Biotechnological conversions of crude glycerol, residue deriving from biodiesel production facilities, by strains of the yeast *Yarrowia lipolytica*

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Abstract
Aim of the present study was to assess the ability of four *Yarrowia lipolytica* strains (ACA-DC 50109, LFMB Y-20, ATCC 20460 and LMBF Y-45) to grow on biodiesel-derived crude glycerol, the principal residue-stream deriving from biodiesel manufacture. Initial trials were carried out in shake-flasks under nitrogen limitation (initial glycerol $Glol_0$~40 g/L, initial nitrogen ~0.35 g/L), that favor the production of cellular lipids and/or extra-cellular secondary metabolites like citric acid (CA). All strains produced appreciable dry cell weight (DCW) quantities (up to 13.0 g/L). The strain ACA-DC 50109 produced CA in concentrations up to 16.0 g/L, while lipid in DCW values of ~15% w/w were recorded. In the next stage, this strain was cultured on media with higher nitrogen limitation ($Glol_0$~50 g/L, initial nitrogen ~0.15 g/L) in batch-bioreactor and shake-flask experiments, and comparable DCW (up to 8.0 g/L) and CA (25-28 g/L) quantities were reported for these trials. Lipid production was higher in the batch-bioreactor experiment. In fed-batch bioreactor trials performed thereafter, a maximum CA quantity of 66.1 g/L (conversion yield 0.66 g per g of glycerol) was obtained. Cellular lipids of all tested strains were mainly composed of the fatty acids Δ9C18:1, Δ9,12C18:2 and C16:0.
Keywords: biodiesel-derived glycerol, citric acid, microbial lipid, *Yarrowia lipolytica*

1. Introduction

Biodiesel fuels, prepared through trans-esterification of conventional or non-conventional (e.g. microbial) oils and fats with short chain alcohols (mostly methanol, ethanol or butanol) are produced at constantly increasing quantities the last decade as a "renewable" response to the decrease of hydrocarbon feedstocks, the continuously rising CO_2 emission, the concerns concerning the problem of the global warming of the planet and the rise in the price of crude oil (Papanikolaou and Aggelis 2009; 2019). Lipid trans-esterification in order biodiesel generation to be carried out is connected with the discharge of a concentrated glycerol-containing water as the principal side-product of this process; with the production of 10 kg of biodiesel deriving from trans-esterification of various oils, *c*. 1 kg of glycerol is generated (Papanikolaou and Aggelis 2009; 2019; Chatzifragkou and Papanikolaou 2012). In 2021, the worldwide biodiesel production only by taking into consideration edible vegetable oils is expected to increase up to 30×106 t., therefore, only from production of the "1st generation" biodiesel, production of 3×10^6 t. of glycerol is expected to occur (Koutinas et al 2014). It can therefore be concluded that conversion of glycerol in order for higher added-value compounds to be generated present a constantly increasing interest.
In the current investigation, a number yeast strains belonging to the species *Y. lipolytica* (4 strains), were tested as regards their potential to convert crude glycerol, the principal "waste" stream deriving from a biodiesel-fabricating unit, in order metabolites of higher added-value (i.e. microbial lipids, intra-cellular polysaccharides, citric acid, etc) to be synthesized.

2. Materials and Methods

The strains used in the current investigation belonged to the species *Y. lipolytica* (strains ACA-DC 50109, LFMB Y-20, LMBF Y-47 and ATCC 20460). Experiments were performed in liquid-submerged cultures. The culture medium used contained (in g/L): KH_2PO_4 7.0; Na_2HPO_4 2.5; $MgSO_4\times7H_2O$ 1.5; $CaCl_2$ 0.15; $FeCl_3\times6H_2O$ 0.15; $ZnSO_4\times7H_2O$ 0.02; $MnSO_4\times H_2O$ 0.06. Yeast extract and peptone were used as nitrogen sources. Crude glycerol was provided from a biodiesel-producing plant was used as the sole carbon source. In the first set of trials, all employed strains were shake-flasked at an initial glycerol (Glol0) concentration into the medium adjusted to c. 40 g/L, while yeast extract and peptone were employed at concentrations 1.0 and 2.0 g/L respectively. Experiments were carried out in 250-mL conical flasks, containing 50±1 mL growth

medium, sterilized at T=115 °C/20 min and inoculated with 1 ml of 24-h yeast pre-culture (yeast pre-culture was carried out on yeast-peptone-dextrose liquid medium, at 190±5 rpm, and T=28±1 °C). All cultures of this set of experiments were incubated at T=28±1 °C in an orbital shaker at 180±5 rpm. In a next approach *Y. lipolytica* ACA-DC 50109 was grown in batch bioreactor experiments, at higher nitrogen limitation imposed (yeast extract and peptone were employed at concentrations 0.5 and 0.75 g/L). $Glol_0$ concentration was adjusted at *c*. 50 g/L, and a bench-tope 3-L bioreactor presenting an initial fermentation volume 1.75 L, was inoculated with 0.05 L of a 24-h pre-culture. The agitation rate was adjusted at 400±20 rpm and the aeration rate was adjusted to 1.0 vvm. The pO_2 evolution was uncontrolled (therefore, there was not any cascade mode applied) but throughout the culture the dissolved oxygen concentration value never dropped below 5% v/v. The pH in the bioreactor trial of this set of experiments was adjusted at 6.0±0.1 and was automatically controlled by addition of 2N NaOH, while the incubation temperature was set at T=28±1 °C. A fed-batch trial was also performed, in order to extent the production of metabolites by the above-mentioned yeast strain. Aeration and agitation in the fed-batch bioreactor experiment.

3. Results and Discussion

All *Y. lipolytica* strains produced appreciable DCW production (up to 13.0 g/L). ATCC 20460 strain produced CA in quantities up to 27.8 g/L. ACA-DC 50109 produced CA in concentrations up to 16.0 g/L, while lipid in DCW values of ~15% w/w were recorded. Batch bioreactor and shale-flask experiments of the strain ACA-DC 50109 demonstrated almost equivalent kinetic results concerning DCW and CA production, but lipid in DCW quantities recorded were higher for the bioreactor experiment. In fed-batch bioreactor trials performed thereafter, a maximum CA quantity of 66.1 g/L (Fig. 1)

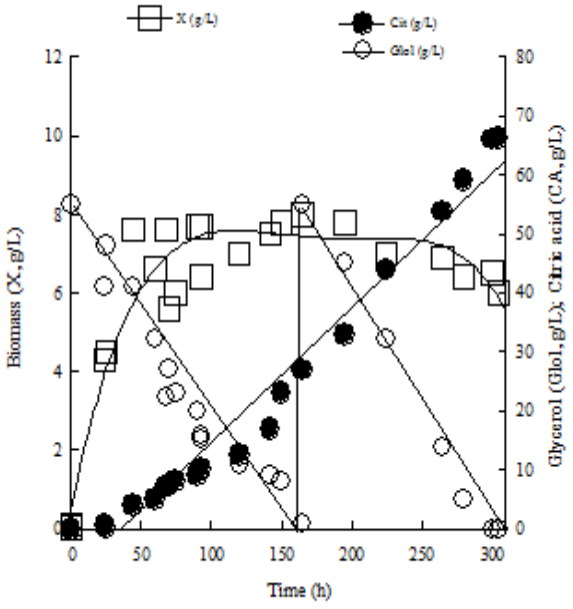

Fig1. Kinetics of biomass (X, g/L) production, glycerol assimilation (Glol, g/L) and citric acid production (CA, g/L) by *Yarrowia lipolytica* ACA DC 50109 during growth on biodiesel-derived glycerol, in fed-batch bioreactor experiment under nitrogen-limited conditions. Culture conditions as in "Materials and Methods". Each point is the mean value of two independent measurements (SE<15%).

Cellular lipids of all tested strains were mainly composed of the fatty acids Δ9C18:1, Δ9,12C18:2 and C16:0.

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