h, 65 h, 72 h, 88 h, 97 h, 113 h, 121 h, and 136 h, respectively. (**b**) Raw glycerol fed-batch fermentations in a 100 L fermenter with a 45 L initial modified FM22 medium. Lane M, low-molecular-weight marker; lanes 1–9, 15 µL of culture supernatant in a 100 L fermenter after 41 h, 49 h, 65 h, 72 h, 88 h, 97 h, 113 h, 121 h, and 136 h, respectively.

Similar to the expression level in 10 L fermentation, the YLL bands thickened and accumulated as the fermentation progressed, suggesting that the rise in YLL productivity increases with the fermentation time in both the 30 L and 100 L fermenters (Figure 6).

In the above flask culture cases, and 10 L, 30 L, and 100 L fed-batch fermentation, YLL was the major protein in the culture supernatants, and extracellular lipase was efficiently produced together with high productivity of single-cell biomass in the *Y. lipolytica* strain.

4. Discussion

Despite intensive research and increasing attention, the *Y. lipolytica* system is still undergoing development compared to *Picha pastoris* and *Saccharomyces cerevisiae systems* [32–34]. To achieve high lipase productivity, the extracellular lipase Lip2 produced by *Y. lipolytica* has been investigated using a fed-batch strategy based on separate phases of cell growth and lipase production in bioreactors [29,35]. Nonetheless, only a studies have concurrently focused on the high production of lipase and SCP. The development of a fed-batch fermentation strategy for the simultaneous production of lipase and cell biomass is economically important.

In this context, the culture conditions of the best engineered *Y. lipolytica* strain were first optimized using a single factorial design of a 500 mL shake flask in the present study. The highest bioactivity of lipase was 880.6 U/mL, when sucrose, a less expensive carbon source, was used, and 32.0 g of dry SCP per liter of fermentation broth was obtained in shake flask. However, *Y. lipolytica* lipase and biomass productivity were low when glycerol was used as the sole carbon source in the shake flasks. At the end of the culture, the pH of the fermentation broth in the shake flask was below 4.0, owing to organic acid production during cell metabolism. It is known that an extremely acidic microenvironment is detrimental to yeast growth and leads to less lipase production, although cells grow most rapidly in a YPG medium [36,37].

The maximal YLL activity was 3044.0 U/mL when feeding glucose and olive oil in a 20 L bioreactor, as reported by Fickers et al. [35]. In contrast, using the above optimal flask culture conditions, the lipase activity reached 2012.5 U/mL, and the final biomass was 102.2 g/L at 101.7 h with our stepwise-feeding strategy involving sucrose and glycerol in 10 L fermentation. Importantly, when raw glycerol was used as an alternative carbon source, the lipase activity and final biomass reached 8083.3 U/mL and 183.1 g dry SCP/L at 94.6 h in 10 L fermentation, respectively. When glycerol alone was used, lipase and dry biomass productivity far exceeded the levels in our stepwise-feeding strategy with sucrose or glycerol. The main reason for these differences in the results in culture between the shake flask and fermenter is that the key parameter, pH, could be regulated automatically by the addition of ammonium hydroxide during 10 L fermentation, whereas, the pHof the fermentation broth in the flask was significantly decreased owing to organic acid production during cell metabolism.

Based on the above results of 10 L fermentation, glycerol was utilized as the sole carbon source in the large-scale fed-batch fermentation of the engineered strain. Turki et al. [38] reported that the stepwise feeding strategy combined with uncoupled cell growth and lipase production phases resulted in lipase activity exceeding 10,000.0 U/mL. The extracellular lipase activity and SCP weight reached 11,100.0 U/mL and 173.3 g of dry SCP per liter, respectively, at 136 h in 30 L of broth in this study. As shown in Table 1, the highest specific activity was 7133.8 U/mg total protein in the culture supernatant at 97 h, which is 7.1-fold higher than the 1000 U/mg specific lipase activity [38], demonstrating that high yields of lipase and SC were achieved simultaneously in large-scale fed-batch fermentation.

As shown in Table 2, the extracellular lipase activity reached 8532.0 U/mL together with the production of 170.3 g of dry SCP/L fermentation broth at 136 h of 100 L fed-batch fermentation, which is slightly lower than that attained with 30 L fermentation. At 97 h of culture, the lipase-specific activity also peaked, 5042.7 U/mg total protein, in the culture supernatant of 100 L, similar to the results of 30 L fermentation, implying that the total duration of fermentation could be shortened. Our results indicate that the crude glycerol-feeding strategy based on modified FM22 is suitable for successful fermentation aimed at simultaneously achieving high productivity of lipase and biomass in the recombinant Y. *lipolytica* strain. As a low-cost and easily available substrate, crude glycerol contains impurities such as fatty acid methyl esters (oleic acid is the main one), salts, soap glycerides, and metal ions. Some ingredients of crude glycerol, such as fatty acid methyl esters, salts, and metal ions, are beneficial for achieving excellent target protein

productivity, as reported by Yan et al. [26] and Imatoukene et al. [39]. An excellent balance between lipase secretion and growth (biomass) in recombinant *Y. lipolytica* cells was achieved with a simple step-feeding operation using cheap crude glycerol as a substrate in large-scale batch fermentation. These results indicate that an appropriate fermentation strategy could provide clues to produce target proteins and biomass with *Y. lipolytica* at a large scale.

Table 1. Yield and specific activity during 30 L fermentation.

30 L Fermentation Period (h)	pH	Total Protein (g/L)	Dry Weight (g/L)	Specific Activity (U/mg)
17.0	5.55			
41.0	5.61	0.68 ± 0.01	89.3 ± 3.8	1591.8 ± 109.7
49.0	5.60	0.78 ± 0.01	108.7 ± 1.5	2412.1 ± 26.5
65.0	5.63	0.74 ± 0.04	115.9 ± 4.9	3719.6 ± 181.0
72.0	5.52	0.75 ± 0.01	132.1 ± 1.7	3895.3 ± 115.2
89.0	5.48	0.91 ± 0.03	143.8 ± 1.5	6611.1 ± 236.3
97.0	5.47	0.97 ± 0.03	155.0 ± 6.9	7133.8 ± 281.1
113.0	5.53	1.30 ± 0.03	161.7 ± 2.5	6501.8 ± 71.4
121.0	5.54	1.95 ± 0.10	170.3 ± 5.7	4490.2 ± 229.5
136.0	5.46	2.06 ± 0.06	173.3 ± 4.7	5393.6 ± 24.7

Table 2. Yield and specific activity during 100 L fermentation.

100 L Fermentation Period (h)	pH	Total Protein (g/L)	Dry Weight (g/L)	Specific Activity (U/mg)
17.0	4.93			
41.0	5.61	0.69 ± 0.01	87.0 ± 10.6	1630.9 ± 41.7
49.0	5.59	0.76 ± 0.01	108.0 ± 4.4	2730.0 ± 38.0
65.0	5.63	0.91 ± 0.03	116.5 ± 2.8	3065.5 ± 101.6
72.0	5.51	1.02 ± 0.03	131.5 ± 7.9	3293.1 ± 138.2
89.0	5.49	1.13 ± 0.03	133.4 ± 6.1	3408.7 ± 143.6
97.0	5.62	1.25 ± 0.01	153.0 ± 5.3	5042.7 ± 11.8
113.0	5.70	2.16 ± 0.10	160.3 ± 8.6	3215.0 ± 191.6
121.0	5.56	2.33 ± 0.15	163.0 ± 8.9	3063.1 ± 145.9
136.0	5.55	2.51 ± 0.05	170.3 ± 7.4	3400.1 ± 79.6

Additionally, yeast–hyphal form transition (dimorphism) was clearly observed in the fermentation process of the nonconventional yeast *Y. lipolytica*, consistent with the findings of previous studies [39–41]. As shown in Supplementary Figure S1, seed *Y. lipolytica* cultured in the YPD medium was a typical ovoid, whereas *Y. lipolytica* cultured in the modified FM22 medium at pH 4.93 (100 L) and pH 5.55 (30 L) after 17 h of fermentation was predominantly hypha. In contrast, most of the dimorphic *Y. lipolytica* cells were cultured with modified FM22 medium as ovoid yeast cells during the bioprocess in 30 L and 100 L fermenters. The relationship between the morphological form and bioactivity of *Y. lipolytica* will be further investigated in microenvironments inside a bioreactor to achieve a good balance of lipase and cell biomass productivity [42,43].

5. Conclusions

In this study, the glycerol feeding strategy with modified FM22 was suitable for successful batch fermentation to simultaneously achieve high lipase and biomass productivity with Y. *lipolytica*. An excellent balance between lipase secretion and growth of *Y. lipolytica* cells was achieved with an easy stepwise operation in batch fermentation. These results indicate that batch fermentation of *Y. lipolytica* could provide clues for achieving a good balance between biomass and target protein productivity by adopting a stepwise-feeding strategy for industrial catalysis.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation9030268/s1, Figure S1: Yeast–hyphal form transition (dimorphism) can be clearly observed in the fermentation process.

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References

1. Madzak, C. Engineering *Yarrowia lipolytica* for use in biotechnological applications: A review of major achievements and recent innovations. *Mol. Biotechnol.* **2018**, *60*, 621–635. [CrossRef]
2. Groenewald, M.; Boekhout, T.; Neuvéglise, C.; Gaillardin, C.; van Dijck, P.W.; Wyss, M. *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. *Crit. Rev. Microbiol.* **2014**, *40*, 187–206. [CrossRef] [PubMed]
3. Nicaud, J.M. *Yarrowia lipolytica*. *Yeast* **2012**, *29*, 409–418. [CrossRef] [PubMed]
4. Abdel-Mawgoud, A.M.; Markham, K.A.; Palmer, C.M.; Liu, N.; Stephanopoulos, G.; Alper, H.S. Metabolic engineering in the host *Yarrowia lipolytica*. *Metab. Eng.* **2018**, *50*, 192–208. [CrossRef] [PubMed]
5. Madzak, C. *Yarrowia lipolytica* strains and their biotechnological applications: How natural biodiversity and metabolic engineering could contribute to cell factories improvement. *J. Fungi* **2021**, *7*, 548. [CrossRef]
6. Larroude, M.; Rossignol, T.; Nicaud, J.M.; Ledesma-Amaro, R. Synthetic biology tools for engineering *Yarrowia lipolytica*. *Biotechnol. Adv.* **2018**, *36*, 2150–2164. [CrossRef]
7. Gündüz Ergün, B.; Hüccetoğulları, D.; Öztürk, S.; Çelik, E.; Çalık, P. Established and upcoming yeast expression systems. *Methods Mol. Biol.* **2019**, *1923*, 1–74. [CrossRef]
8. Liu, H.H.; Ji, X.J.; Huang, H. Biotechnological applications of *Yarrowia lipolytica*: Past, present and future. *Biotechnol. Adv.* **2015**, *33*, 1522–1546. [CrossRef]
9. Markham, K.A.; Alper, H.S. Synthetic biology expands the industrial potential of *Yarrowia lipolytica*. *Trends Biotechnol.* **2018**, *36*, 1085–1095. [CrossRef]
10. Celińska, E.; Nicaud, J.M. Filamentous fungi-like secretory pathway strayed in a yeast system: Peculiarities of *Yarrowia lipolytica* secretory pathway underlying its extraordinary performance. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 39–52. [CrossRef]
11. Niehus, X.; Crutz-Le Coq, A.M.; Sandoval, G.; Nicaud, J.M.; Ledesma-Amaro, R. Engineering *Yarrowia lipolytica* to enhance lipid production from lignocellulosic materials. *Biotechnol. Biofuels.* **2018**, *11*, 11. [CrossRef]
12. Xie, D.; Miller, E.; Sharpe, P.; Jackson, E.; Zhu, Q. Omega-3 production by fermentation of *Yarrowia lipolytica*: From fed-batch to continuous. *Biotechnol. Bioeng.* **2017**, *114*, 798–812. [CrossRef] [PubMed]

13. Sun, M.L.; Shi, T.Q.; Lin, L.; Ledesma-Amaro, R.; Ji, X.J. Advancing *Yarrowia lipolytica* as a superior biomanufacturing platform by tuning gene expression using promoter engineering. *Bioresour. Technol.* **2022**, *347*, 126717. [CrossRef]
14. Wang, K.; Shi, T.Q.; Lin, L.; Wei, P.; Ledesma-Amaro, R.; Ji, X.J. Engineering *Yarrowia lipolytica* to produce tailored chain-length fatty acids and their derivatives. *ACS Synth. Biol.* **2022**, *11*, 2564–2577. [CrossRef] [PubMed]
15. Jach, M.E.; Malm, A. *Yarrowia lipolytica* as an alternative and valuable source of nutritional and bioactive compounds for humans. *Molecules* **2022**, *27*, 2300. [CrossRef]
16. Cao, L.; Yin, M.; Shi, T.Q.; Lin, L.; Ledesma-Amaro, R.; Ji, X.J. Engineering *Yarrowia lipolytica* to produce nutritional fatty acids: Current status and future perspectives. Synth. *Syst. Biotechnol.* **2022**, *7*, 1024–1033. [CrossRef] [PubMed]
17. Wang, K.; Shi, T.Q.; Lin, L.; Wei, P.; Ledesma-Amaro, R.; Ji, X.J.; Huang, H. Advances in synthetic biology tools paving the way for the biomanufacturing of unusual fatty acids using the *Yarrowia lipolytica* chassis. *Biotechnol. Adv.* **2022**, *59*, 107984. [CrossRef]
18. Zinjarde, S.S. Food-related applications of *Yarrowia lipolytica*. *Food Chem.* **2014**, *152*, 1–10. [CrossRef]
19. Carsanba, E.; Papanikolaou, S.; Erten, H. Production of oils and fats by oleaginous microorganisms with an emphasis given to the potential of the nonconventional yeast *Yarrowia lipolytica*. *Crit. Rev. Biotechnol.* **2018**, *38*, 1230–1243. [CrossRef]
20. Galvagno, M.A.; Iannone, L.J.; Bianchi, J.; Kronberg, F.; Rost, E.; Carstens, M.R.; Cerrutti, P. Optimization of biomass production of a mutant of *Yarrowia lipolytica* with an increased lipase activity using raw glycerol. *Rev. Argent. Microbiol.* **2011**, *43*, 218–225. [CrossRef]
21. Vandermies, M.; Fickers, P. Bioreactor-scale strategies for the production of recombinant protein in the yeast *Yarrowia lipolytica*. *Microorganisms* **2019**, *7*, 40. [CrossRef]
22. Yuzbasheva, E.Y.; Taratynova, M.O.; Fedyaeva, I.M.; Dementev, D.A.; Korobov, V.S.; Fedorov, A.S.; Vidal, L.S.; Yuzbashev, T.V.; Sineoky, S.P.; Mikheev, M. Large-scale bioproduction of natural astaxanthin in *Yarrowia lipolytica*. *Bioresour. Technol. Rep.* **2023**, 101289. [CrossRef]
23. Delvigne, F.; Zacchetti, B.; Fickers, P.; Fifani, B.; Roulling, F.; Lefebvre, C.; Neubauer, P.; Junne, S. Improving control in microbial cell factories: From single-cell to large-scale bioproduction. *FEMS Microbiol. Lett.* **2018**, *365*, fny236. [CrossRef] [PubMed]
24. Spagnuolo, M.; Hussain, M.S.; Gambill, L.; Blenner, M. Alternative substrate metabolism in *Yarrowia lipolytica*. *Front. Microbiol.* **2018**, *9*, 1077. [CrossRef] [PubMed]
25. Fickers, P.; Benetti, P.H.; Waché, Y.; Marty, A.; Mauersberger, S.; Smit, M.S.; Nicaud, J.M. Hydrophobic substrate utilization by the yeast *Yarrowia lipolytica*, and its potential applications. *FEMS Yeast Res.* **2005**, *5*, 527–543. [CrossRef]
26. Yan, J.; Han, B.; Gui, X.; Wang, G.; Xu, L.; Yan, Y.; Madzak, C.; Pan, D.; Wang, Y.; Zha, G.; et al. Engineering *Yarrowia lipolytica* to simultaneously produce lipase and single cell protein from agro-industrial wastes for feed. *Sci. Rep.* **2018**, *8*, 758. [CrossRef] [PubMed]
27. Stratton, J.; Chiruvolu, V.; Meagher, M. High cell-density fermentation. In *Methods in Molecular Biology: Pichia Protocols*; Higgins, D.R., Cregg, J.M., Eds.; Humana Press: Totowa, NJ, USA, 1998; Volume 103, pp. 107–120.
28. Thorpe, E.D.; d'Anjou, M.C.; Daugulis, A.J. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnol. Lett.* **1999**, *21*, 669–672. [CrossRef]
29. Wang, X.; Sun, Y.; Shen, X.; Ke, F.; Zhao, H.; Liu, Y.; Xu, L.; Yan, Y. Intracellular expression of *Vitreoscilla hemoglobin* improves production of *Yarrowia lipolytica* lipase LIP2 in a recombinant *Pichia pastoris*. *Enzyme Microb. Technol.* **2012**, *50*, 22–28. [CrossRef] [PubMed]
30. Braford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
31. Barnett, W.; Spragg, S. Estimation of molecular weights of protein-SDS Complexes. *Nat. New Biology.* **1971**, *234*, 191–192. [CrossRef]
32. Koch, B.; Schmidt, C.; Daum, G. Storage lipids of yeasts: A survey of nonpolar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*. *FEMS Microbiol. Rev.* **2014**, *38*, 892–915. [CrossRef] [PubMed]
33. Baghban, R.; Farajnia, S.; Rajabibazl, M.; Ghasemi, Y.; Mafi, A.; Hoseinpoor, R.; Rahbarnia, L.; Aria, M. Yeast expression systems: Overview and recent advances. *Mol. Biotechnol.* **2019**, *61*, 365–384. [CrossRef]
34. Patra, P.; Das, M.; Kundu, P.; Ghosh, A. Recent advances in systems and synthetic biology approaches for developing novel cell-factories in non-conventional yeasts. *Biotechnol. Adv.* **2021**, *47*, 107695. [CrossRef]
35. Fickers, P.; Destain, J.; Thonart, P. Improvement of *Yarrowia lipolytica* lipase production by fed-batch fermentation. *J. Basic Microbiol.* **2009**, *49*, 212–215. [CrossRef]
36. Mamaev, D.; Zvyagilskaya, R. *Yarrowia lipolytica*: A multitalented yeast species of ecological significance. *FEMS Yeast Res.* **2021**, *21*, foab008. [CrossRef] [PubMed]
37. Mears, L.; Stocks, S.M.; Albaek, M.O.; Cassells, B.; Sin, G.; Gernaey, K.V. A novel model-based control strategy for aerobic filamentous fungal fed-batch fermentation processes. *Biotechnol. Bioeng.* **2017**, *114*, 1459–1468. [CrossRef] [PubMed]
38. Turki, S.; Ayed, A.; Chalghoumi, N.; Weekers, F.; Thonart, P.; Kallel, H. An enhanced process for the production of a highly purified extracellular lipase in the non-conventional yeast *Yarrowia lipolytica*. *Appl. Biochem. Biotechnol.* **2010**, *160*, 1371–1385. [CrossRef]
39. Imatoukene, N.; Back, A.; Nonus, M.; Thomasset, B.; Rossignol, T.; Nicaud, J.M. Fermentation process for producing CFAs using *Yarrowia lipolytica*. *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 403–412. [CrossRef]

40. Wu, H.; Shu, T.; Mao, Y.S.; Gao, X.D. Characterization of the promoter, downstream target genes and recognition DNA sequence of Mhy1, a key filamentation-promoting transcription factor in the dimorphic yeast *Yarrowia lipolytica*. *Curr. Genet.* **2020**, *66*, 245–261. [CrossRef]
41. Tsirigka, A.; Ntoula, M.; Kontogiannopoulos, K.N.; Karabelas, A.J.; Patsios, S.I. Optimization of solvent extraction of lipids from *Yarrowia lipolytica* towards industrial applications. *Fermentation* **2023**, *9*, 35. [CrossRef]
42. Pomraning, K.R.; Bredeweg, E.L.; Kerkhoven, E.J.; Barry, K.; Haridas, S.; Hundley, H.; LaButti, K.; Lipzen, A.; Yan, M.; Magnuson, J.K.; et al. Regulation of yeast-to-hyphae transition in *Yarrowia lipolytica*. *mSphere* **2018**, *3*, e00541-18. [CrossRef] [PubMed]
43. Shu, T.; He, X.Y.; Chen, J.W.; Mao, Y.S.; Gao, X.D. The pH-responsive transcription factors YlRim101 and Mhy1 regulate alkaline pH-induced filamentation in the dimorphic yeast *Yarrowia lipolytica*. *mSphere* **2021**, *6*, e00179-21. [CrossRef] [PubMed]