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Study of Lipase Production by *Yarrowia Lipolytica* Grown in High Concentration of Hydrophobic Carbon Sources

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Yarrowia lipolytica is one of the most studied “non-conventional” oleaginous yeasts. It is able to produce a number of valuable proteins and metabolites such as lipases and other hydrolytic enzymes, single cell oils (SCOs), single cell proteins (SCPs), carboxylic acids, erythritol and γ-decalactone (Nicaud, 2012). Scientific research is focusing on *Y. lipolytica* due to its ability to grow on different carbon sources, both hydrophobic and hydrophilic, and to its high tolerance to broad pH ranges and salt concentrations (Miller and Halper, 2019). Lipases (EC 3.1.1.3) are a class of hydrolases which naturally catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at the oil–water interface in the presence of emulsions. Furthermore, lipases are very important for the synthesis of esters through esterification and transesterification reactions in anhydrous conditions, with high enantioselectivity. For their ability to catalyze specific biotransformations in different reaction media, lipases have been employed in several industrial sectors, from the pharmaceutical to food and chemical industries (Treichel et al., 2010). *Y. lipolytica* use as a sustainable platform for lipase production has been investigate using waste cooking oils (WCOs). The term WCO refers to oils and fats from food processing and storage at a commercial, industrial or household level. In Europe, WCOs annual production accounts for approximately 4 million tons/y (EBIA, 2021). If not collected and properly disposed of, WCOs can form the so-called “fatbergs” that can damage and clog the sewage system and pollute water and soil (Foo et al., 2014).
In this work, the capabilities of *Y. lipolytica* to grow on high concentrations of different hydrophobic carbon sources, such as Olive Oil and WCOs, among others, were investigated to evaluate their potential as inducers for lipase production.

1. Introduction

Yarrowia lipolytica is an ascomycete yeast classified in the family of the Dispodascaceae, it can be found in nature in several different environments ranging from processed foods (*e.g.*, cheese, processed meats) (Suzzi *et. al*, 2001; Barth and Gaillardin, 1996) to lipid-rich media (Smita, *et. al*, 1998) and marine environments with high saline concentrations (Beopoulos et al., 2010). Most strains of this species are unable to grow at temperatures higher than 32 °C and their metabolism is strictly aerobic. This feature contributed to its classification as non-pathogenic and Generally Regarded As Safe (GRAS) by the American Food and Drug Administration (Nicaud, 2012).
Y. lipolytica gained interest in the scientific community in the last 20 years due to its ability to grow on unconventional media such as hydrophobic carbon sources, at broad pH ranges, high saline concentrations (Miller, 2019) and due to the recent development of molecular tools for its efficient genetical manipulation (Beopoulos et al., 2010; Zhu and Jackson, 2015).
Nowadays *Y. lipolytica* is used in several processes for the production of industrially interesting molecules and enzymes such as lipases, which are gaining importance due to their natural function in the yeast for triacylglycerols (TAG) metabolism. These enzymes have a role in the accumulation of TAG in the cell, reaching up to 50% of cell weight, and on the conversion of media containing lipid rich carbon sources to other metabolites (Beopoulos et al., 2009).

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Lipases (EC 3.1.1.3) belong to the serine hydrolase family that act on carboxylic ester bonds and their natural function is to hydrolyze TAGs in diglycerides (DAG), monoglycerides (MAG), fatty acids (FA) and glycerol. Lipases primarily act at the water-oil interface with a kinetic model that involves two distinctive equilibria: the first equilibrium implicates a reversible adsorption of the enzymes to the interface, the second equilibrium involves the formation of an enzyme-substrate complex and it is characterized by a typical Michaelis-Menten kinetic (Verger, et al., 1973; Verger and Haas, 1976). Lipases also catalyze other reactions in addition to hydrolysis such as esterification and transesterification with high enantioselectivity and regioselectivity on TAGs and other substrates also in unconventional media. In light of these features, commercially produced microbial lipases find applications in various industrial processes (*e.g.*, food processing, pharmaceuticals, detergents).
Following the sequencing of the genome of *Y. lipolytica* haploid strain E150 (CLIB99) 16 lipase encoding genes were identified, which were grouped in the GL3R0084 family (Casaregola et al., 2000; Dujon et al., 2004). Lip2p (311 aa) encoded by the *LIP2* gene, Lip7p (341 aa) encoded by the *LIP7* gene and the Lip8p (371 aa) encoded by the *LIP8* gene are the most characterized lipases among all the identified genes. Lip2p has been identified as the most active extracellular lipase, while Lip7p and Lip8p are suggested to be mainly cell-bound (Fickers, et al., 2011). These lipases have enlarged the applications of *Y. lipolytica* in the field of oil-rich waste treatment, valorization and bioremediation. Several studies were conducted on the treatment of Oil-Mill Wastewaters (OMW) characterized by high Chemical Oxygen Demand (COD) and generated as an effluent from the olive oil industry (Ben Sassi et al., 2006; Marques I.P., 2001). *Y. lipolytica* ATCC 20255 was able to reduce COD levels by 80% in 24h, producing also extracellular lipases and single-cell proteins during the culture. (De Felice et al., 1997). The W29 (ATCC20460) strain tested by Lopes et. al (2008) also reduced the COD of OMW by 80% and the phenol composition by 70% while producing lipases. Among waste products, WCOs showed promising results as carbon source, in the orders of tens of g/L, for the production of valuable compounds by *Y. lipolytica* such as erythritol, lipases (Xiaoyan et al., 2017) and limonene (Li et al., 2022). In this work, higher concentrations of WCO have been tested and compared to free fatty acids (FFA), and Olive Oil (OO) to investigate the potential for lipase production using *Y. lipolytica* W29 strain.

2. Materials and Methods

2.1 Strains, Media and Culture Conditions

Yarrowia lipolytica W29 (ATCC20460) strain was used in this work. The strain was maintained in YPDA medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) at 4 °C and restreaked every week. A single colony was inoculated in 10 mL culture tubes containing 4 mL YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) and pre-cultivated overnight at 180 rpm and 29°C. Pre-cultures were collected and inoculated at a final OD_{600} of 0.1 in 50 mL Erlenmeyer flasks containing 20 mL YP medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 1% (v/v) TWEEN® 80 and different hydrophobic carbon sources (WCO, FFA and OO) at concentrations of 20 g/L, 100 g/L and 200 g/L. The cultures were incubated at 180 rpm for 216 h at 29°C. Samples were collected at different time points and centrifuged at 4500 rpm for 5 min. The cell pellets were collected, washed two times with sterile water to remove residual hydrophobic substances that could interfere with the spectrophotometric analysis and measured for the OD_{600}. The supernatants were, instead, diluted if necessary and used for the lipase activity assays.
OD_{600} were measured using a Thermo Spectronic Genesys 20 spectrophotometer (Thermo Fisher, USA). All materials were purchased from Sigma-Aldrich, WCO was kindly provided by Greenoil s.r.l. (Italy). The FFA mixture was produced from WCO as previously described (Salimon et al., 2011).

2.2 Lipase assay

Extracellular lipase activity was monitored at different time intervals by analyzing the supernatants with a 96-wells microplate spectrophotometric method using *p*-Nitrophenyl laurate as substrate following a previously described method with minor modifications (Biundo et al., 2015). The time course of absorbance was measured at 405 nm for 15 min using a Tecan Sunrise™ plate reader (TECAN, Switzerland) at pH 7 and 25 °C. The final substrate concentration was 100 µM in DMSO, in 220 µL final volume of the reaction mixture (20 mM K_2HPO_4, 150 mM NaCl, 0,01% Triton X-100, 22 µL supernatant sample). The measurements were done in triplicates and corrected with blanks that contained the same media conditions and buffer as the samples. One unit (U) of enzyme activity was expressed as the amount of enzyme releasing 1 µmol of *p*-Nitrophenol per minute at experimental conditions. All materials were purchased from Sigma-Aldrich (USA).

3. Results and discussion

3.1 Cell growth

Y. lipolytica W29 cell growth in different media was monitored through sampling and OD_{600} measurements at different time points. Growth data showed that *Y. lipolytica* accepted WCO, OO and FFA as carbon sources (*Table 1*). Higher concentrations of substrates correspond to higher measurements of OD_{600} for the 3 tested substrates. Particularly, at 100 g/L and 200 g/L OO concentration, after 168 h, the cells reached their highest OD_{600} signal of 53 and 60.5 respectively. OO in a concentration of 20 g/L induced a slower and lesser growth when compared to the other substrates at the same concentration. Similar growth profiles were shown among cells cultivated on media containing WCO and FFA. However, a higher biomass production on WCO was shown compared to FFA.

Table 1: OD_{600} measurements of Y. lipolytica cells grown on WCO, FFA and OO. Values are obtained from triplicates; all standard deviations were found to be < 10%.

Time (h)	24	72	96	144	168	192	216
WCO							
20 g/L	10.7	26.7	25.5	34.8	36.5	31.6	27
100 g/L	16	27.1	32.9	35.2	37.1	40.8	41.4
200 g/L	22.8	38.7	40	42	45.2	51.1	43.3
FFAs							
20 g/L	6.6	21.5	22.4	24.7	25.9	25.3	20.6
100 g/L	6.8	18.8	20.2	24.3	29.6	25.2	27.5
200 g/L	7.3	25.8	26.7	27.8	31.5	31.1	36.7
OO							
20 g/L	8.1	10.1	9	11.5	12.6	12	12.8
100 g/L	9	26.4	25.4	42.3	53	47.2	53.2
200 g/L	9.7	36.8	42.6	46.7	60.5	59.3	55

3.2 Lipase activity

Extracellular lipase activity was measured at different time points (*Figure 1)*.
Contrarily to biomass production (*Table 1*), higher lipase activity was shown in supernatants derived from WCO-containing cultures compared to OO and FFA-containing cultures despite other findings that suggest a significant correlation between high level of biomass production and lipase activity (Corzo and Revah, 1999). The highest lipase activity was found in the supernatants of the 200 g/L WCO-containing cultures after 192 h with a value of 0.925 ± 0.080 U/mL. The latter was similar to the highest activity measured for supernatants obtained in 100 g/L of WCO at the same time point. These results show that *Y. lipolytica* was not negatively affected by the higher concentration of this hydrophobic substrate.
Supernatants from OO cultures had the lowest activities when compared to cultures containing other hydrophobic substrates, reaching a maximum activity of 0.440 ± 0.060 U/ml after 216 h with a 200 g/L substrate concentration.
Results showed a direct correlation between carbon sources concentration and lipase activity with a few exceptions (lipase activity at 48 h in FFA). Lipase activity in the supernatant of cells grown on 20 g/L of FFA reached its maximum at 48 h, which then constantly decreased over time. This difference could be imputable to a higher availability of unesterified fatty acids in the FFA-containing media that could more promptly act as inducers on extracellular lipase production. Oleic acid, for instance, was reported to be one of the main inducers for the *LIP2* gene promoter (Fickers et al., 2004) and is capable of freely diffusing in the cell at concentrations higher than 10 µM (Beopoulos et al., 2009).
Notably, the activity peaks for different substrates were not recorded at the same time. For 100 g/L WCO, a first peak in activity was noted at 48 h and a second peak at 192 h. In the case of 200 g/L WCO, it can be observed that a first peak in activity occurs at the same time as the 100 g/L WCO which is quickly followed by a second activity peak at 96 h, which is not present in the 100 g/L WCO, and a final third peak at 192 h. The first two peaks showed activities that are 78% and 44% lower than the highest recorded peak at 192 h, respectively.

250

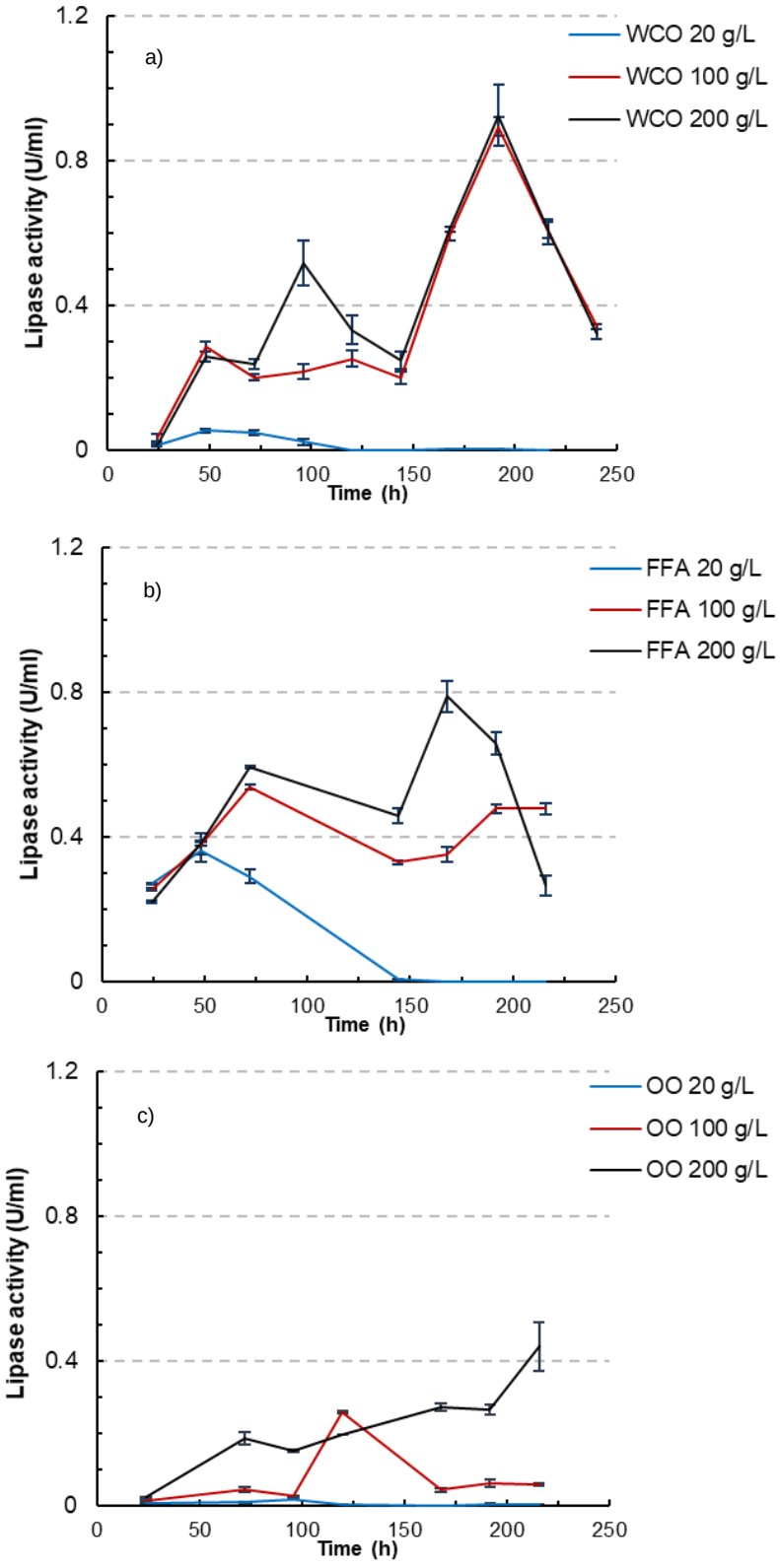

Figure 1: Lipase activity over time of supernatants obtained from a) WCO, b) FFA and c) OO cultures at different concentrations. All the values derive from triplicates; error bars are standard deviations.

FFA-containing cultures showed peaks in activity at later time points if compared to WCO. A first peak for 100 and 200 g/L FFA-containing cultures can be observed at 72 h. A second peak was observed at 192 h for the

100 g/L condition. For the culture containing 200 g/L FFA, instead, the second and highest peak reported for this substrate (0.788 ± 0.043 U/mL) appeared 24 h earlier. OO-containing cultures displayed lower lipase activities. 100 g/L and 200 g/L OO supernatants presented a first peak at 72 h. A second activity peak was observed at 120 h for the 100 g/L OO, followed by a decrease in activity. The 200 g/L OO showed, alternatively, the second and highest activity peak at the 216h time point (0.440 ± 0.067 U/mL).
In the cultures containing 20 g/L concentration of carbon sources, a single activity peak was observed, followed by the reduction of lipase activities at later time points that could possibly coincide with the total consumption of the carbon sources and, therefore, lack of *LIP2* inducers. Similar trends in lipase productivity have been previously noted in literature. Namely, on different concentrations of oleic acid used as a carbon source, *Y. lipolytica* presented different phase shifts in lipase productivity. When 30 g/L of oleic acid was used, a peak in activity around the same time point as the one found in the present study for the 20 g/L FFA-containing cultures was recorded (Fickers et al., 2004). This result was probably due to the prompt induction by FFA, among which oleic acid, on the *LIP2* promoter. As for lipases, also proteases can be expressed by *Y. lipolytica* in the culture media in the presence of inducers (Braga et al., 2012). These proteases could degrade extracellular lipases reducing their actual activity in time, determining the visible diminishing trend in activities after the peaks.
Similar lipase production by the W29 strain have also been reported. Notable examples come from the works of Braga et al. (2012) and Lopes et al. (2008). In the former, 70 g/L OO were used in an optimized medium as a carbon source and inducer, obtaining an extracellular lipase activity of 0.5 U/mL which resembles the results obtained in this work with 200 g/L OO (0.45 U/mL at 216 h). Accordingly, the work of Lopes showed similar activities (0.45 U/mL) in 600 mL bioreactors and lower OO titres (7 g/L) after fermentation optimization.
Only a few studies are available on WCO valorization for lipase and erythritol production using *Y. lipolytica*. In one of them, using *Y. lipolytica* M53, an atmospheric and room temperature plasma (ARTP) mutated strain, in the presence of 50 g/L WCO, extracellular lipase activities of 8 U/mL and 11.3 U/mL were obtained in a flask fermentation after 7 days and in a 3-L bioreactor after 72 h, respectively (Xiaoyan et al. 2017). Nunes et al. (2014) evaluated lipase production by *Y. lipolytica* 50682 using lower WCO and OO concentrations (10 g/L) showing extracellular activities (0.034 U/mL and 0.153 U/mL respectively) similar to those reported in this work at 24h of culture. The results obtained show that higher activities of extracellular lipases can be achieved with cultures longer than 24 h.To the best of our knowledge, this is the first report which shows concentrations of WCO higher than 100 g/L used for lipase and biomass production for *Y. lipolytica*.

4. Conclusions

In summary, it has been demonstrated that WCO can be used to produce lipases in *Yarrowia lipolytica* W29 under high concentration of hydrophobic substrates. The yeast could grow to optimal levels in the presence of high concentrations of hydrophobic substrates. Moreover, cell growth was shown to be enhanced in these conditions, triggering at the same time the production of lipase whose maximal recorded extracellular activity was 0.925 ± 0.08 U/mL after 198 h of culture on 200 g/L WCO supplemented media. Future analysis on media and cell lipid composition during the culture may bring new information capable of improving the results of this work for lipase and biomass production for the valorization of a waste product such as WCO. Further supernatant characterization could help identify the presence of different proteases which could be inhibited, probably increasing lipase activities. Media design approaches and genetic engineering could also improve the preliminary results showed in this work to promote the industrial feasibility of the process.
Overall, it can be concluded that WCO revealed itself as the best substrate, among the tested ones, for lipase production in *Y. lipolytica* and it is, thus, a suitable alternative to other carbon sources due to its cheap nature, availability and need of disposal. Its use as feedstock for cultures involving *Y. lipolytica* could bring interesting solutions to pollution problems and alternative approaches to the field of waste valorization.

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