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Bio-valorization of olive mill wastewaters and crude glycerol blends with the use of a *Yarrowia lipolytica* strain to produce citric acid, polyols and lipids

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Abstract

In the present study, crude glycerol, waste discharged from bio-diesel production, was used as substrate for screening eleven natural *Yarrowia lipolytica* strains when grown in nitrogen-limited submerged shake-flask experiments. In media with initial glycerol concentration of 40 g/L, all strains presented satisfactory microbial growth and almost complete glycerol uptake. The principal metabolic product was citric acid (Cit_{max}~30.0 g/L, yield 0.30–0.80 g per g of glycerol consumed) simultaneous with the accumulation of storage lipid. Polyols mannitol, arabitol and erythritol were also synthesized as strain dependent compounds. *Y. lipolytica* strain ACA-YC 5031 produced sufficient amount of citric acid and presented the highest (amongst the screened strains) production of the polyol erythritol (~4.0 g/L, $Y_{Ery/Glol}$~0.18 g/g) as also non negligible amounts of the polyols mannitol (~5.6 g/L, $Y_{Man/Glol}$~0.25 g/g) and arabitol (~2.2 g/L, $Y_{Ara/Glol}$~0.15 g/g). This strain was further grown on increased concentration of crude glycerol nitrogen-limited experiments of ~70 g/L (blank experiment) and on blends of crude glycerol and olive mill wastewaters (OMW). Specific volume of OMW was used with the rationale to partially substitute process tap water, thus giving initial phenolic compounds concentration of 1.0 g/L. The metabolism seemed to be shifted towards citric acid production at expense of erythritol production, due to the addition of OMW into the medium. The maximum production of biomass (X_{max}=10.2 g/L, $Y_{X/Glol}$=0.14 g/g) as also the accumulation of both citric acid (Cit_{max}=37.1 g/L, $Y_{Cit/Glol}$=0.51 g/g) and cellular lipids (L_{max}=2.8 g/L, $Y_{L/X}$=0.25 g per g of dry cell weight) was favored by OMW addition, compared to blank experiment. On the other hand erythritol production presented significantly lower values with OMW addition (Ery_{max}=7.4 g/L, $Y_{Ery/Glol}$=0.11 g/g) whereas mannitol and arabitol production showed no significant difference (Man_{max}=11.6 g/L, $Y_{Man/Glol}$=0.16 g/g; Ara_{max}=2.6 g/L, $Y_{Ara/Glol}$=0.04 g/g) compared to blank experiment. Finally, removal of medium color occurred (up to ~20%).

Introduction

Crude glycerol (the major side-product of biodiesel production) is considered as a renewable resource generated in continuously increasing quantities worldwide [1, 2]. The potential of eukaryotic microorganisms to convert glycerol into various metabolic compounds of added-value like microbial mass, single cell oil (SCO), citric acid and polyols is highly desired [3]. Olive mill wastewaters (OMW) are one of the most difficult to treat agro-industrial residues produced in the Mediterranean base. The phenolic content of this effluent is responsible for its (phyto)-toxic effect and dark color [4]. Blends of OMW and crude glycerol could be a promising substrate which might limit environmental pollution and produce useful products with lower process cost.

Aim of the present study was to investigate the biochemical behavior of a *Y. lipolytica* strain when cultivated in crude glycerol (issued from a bio-diesel manufacturing unit) and in blends of crude glycerol and OMW (used as partial substitute to process tap water) media, in nitrogen-limited submerged shake-flask cultures. Kinetic interpretations concerning the yeast behavior were also considered and discussed. Potential decolorization of the media (color existence, due to OMW presence) by the yeast strain used, was simultaneously assessed.

Methods and Materials

Yeast strain: *Yarrowia lipolytica* strains ACA-YC 50109, ACA-YC 5029, ACA-YC 5030, ACA-YC 5031, ACA-YC 5032, ACA-YC 5033, W29, Y45, Y46, Y47 and LFMB 20.

Media: Crude glycerol (purity ~88% w/w). OMW obtained from three phase decanter manufactures with initial total phenol content of ~3.5 g/L, subsequently centrifuged (9000 rpm, 15 min, 4ºC) and filtrated. The glycerol concentration used was adjusted at initial value of ~40 g/L regarding nitrogen-limited screening experiments. The selected strain *Y. lipolytica* ACA-YC 5031 was further grown on increased initial concentration of crude glycerol (nitrogen-limited) experiments of ~70 g/L (blank experiment) and on blends of crude glycerol and OMW. Specific ratio of OMW and water was used in order to result in initial phenolic compounds concentration of 1.0 g/L. In all cases, the nitrogen source used were yeast extract and peptone (1.0 g/L each). The salt composition of the media contained (in g/L): KH_2PO_4, 7.0; Na_2HPO_4, 2.5; $MgSO_4x7H_2O$, 1.5; $FeCl_3x6H_2O$, 0.15; $CaCl_2x2H_2O$, 0.15; $ZnSO_4x7H_2O$, 0.02; $MnSO_4xH2O$, 0.06.

Cultivation: Erlenmeyer flasks (250 mL) of 50 mL liquid medium were inoculated with 1 mL of exponential pre-culture. Flasks were incubated aerobically in an orbital shaker (180±5 rpm, 28±1 ºC). The pH was maintained in 6.0.

Biomass determination: Biomass was harvested by centrifugation (9000 rpm, 10 min, 4 ºC), washed once with distilled water and centrifuged again. Biomass concentration was determined from dry weight (85±5 ºC until constant weight).

Lipid determination: Cellular lipids were determined gravimetrically using a mixture of chloroform/methanol 2/1 (v/v) as the extracting solvent.

Analyses: Glycerol, citric acid and polyols mannitol, arabitol and erythritol were analyzed with the aid of a HPLC apparatus. Filtered aliquots of the culture medium were determined by a Waters Association 600E apparatus at a 30.0 cm × 7.8 mm column Aminex HPX-87H (Bio-Rad, USA), coupled to a differential refractometer (RI; Waters 410) and a UV detector (Waters 486). Operating conditions were as follows: sample volume 20 μL; mobile phase 0.005M H_2SO_4; flow rate 0.6 mL/min; column temperature T=65 °C. Glycerol and polyols were detected by the RI detector while citric acid by the UV detector. The decolorization of the treated media was measured according to Sayadi and Ellouz [5].

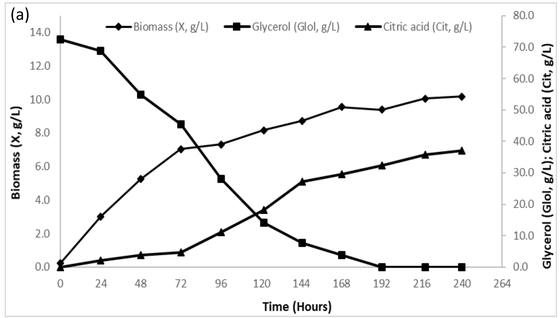
Results

All screened strains presented satisfactory production of biomass (X_{max}=6.3 g/L, $Y_{X/Glol}$=0.18 g/g) and almost complete glycerol uptake. The principal metabolic product was citric acid (Cit_{max}=31.7 g/L, $Y_{Cit/Glol}$=0.84 g/g) simultaneous with the accumulation of storage lipid (L_{max}=0.8 g/L, $Y_{L/X}$=0.13 g/g). Polyols mannitol, arabitol and erythritol were also synthesized as strain dependent compounds (data not shown). The selected strain *Y. lipolytica* ACA-YC 5031 presented the highest production of erythritol (Ery~4.0 g/L, $Y_{Ery/Glol}$~0.18 g/g) as also non negligible amounts of mannitol (Man~5.6 g/L, $Y_{Man/Glol}$~0.25 g/g) and arabitol (Ara~2.2 g/L, $Y_{Ara/Glol}$~0.15 g/g). This strain was further grown on increased concentration of crude glycerol experiments of ~70 g/L (blank experiment, no OMW addition) and on blends of crude glycerol and OMW (initial phenolic compounds concentration of 1.0 g/L). In the blank experiment, the strain presented sufficient production of both biomass (X_{max}=6.8 g/L, $Y_{X/Glol}$=0.09 g/g) and SCO (L_{max}=1.8 g/L, $Y_{L/X}$=0.27 g/g) as also surprisingly high production of erythritol (Ery_{max}=22.3 g/L, $Y_{Ery/Glol}$=0.37 g/g) (Table 1).

The adaptation of the strain when OMW added in the media, was sufficient resulting in higher biomass (X_{max}=10.2 g/L, $Y_{X/Glol}$=0.14 g/g), lipid production (L_{max}=2.8 g/L, $Y_{L/X}$=0.28 g/g) and significantly higher production of citric acid (Cit_{max}=37.1 g/L, $Y_{Cit/Glol}$=0.51 g/g), compared to blank experiments (Table 1). Mannitol and arabitol production showed no significant difference compared to blank experiment, whereas erythritol production presented significantly lower values when OMW added (Table 1). Moreover, kinetics of the fermentation of OMW and crude glycerol blends showed that after depletion of glycerol in the medium, erythritol is totally consumed in favor of citric acid production [Chart 1, (a) and (b)].

Finally, removal of medium color occurred up to ~20% (data not shown).

Chart 1. In g/L: (a) Biomass (X), glycerol (Glol), citric acid (Cit) and (b) mannitol (Man), arabitol (Ara) and erythritol (Ery) evolution during growth of *Y. lipolytica* strain ACA-YC 5031 on blends of OMW and crude glycerol (initial phenolic compounds concentration 1.0 g/L and initial glycerol concentration ~70 g/L). Culture conditions as described in methods and materials.



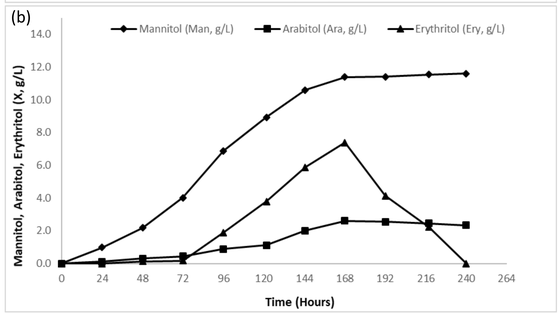


Table 1. Kinetics of *Y. lipolytica* strain ACA-YC 5031 when cultivated on crude glycerol and on blends of OMW and crude glycerol. Representations (in g/L) of maximum values of biomass (X), cellular lipid (L), citric acid (Cit), mannitol (Man), arabitol (Ara) erythritol (Ery) and (in g/g) respective yield of biomass on glycerol consumed ($Y_{X/Glol}$), yield total lipid in biomass ($Y_{L/X}$), conversion yield of citric acid produced per glycerol consumed ($Y_{Cit/Glol}$), conversion yield of mannitol produced per glycerol consumed ($Y_{Man/Glol}$), conversion yield of arabitol produced per glycerol consumed ($Y_{Ara/Glol}$) and conversion yield of erythritol produced per glycerol consumed ($Y_{Ery/Glol}$). Culture conditions as described in methods and materials.

Initial Phenolics (g/L)	X_{max} (g/L)	L_{max} (g/L)	Cit_{max} (g/L)	Man_{max} (g/L)	Ara_{max} (g/L)	Ery_{max} (g/L)	$Y_{X/Glol}$ (g/g)	$Y_{L/X}$ (g/g)	$Y_{Cit/Glol}$ (g/g)	$Y_{Man/Glol}$ (g/g)	$Y_{Ara/Glol}$ (g/g)	$Y_{Ery/Glol}$ (g/g)
0.0	6.8	1.8	13.9	12.0	2.8	22.3	0.09	0.27	0.18	0.16	0.04	0.37
1.0	10.2	2.8	37.1	11.6	2.6	7.4	0.14	0.28	0.51	0.16	0.04	0.11

Discussion

The significant increase of bio-diesel production and therefore of crude glycerol as its main by-product, imposes the necessity of discovering various (bio)-processes for valorizing this residue. The potential of a *Y. lipolytica* strain to grow on crude glycerol and on blends of OMW and crude glycerol, was assessed. As far as the authors are concerned, this is the first report in literature which deals with the fermentation of OMW and crude glycerol blends, by a yeast, to produce (high-)added value metabolites. Whereas, there are a little reports dealing with the secretion of polyols into the culture medium together with citric acid, when glycerol was utilized as the sole carbon source in nitrogen-limited submerged experiments [3, 6, 7].

The fact that the addition of OMW in the medium favored the accumulation of storage lipids, suggesting that OMW seemed to be a “lipogenic” substrate, is in accordance with literature [4, 8, 9].

Conclusions

All strains presented satisfactory microbial growth and almost complete glycerol uptake, in screening experiments. The principal metabolic product was citric acid simultaneous with the accumulation of storage lipid. Polyols mannitol, arabitol and erythritol were also synthesized as strain dependent compounds. The selected strain *Y. lipolytica* ACA-YC 5031, produced sufficient amount of citric acid and presented the highest production of erythritol. This strain presented the ability to grow very satisfactory on higher initial glycerol concentration cultures (compared to screening experiments) as also on media with blends of OMW and crude glycerol. The metabolism seemed to be shifted towards citric acid production at expense of mainly erythritol production (rather than mannitol and arabitol), due to the addition of OMW into the medium. *Y. lipolytica* strain ACA-YC 5031 can be considered as a satisfactory candidate grown in high concentrations of crude glycerol and on blends of OMW and crude glycerol for the production of citric acid, SCO and polyols and for simultaneous (partial) decolorization of the media.

Future Directions

It would be of great interest to perform experiments with higher initial phenolic compounds concentrations, by means of total substitution of process tap water by OMW. Moreover, fermentations with higher initial glycerol concentration leading in the production of higher concentrations of metabolic products could attract interest. Last but not least, scale-up of the process studying batch or fed-batch cultures in lab-scale bioreactors could lead to promising results.

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