



BIOCONVERSION OF VOLATILE FATTY ACIDS INTO MICROBIAL LIPIDS BY *YARROWIA LIPOLYTICA*

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Abstract

Volatile fatty acids (VFAs) are short chain fatty acids that can be obtained from organic wastes through acidogenic fermentation and can be used as carbon source for microbial lipids production. The bioconversion of acetate, propionate and butyrate into microbial lipids by *Yarrowia lipolytica* W29 was evaluated, and the yeast shown the ability to grow using VFA as carbon source and accumulate lipids intracellularly (around 5 % of dry cell mass). The addition of a co-substrate (glucose or glycerol) to VFA-based medium led to an enhancement of cellular growth and lipid content of the cells. The highest lipids concentration of around 1 g/L was obtained in batch cultures carried out with a mixture of VFAs and glycerol. Notwithstanding the low lipid content obtained in this work, *Y. lipolytica* demonstrated the ability to metabolize VFAs and convert them to microbial lipids, which can be used for biodiesel production.

INTRODUCTION

The global increase in energy demands, the depletion of fossil fuel reserves, climate change and increase in environmental pollution have attracted attention of researchers to find renewable and environmentally friendly energy source. Biodiesel is one of the most attractive alternatives for replacement of conventional fossil fuels due to its biodegradable, non-toxic and energy efficient properties. Conventionally, biodiesel is produced from a variety of feedstock, including plant/vegetable oils, but the high cost and limited supply of these feedstocks are the major problems for commercial production of biodiesel. Oleaginous microorganisms, which can accumulate lipids at more than 20% of their dry weight under specific culture conditions, are now considered as promising candidates for biodiesel production, since the composition of these microbial lipids are similar to that of



vegetable oils (Sitepu *et al.*, 2014). In the process of microbial lipids production by yeast, several advantages are recognized: requires few labor, can be accomplished under controlled conditions that are not dependent on venue, season or climate, has a short life cycle, can be cultivated on a wide variety of wastes and is easy to scale up (Beopoulos and Nicaud, 2012). Currently, the production cost of microbial oils is not economically competitive, so it is important to produce them from wastes or renewable feedstocks. Volatile fatty acids, produced during the acidification step of anaerobic digestion of organic wastes, can be used as new alternative carbon sources for the cost-effective production of microbial lipids (Gao *et al.*, 2017). The oleaginous yeast *Yarrowia lipolytica* has the ability to use a wide range of substrates, including VFAs, and has been used as a cell factory for the production of enzymes and microbial lipids from low-cost renewable feedstocks (Lopes *et al.*, 2018; Gao *et al.*, 2017). In this work, the ability of *Y. lipolytica* W29 to grow and accumulate lipids intracellularly using VFAs as carbon source (acetic, propionic and butyric acids) was evaluated in small scale batch cultures.

METHODOLOGY

Yeast strain preservation

Cryo-stocks of *Y. lipolytica* W29 (ATCC 20460) were prepared adding 800 μ L of yeast culture pre-grown overnight in YPD medium (peptone 20 g/L, yeast extract 10 g/L and glucose 20 g/L) to a 200 μ L of glycerol (purity 99.5 %) in a sterile microtube and stored at -80 °C.

Erlenmeyer flask experiments

Yeast cells were pre-grown overnight in a 500 mL Erlenmeyer flask filled with 200 mL of YPD medium inoculated with 1 cryo-stock, at 27 °C and 170 rpm on an orbital incubator (pre-inoculum). Batch cultures were carried out in 500 mL baffled Erlenmeyer flasks containing 200 mL of VFA-based medium, composed of 5 g/L VFA, 0.5 g/L yeast extract, 1.7 g/L YNB (without amino acids and ammonium sulfate) and ammonium sulfate in variable concentrations to obtain a C/N ratio of 75. Medium pH was adjusted to 6. Pre-inoculum was centrifuged and resuspended (at an initial cell density of 0.5 g/L) in batch cultivation medium, which were maintained on an orbital incubator for 48 h at 27 °C and 170 rpm. The addition of glucose (5 g/L) to each VFA-based medium was carried out to



study the effect of a co-substrate on yeast growth and lipids accumulation, as well as the use of a mixture of VFAs (2 g/L of each VFA) and glucose or glycerol (20 g/L), were tested in batch cultures of *Y. lipolytica*.

Analytical methods

Cellular concentration was quantified by optical density at 600 nm and converted to cell dry weight (g/L) by a calibration curve. Glucose, glycerol and VFAs (acetate, propionate and butyrate) were quantified by high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H column (300mm x 7.8mm, 8 μ m particle size) at 60 °C and with refractive index and ultra-violet detectors. The mobile phase used was sulfuric acid 5 mM at 0.5 mL/min. Intracellular lipids were quantified by phospho-vanillin colorimetric method from lyophilized cells, after extraction with methanol and chloroform (1:1, v/v), as described by Lopes *et al.* (2018). Microbial lipids were also visualized by fluorescence microscopy after staining with Nile red (excitation wavelength 470 - 490 nm). A cellular suspension (1/10, v/v) was mixed with a solution of Nile red (0.1 mg/mL in acetone) and incubated for 1 h at room temperature. After this time, cells were centrifuged, washed twice with PBS buffer 0.1 M, resuspended in PBS buffer 50 mM to a final absorbance (λ = 600 nm) of 5 and visualized in an Olympus BX 51 fluorescence microscope.

RESULTS

In batch cultures of *Y. lipolytica* W29 using each VFA as sole carbon and energy source, the maximum biomass concentration and lipid content were obtained in butyrate-based medium, followed by acetate and propionate (Table 1). The lowest values reached in propionate-based medium may be justified by the lower consumption rate (0.19 g/L·h) comparing to acetate (0.24 g/L·h) and butyrate (0.23 g/L·h) (Figure 1a). The addition of glucose (5 g/L) to each VFA-based medium led to an increase on biomass and lipid content, regardless of the VFA tested. Particularly for the experiment with butyrate, a 2.4-fold improvement on lipid content was attained with the addition of a co-substrate. The highest intracellular lipid concentration (0.55 grams of lipids per liter of medium) was reached in cultures using butyrate and glucose as carbon sources. In fact, the highest biomass and lipid concentration were obtained in butyrate-based medium, either used individually or combined with glucose. As occurred in experiments using each VFA



individually, also in the cultures supplemented with glucose the consumption rate of propionate was lower than the other VFAs (Figure 1b). The consumption of glucose was simultaneous with the consumption of VFA and a complete depletion of glucose was observed after 24 h of cultivation (data not shown). However, it was previously reported that lipids accumulation by *Y. lipolytica* is favored in acetate-based medium, since this VFA is directly converted to acetyl-CoA (a central intermediate in lipid synthesis), while propionate and butyrate undergo more metabolic transformations (Gao *et al.*, 2017; Kolouchová *et al.*, 2015; Fontanille *et al.*, 2012).

Table 1. Values of final biomass, biomass yield, lipid content (expressed as the ratio of lipids mass and dry cellular mass) and lipid concentration obtained in batch cultures of *Y. lipolytica* W29. Data are average \pm standard deviation for two independent replicates.

Carbon source	Biomass (g/L)	$Y_{x/s}$ (g/g)	Lipid content (% w/w)	Lipid concentration (g/L)
Acetate 5 g/L	3.3 ± 0.1	0.48 ± 0.01	3.2 ± 0.7	0.11 ± 0.02
Propionate 5 g/L	3.3 ± 0.1	0.54 ± 0.03	2.8 ± 0.5	0.09 ± 0.02
Butyrate 5 g/L	5.1 ± 0.1	0.86 ± 0.01	4.5 ± 0.5	0.23 ± 0.03
Acetate 5 g/L + Glucose 5 g/L	4.6 ± 0.8	0.50 ± 0.01	7.0 ± 0.5	0.32 ± 0.05
Propionate 5 g/L + Glucose 5 g/L	3.4 ± 0.1	0.48 ± 0.01	7.8 ± 0.9	0.27 ± 0.04
Butyrate 5 g/L + Glucose 5 g/L	7.4 ± 0.3	0.69 ± 0.01	7.5 ± 0.7	0.55 ± 0.04
Glucose 20 g/L + Mixture VFAs*	12.0 ± 0.5	0.53 ± 0.01	8.2 ± 0.7	0.99 ± 0.09
Glycerol 20 g/L + Mixture VFAs*	10.3 ± 0.3	0.39 ± 0.01	10.1 ± 1.1	1.04 ± 0.11

*Mixture of acetate, propionate and butyrate (2 g/L of each VFA).

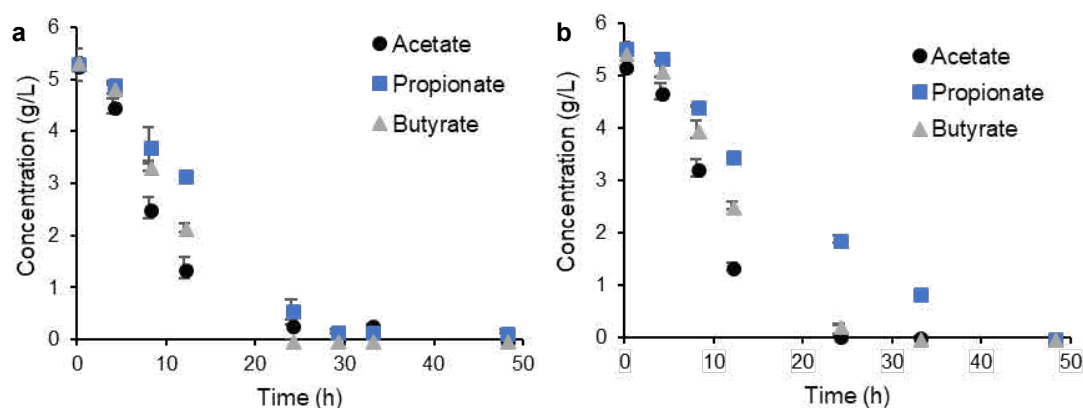


Figure 1. Profile of VFAs consumption by *Y. lipolytica* W29 on experiments performed with VFAs individually (5 g/L) (a) and supplemented with glucose (5 g/L) (b).



As the addition of glucose (5 g/L) led to an enhancement of final biomass and lipid concentration, and the product of a real acidogenic fermentation is a mixture of VFAs, additional experiments with higher glucose concentration and a mixture of VFAs were carried out. Additionally, the replacement of glucose by glycerol was also tested. In these experiments, higher biomass and lipid concentration were attained: a 4.5- and 1.9-fold improvement on intracellular lipid concentration was achieved comparatively with the experiments carried out with butyrate as sole carbon source and supplemented with glucose, respectively. Glucose and glycerol were consumed simultaneously with VFAs, and the consumption rate of propionate was lower than that of other VFAs (data not shown). The microscopic visualization of yeast cells growing on VFAs showed that yeast cells remained in a typical oval form (Figure 2). Moreover, it was possible to visualize the microbial lipids accumulated intracellularly by *Y. lipolytica* cells as lipid bodies (indicated by arrows). Some cells had more than one lipid body in the cytoplasm. These lipid bodies were smaller when yeast grown only on VFAs and increased in size with the addition of a co-substrate (glucose or glycerol).

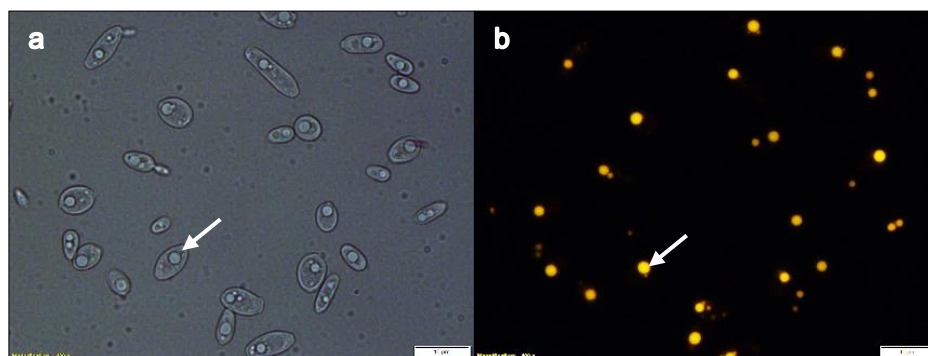


Figure 2. Light microscopy (a) and florescence microscopy images (b) of *Y. lipolytica* W29 cells stained with Nile red: lipid bodies visualization after growth on a mixture of VFAs supplemented with glucose (Magnification 1000 x).

CONCLUSIONS

This work demonstrates the possibility of using VFAs (acetic, propionic and butyric acid), commonly obtained by acidogenic fermentations of wastes, for cell growth and lipid accumulation by *Y. lipolytica* W29. The use of co-substrates (glucose or glycerol) allowed obtaining more biomass and lipids concentration than with only VFAs, demonstrating that the feasibility of this process may be improved by the presence of simple sugars resulted



from wastes hydrolysis and by mixing with others industrial by-products such as crude glycerol from biodiesel production, closing the loop of bioenergy production and favoring the circular economy.

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REFERENCES

- Beopoulos, A., Nicaud, J. (2012). Yeast: A new oil producer?. *OCL - Oilseeds and Fats, Crops and Lipids* 19(1), 22–28.
- Fontanille, P., Kumar, V., Christophe, G., Nouaille, R., Larroche, C. (2012). Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica*. *Bioresource Technology* 114, 443–449.
- Gao, R., Li, Z., Zhou, X., Cheng, S., Zheng, L. (2017). Oleaginous yeast *Yarrowia lipolytica* culture with synthetic and food waste - derived volatile fatty acids for lipid production. *Biotechnology for Biofuels* 10(247), 1–15.
- Kolouchová, I., Schreiberová, O., Sigler, K., Masak, J., Řezanka, T. (2015). Biotransformation of volatile fatty acids by oleaginous and non-oleaginous yeast species. *FEMS Yeast Research* 15(7).
- Lopes, M., Gomes, A.S., Silva, C.M., Belo, I. (2018). Microbial lipids and added value metabolites production by *Yarrowia lipolytica* from pork lard. *Journal of Biotechnology* 265, 76–85.
- Sitepu, I.R., Garay, L.A., Sestric, R., Levin, D., Block, D.E., German, J.B., Boundy-mills, K.L. (2014). Oleaginous yeasts for biodiesel: current and future trends in biology and production. *Biotechnology Advances* 32(7), 1336–1360.