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Physiological Characterisation of *Yarrowia lipolytica* Cultures Grown on Alternative Carbon Sources to Develop Microbial Platforms for Waste Cooking Oils Valorisation

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With the economic development, the non-renewable resources consumption and the energy crisis, microbial-based platforms represent a promising alternative route for the production of valuable chemicals. *Yarrowia lipolytica* is a non-conventional oleaginous yeast, which is able to grow on hydrophobic substrates, such as triglycerides, as carbon source. Its growth is associated with the production of different high-value bio-products, such as enzymes, organic acids, and lipids. In recent years, both basic and applied research have been carried out for improving its genetic manipulation and industrial use. The thorough knowledge of *Y. lipolytica* biolipid conversion and production pathways makes it a good candidate for its use as a cell factory for the design of alternative bioprocesses based on renewable substrates. Waste cooking oils (WCOs) are vegetable oils and animal fats that are discarded after food processing. Globally, high amounts of this waste product are produced every year. Due to their composition in triglycerides, WCO can be used as feedstock for microbial growth to create novel routes for the so-called "industrial symbiosis" following the circular economy approach, to upgrade a waste as a renewable feedstock for the bio-based industry. In particular, the goal of this study was the creation of new value chains from waste cooking oil (WCO) for the sustainable production of added-value compounds to valorize this type of waste and to reduce its incorrect disposal. Specifically, *Y. lipolytica* was investigated to evaluate its ability to grow in the presence of different concentrations of WCO compared to glucose as carbon source and determine its response both in terms of industrially-relevant compounds production and cell robustness. Flow cytometry analysis was performed to investigate the response of *Y. lipolytica* at increasing WCO concentrations in terms of intracellular lipids quantification and cellular viability.

1. Introduction

In the current scenario characterised by over-exploitation and mismanagement of resources, destruction of the ecosystems and climate change, a major challenge for our society is to achieve the transition to a circular bio-economy model. This new system is based on the development of innovative production strategies that are more environmentally friendly than ones based on fossil fuels. In this new approach, the identification of alternative renewable resources and the elimination of the concept of waste along the supply chain are mandatory (Madzak, 2021). White biotechnology, that uses microbial cell factories, plays a key role in the transition process due to utilisation of side-stream biomasses to manufacture compounds of industrial interest (Lee et al., 2019). Among the microorganisms suitable for industrial biotechnology, non-conventional yeasts, *i.e.* metabolically and biochemically different microorganisms than well-established model *Saccharomyces cerevisiae,* represent excellent cell factories due to high growth capacity, utilisation of different carbon source, resistance to extreme environmental conditions, and good tolerance to inhibitors (Rebello et al., 2018). *Yarrowia lipolytica* is a non-conventional oleaginous yeast, generally recognized as safe (GRAS), that have received increasing interest in the last decades for its characteristics. *Y. lipolytica* grows in the optimal temperature range of 25 to 30 °C and tolerates a wide range of pH values, from 2.0 to 9.7, even if the growth is optimal at neutral pH (Sutherland et al., 2014). This yeast shows a dimorphic switch, from round budding cells to mycelia with

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hyphae and *vice versa*, caused by physico-chemical conditions of growth. The control of its morphology is crucial when *Y. lipolytica* is used as cell factory at industrial scale (Timoumi et al., 2018). *Y. lipolytica* is an efficient producer of enzymes such as lipases, proteases, and RNases, that are easily secreted in the culture medium. This yeast also produces a wide range of metabolites, from organic acids, such citric (Carsanba et al., 2019), isocitric (Morgunov et al. 2019), succinic, α-ketoglutaric, acetic acids (Fickers et al., 2020) to polyols, mainly erythritol and mannitol (Bilala et al., 2020). In addition to these products, *Y. lipolytica,* as an oleaginous yeast, accumulates high quantities of lipids, mainly triacylglycerols (TAGs), stored in intracellular lipid bodies that represent up to 30 % to 50 % of cell dry weight mass. Lipids are produced through two different metabolic pathways, *de novo* and *ex novo* synthesis, which are activated by different starting substrates. When *Y. lipolytica* grows on hydrophilic substrates, such as small carbon molecules (*i.e.,* sugars, alcohols, organic acids), accumulates acetyl-CoA, when nitrogen in the nutrient medium is scarce. Acetyl-CoA is used as precursor for the fatty acids biosynthesis (Lazar et al., 2018). Instead, when the oleaginous yeast uses hydrophobic substrates (*i.e.* TAGs, methyl-esters, oils), they are converted into free fatty acids and transported inside the cell where they are transformed, thereby making a different fatty acids profile than the one present in the substrate. These new fatty acids are degraded into short chain acyl-CoAs via β-oxidation and are used both for cell energy requirements and for the synthesis of new cellular material, including lipid bodies. *Ex novo* synthesis is a pathway associated to growth, and it is independent from nitrogen concentration in the nutrient medium, while *de novo* pathway typically occurs during stationary growth phase when a medium component is exhausted (Carsanba et al., 2018). Over the last few years, there have been many research works regarding *Y. lipolytica* oil production, known as single-cell oils (SCOs), which have a huge potential both in the new-generation biodiesel industry (Darvishi et al., 2017) and in the food and healthcare industry (Beligon et al., 2016). The lipid production pathways point out that *Y. lipolytica* uses a wide array of substrate for its growth and metabolism, thereby showing a high potential for the valorisation of waste and by-products from various origins. Among the low-cost substrates, Waste Cooking Oils (WCOs), which are considered as one of the major streams of the food waste, are oil-based substances consisting of edible matter that has been used in the preparation of foods and is no longer suitable for human consumption (Falade et al., 2017). Due to huge amount of WCO generated (about 4 Mtons/year in European Union) (EUBIA, 2021), this waste represents a valuable asset for the development of bioprocesses for SCOs production, thereby avoiding its polluting disposal in the environment. To further explore this potential, in the present work, WCO was used as substrate for *Y. lipolytica* W29 growth. High-throughput growth screening was assayed to evaluate effects of hydrophilic (glucose) and hydrophobic (WCO) substrate at various concentrations. This screening was useful to provide information to support decision-making in fermentation scale-up bioprocess, that was further carried out in 50 mL-flasks. Specifically, WCO concentrations investigated in the flasks were higher than the ones used in other studies so far, in which the maximal concentration investigated was 100 g/L (Katre et al., 2012). In the batch cultivations system, physiological properties of *Y. lipolytica* W29 were analysed with flow cytometry. A fast investigation of lipid accumulation and cells viability was carried out, thereby providing a growth and metabolic profile of *Y. lipolytica* W29 as microbial platform for WCO valorisation.

2. Materials and methods

2.1. Strains and media

Yarrowia lipolytica W29 (ATCC20460) strain was used in this work. For strain preservation, the yeast was maintained at 4 °C on YPDA plates which contained yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L), and agar (20 g/L) at pH 4.8-5, and the culture was transferred to fresh YPDA plate every week to maintain viability. For the pre-culture of the seed cells, a single colony of yeast cells was inoculated in 10 mL culture tube containing 2 mL YPD medium and then incubated in a rotary shaker set to 180 rpm at 29 °C for 24 h. The nutrient media used for the cultures were rich and basal media. The rich medium was YP which contained 10 g/L yeast extract and 20 g/L peptone, while the basal medium was yeast nitrogen base (YNB) without amino acids and with 2 g/L of ammonium sulfate. Glucose, Linoleic Acid (C18:2, LA) or Waste Cooking Oil (WCO) were used as carbon source at different concentrations. When LA or WCO were used as substrate, TWEEN®80 was added in the nutrient medium at concentration of 1 % (v/v) as surfactant to improve fatty acids emulsification and cell membranes permeabilisation. The media were sterilized at 121 °C for 15 min. All materials were purchased from Sigma-Aldrich (USA) and WCO was kindly provided by Greenoil s.r.l. (Italy).

2.2 Sunrise Tecan™ growth conditions

Y. lipolytica W29 growth was detected in the Sunrise Tecan™ plate reader (TECAN, Switzerland) complemented by Magellan™ software using a 96-well microplate. Each experimental condition was performed in triplicate. For growing conditions optimisation, different working volumes (350 and 200 µL) and different values of the starting inoculum (OD_{600}: 0.1, 0.15, 0.3) from the 24 h pre-culture of the seed cells were investigated. Both non-diluted

and tenfold-diluted media were used to optimize Sunrise Tecan™ performances. After cells inoculum, the 96-well plate was covered with SealPlate®ColorTab™ film (Sigma-Aldrich, USA) to ensure sterility and aeration. The experiments were performed at the temperature of 29°C under constant wide shaking. Growth was monitored by measuring OD_{600} from the centre of each well every 17 min for about 48 h.

2.3 Flasks growth conditions and flow cytometry analysis

Batch cultivation of *Y. lipolytica* W29 was performed in 50 mL Erlenmeyer flasks with a working volume of 20 mL in rich medium with glucose or WCO as substrate at different concentrations. The medium was inoculated using a 24 h-pre-culture of the seed cells at initial OD_{600} of 0.1. Batch cultivations were carried out at 29 °C and 180 rpm in an orbital incubator for about 144 h. At appropriate intervals, aliquots from flasks were collected for the determination of cells growth, lipid production and cells viability. Cell growth was quantified by measuring OD_{600} with Thermo Spectronic Genesys 20 spectrophotometer (Thermo Fisher, USA). Lipid production and cells viability were analysed by Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher, USA) using specific fluorescent dyes. Nile Red (NR), 9-diethylamino-5H-benzo[a]phenoxazine-5-one, is a stain for the detection of intracellular lipid droplets by flow cytometry. Yeast cells with OD_{600} of 1 were stained with 3.8 µg/mL NR in 500 µL of volume reaction and incubated at 30 °C in the dark for 15 min. After a washing with cold H_2O, the yeast cell sample was diluted to obtain a final OD_{600} of about 0.1, that is the adequate cell density to obtain optimum fluorescence measurements. Cells viability was investigated with 7-aminoactinomycin (7-AAD), a fluorescent DNA intercalator, that penetrates only membrane-compromised yeast cells. Yeast cells sample with OD_{600} of 0.1 were stained with 1 µg/mL 7-AAD in 500 µL of volume reaction and incubated at 4 °C in the dark for 45 min. The flow cytometry analysis uses an exciting wavelength of 488 nm and an emission wavelength of both 695 ± 40 nm (BL-3) and 574 ± 26 nm (BL2), which detect red and orange fluorescence, respectively.

3. Results and discussion

3.1 Optimisation of high-throughput growth conditions

Y. lipolytica W29 is known for its ability to grow on both hydrophilic and hydrophobic substrates. In this study, different carbon sources were investigated as substrates for the oleaginous yeast growth. The Sunrise Tecan™ system was chosen for carrying out a preliminary study because it allows a high-throughput screening of different conditions. Therefore, the first growth study carried out in Sunrise Tecan™ system was conducted by using glucose and LA as substrates, at concentration of 20 and 25 g/L, respectively. The nutrient media used for this study were the rich medium and the basal medium, inoculated with OD_{600} of 0.1 of yeast cells. The final volume of each well was 350 µL. The growth curves (Figure 1) showed that glucose is a better substrate than LA. In fact, after 15 h, yeast cells reached an OD_{600} of about 0.7 in the presence of glucose, which is 57.2% higher than final OD_{600} reached with LA (Figure 1a and 1b). Moreover, growth is higher in YP than in YNB when glucose is used as substrate, while there is no difference between the two cultivation media with LA.

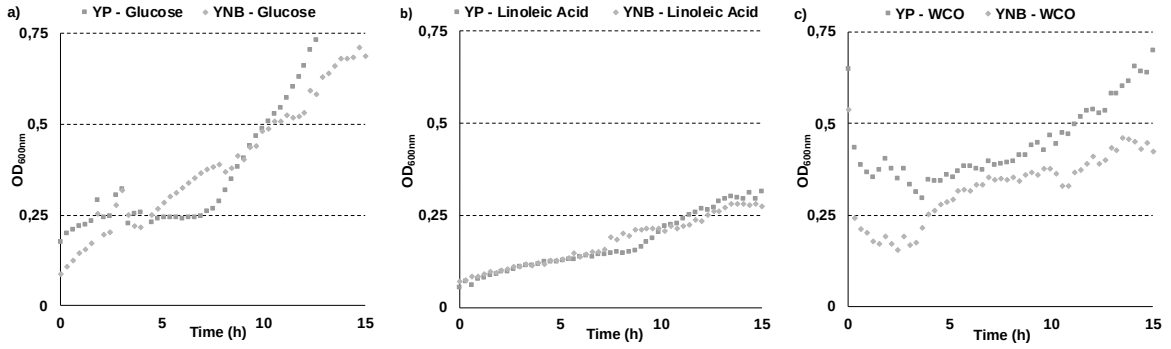

Figure 1: Cell growth profiles in Sunrise Tecan™ system *of Y. lipolytica W29 in rich (YP) and basal (YNB) medium with different substrates: a) Glucose 20 g/L; b) LA 25 g/L; c) WCO 10 g/L.*

These findings highlighted that probably hydrophobic substrate assimilation by *Y. lipolytica* W29 requires metabolic and morphological adaptations, thereby slowing down the growth. In fact, Bouchedja et al. (2017), which used a mixture of glucose and oleic acid for *Y. lipolytica* JMY 775 growth and lipid production, showed that oleic acid induced the formation of small protrusions across cell surfaces. Moreover, oleic acid consumption started only after glucose depletion, showing that glucose is a preferred substrate. As shown in other studies, WCO is a low-cost hydrophobic substrate that can be efficiently used because *Y. lipolytica*, through its lipases, hydrolyses lipid into glycerol and fatty acids. Therefore, WCO is useful for product synthesis, such as lipases (Nunes et al., 2014), lipids (Lopes et al., 2019), citric acid (Xiaoyan et al., 2015) and erythritol (Xiaoyan et al.,

2017). In this study, therefore, WCO was preliminary investigated as hydrophobic substrate at initial concentration of 10 g/L, both in rich medium and basal medium (Figure 1c). The concentration investigated was lower than the concentration of LA, to avoid growth inhibition as shown above. The results showed that biomass production was improved to OD_{600} 0.7 in YP supplemented with WCO, showing that *Y. lipolytica* W29 uses more efficiently WCO than LA. The growth curve on WCO was comparable to the curve obtained in YP with 20 g/L glucose, while in YNB yeast growth with WCO was slightly lower. However, the Sunrise Tecan™ system showed a low performance due to interferences of WCO when it was used as substrate. This problem was probably caused by low lipid emulsification in the small volume of each well, despite TWEEN®80 was added in the nutrient media as surfactant. In order to optimize growth conditions on WCO in the Sunrise Tecan™ system, media dilutions, higher initial OD_{600} and lower well volume were investigated. When *Y. lipolytica* W29 is inoculated at initial OD_{600} 0.15 in a final volume of 200 µL of tenfold diluted medium, the growth curves did not show any interference (Figure 2a). This better performance could mainly be ascribed to the dilution, which included both nutrient medium and substrate. The highest biomass in the rich medium reached a final OD_{600} of 0.7, instead it was only 0.3 in YNB medium. Similar results were obtained when an initial OD_{600} of 0.3 was used (data not shown). Due to higher growth in YP medium compared to YNB medium, YP medium was chosen for further experiments. The effect of different WCO concentrations (10, 20 and 40 g/L) were evaluated, using tenfold-diluted YP medium. As shown in Figure 2b, by increasing the initial WCO concentration from 10 to 40 g/L, the lag phase decreased from 5-6 h to 0 h, highlighting *Y. lipolytica* W29 ability to efficiently use higher amounts of WCO. To date, most research studies on transforming WCO into high-value products identified optimal initial concentration values for the substrate in the range from 10 to 30 g/L.

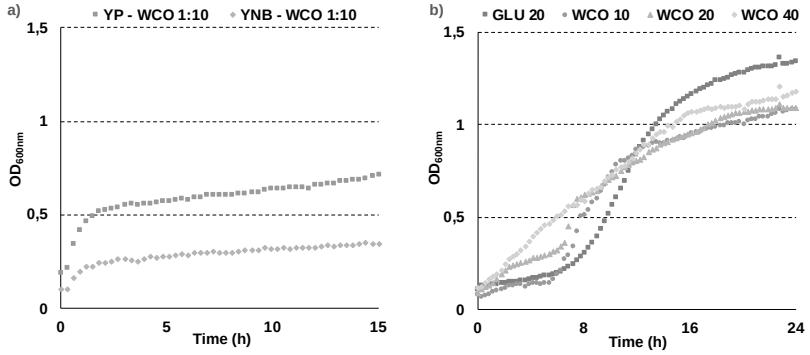

Figure 2: Cell growth profiles in Sunrise Tecan™ system of Y. lipolytica W29: a) in tenfold-diluted rich (YP) and basal (YNB) medium with WCO 10 g/L and b) in ten-fold diluted rich medium with Glucose 20 g/L, WCO 10, 20 and 40 g/L.

Xiaoyan et al. (2017) showed that the higher erythritol production (18.3 g/L) was reached at an initial WCO concentration of 30 g/L, with a biomass concentration of 8.1 g/L and lipase activity of 11.3 U/mL. Spalvins et al. (2020) highlighted that the maximal lipase activity was obtained a concentration of 10 g/L of WCO, while the higher lipid content was reached at 30 g/L of WCO. The findings of this preliminary study represent a proof of concept that can support further investigation of higher concentrations of WCO in the scale-up process.

3.2 Batch cultivation system scale-up

The first step for scaling-up the bioprocess for WCO valorisation was carried out in 50 mL Erlenmeyer flasks with a working volume of 20 mL. The conditions investigated were 20, 100 and 200 g/L of WCO concentration in the rich medium. Glucose (20 g/L) in YP was used as control as a comparison basis to evaluate the performance of other carbon substrates, including waste. These conditions will be indicated as WCO20, WCO100, WCO200 and GLU20, respectively. The growth was monitored by measuring OD_{600} at three different intervals of time from the yeast cells inoculum: 48, 72 and 144 h (Figure 3a). Growth of *Y. lipolytica* W29 in WCO20 showed 4 times and 3 times higher values than GLU20 at 48 and 72 h, respectively. The latter was maintained also for 144 h. Although, in WCO100 and WCO200, at 48 and 72 h growth was slightly lower than WCO20, while the OD_{600} in WCO100 and WCO200 at 144 h were 73.5 and 84, respectively, which are 26.3 % and 44.3 % higher than WCO20. These data probably indicated a lag phase due to metabolic adaptations at high concentration of hydrophobic substrate. To further investigate high WCO concentrations effects on *Y. lipolytica* W29, flow cytometry analysis was carried out. This is a powerful instrument that allows multi-parameters analysis using specific fluorescent dyes. The analysis with 7-AAD showed that yeast cells

maintained their viability at 144 h at the WCO concentrations tested (Figure 3b), highlighting those high concentrations of WCO did not have a negative effects on *Y. lipolytica* W29 viability.

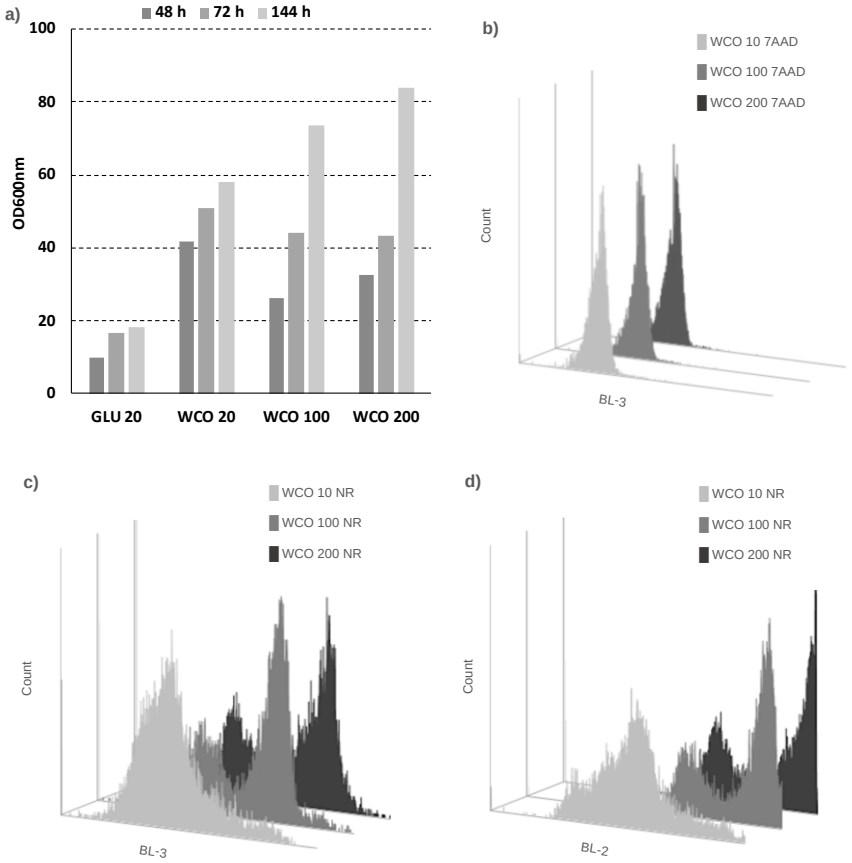

Figure 3: Batch cultivation system Y. lipolytica W29 in rich medium with Glucose 20 g/L, WCO 20, 100 and 200 g/L: a) cell growth profiles (OD_{600}) at three time intervals (48, 72 and 144 h); b) 7-AAD fluorescence intensity (BL-3); c) NR fluorescence intensity (BL-3); d) NR fluorescence intensity (BL-2). Fluoresce intensities for each dye were determined at 144 h in rich medium with WCO 20, 100 and 200 g/L.

When we used NR as dye, the red fluorescence (BL-3) is associated to phospholipids, while the orange one (BL-2) to TAGs. The histograms showed that fluorescence intensity revealed both by BL-3 and BL-2 channels at 144 h, increase with higher WCO concentrations (Figure 3c and 3d). The data from the flow cytometry analysis revealed that the higher amounts of lipid storage, in the form of lipid bodies, occur when *Y. lipolytica* W29 growth was carried out on 200 g/L WCO supplemented medium. Kameda et al. (2014) showed that red and orange fluorescence profile are similar because both lipids, polar and neutral, are into the lipid bodies. In the present study, however, orange fluorescence intensity is tenfold higher than red one, showing that TAGs are the predominant lipid type respect to phospholipids. To date, only in the research work of Katre et al. (2012) high concentrations of WCO were explored (up to 100 g/L), showing that only the *Y. lipolytica* NCIM 3589 was able to tolerate these WCO concentrations without any significant inhibition. In this study, it was demonstrated that the *Y. lipolytica* W29 is able to grow at higher concentrations of WCO up to 200 g/L without any detectable viability inhibition, showing how this yeast is a valuable microbial platform for lipid production.

4. Conclusions

In the present study, a physiological characterisation of *Y. lipolytica* W29 grown on high WCO concentrations was carried out. The Sunrise Tecan™ system was a useful instrument for the screening of different experimental conditions. The preliminary data from this system highlighted that the best grow conditions for *Y. lipolytica* W29 was the rich medium supplemented with WCO. These findings were confirmed in the batch cultivation system. Through flow cytometry, it was possible to analyse the viability and the lipid accumulation process of *Y. lipolytica* W29 grown on WCO. The data highlighted that 200 g/L WCO supplemented medium represents the optimal condition for lipid production, without any detectable inhibitory effects on growth. Therefore, it was demonstrated that *Y. lipolytica* W29 is a valuable microbial platform that can be employed in the WCO valorisation process,

for the production of industrially-relevant compounds, such as lipids. Further investigations on medium optimisation and quantification and characterisation of lipids will be an interesting process improvement in the industrial prospective.

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