

Bioconversions of olive oil mill wastewaters blends

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Abstract

The ability of the anaerobic mixed bacterial culture from an upflow anaerobic sludge blanket (UASB) bioreactor to convert blends of olive mill wastewaters (OMWs) with synthetic glucose medium or molasses into ethanol simultaneously with organic acids (OAs) was studied in the present work. All fermentations were conducted by free cells under non-aerated conditions at 37 °C and the effect of OMWs concentration on ethanol and OAs production was investigated. The highest amount of bioethanol (14.7 g L⁻¹) was produced during fermentation of 45% v/v OMWs mixed with synthetic glucose medium (5% w/v) in only 28 hours. In mixtures of 30% v/v OMWs and molasses solution (3 °Be) 13.4 g L⁻¹ of bioethanol were produced. Also, 16.2 g L⁻¹ of succinic acid were produced, when 65% v/v OMWs mixed with synthetic glucose medium. Moreover, totally 11.6 g L⁻¹ of varied OAs (succinic, malic, butyric and acetic) observed in mixtures of 35% OMWs with molasses. Finally, it has been shown that the ¹⁴C-labelled glucose uptake rate (GUR) by biomass was strongly correlated to fermentation rate.

Keywords: Olive mill wastewaters (OMWs), fermentation, culture of upflow anaerobic sludge blanket (UASB) bioreactor, molasses, ethanol, ¹⁴C-labeled glucose uptake rate.

1. Introduction

The olive mill wastewaters (OMWs) are produced during the extraction of olive oil via the three-phase method in modern centrifugal units. These wastes are very serious pollutants of aqueous receptors and soils mainly in Mediterranean countries (Aggelis *et al.*, 2003; McNamara *et al.*, 2008; Karaouzas *et al.*, 2011), due to their high organic load. They are dark coloured solutions with characteristic odour and solid content up to 6% w/v the precise composition of which depends on the region of origin, the variety, the maturation of olives, etc. (Roig *et al.*, 2006). Mainly, the OMWs contain phenolic compounds, tannins, polysaccharides, fatty acids, polyalcohols (Lesage-Meessen *et al.*, 2001) and are characterized by a high polluting load of 14-110 and 41.4-130 g L⁻¹ in organic (BOD₅) and chemically required oxygen (COD) values respectively (Blika, 2009).

Over the last twenty years, many researchers have been studied with the possibility of decontaminating OMWs either by decomposing their components, such as polyphenols or by processing them to produce high added value products, e.g. ethanol, organic acids, enzymes etc. (Tsagaraki *et al.*, 2006; Sarris *et al.*, 2013, 2014). To this end, many physico-chemical and biotechnological processes have been proposed in order these wastes to be treated. Occasionally, oxidation, reverse osmosis, etc. (Scoma *et al.*, 2011; Ena *et al.*, 2012) as well as aerobic and anaerobic digestion, composting etc. (Tortosa *et al.*, 2012; Dermeche *et al.*, 2013) have been used for the OMWs decontamination. However, these wastes should be considered as a good substrate for a variety of bio-processes (Lanciotti *et al.*, 2005; Zanichelli *et al.*, 2007), which are generally low-cost, environmentally friendly and in line with Green Development.

The production of biofuels, such as ethanol, by fermentation of OMWs with microorganisms, has widely been investigated (Bellou *et al.*, 2014; Mateo and Maicas, 2014). Often the mixing of OMWs with sugar-rich waste or by-products of the food industry, such as molasses, glucose etc. (Sarris *et al.*, 2014; Dourou *et al.*, 2016) was proposed for increasing the products yields.

In the present work the treatment of OMWs modified with molasses or glucose for the production of ethanol simultaneously with organic acids (OAs) by anaerobic fermentation was investigated. In an effort to discover new natural microorganisms capable of producing ethanol and OAs at high final concentrations or producing valuable compounds (i.e. succinic acid) in sufficient quantities, the mixed bacterial culture from an upflow anaerobic sludge blanket (UASB) bioreactor was used. According to our knowledge this is the first investigation indicating the use of the culture UASB for the valorization of OMWs or OMWs-based media for the production of ethanol and organic acids. The effect of OMWs concentration on the amount of ethanol and OAs produced during fermentation of OMWs blends by UASB mixed culture free cells was studied too. Finally, in the optimal fermentation conditions, the rate of ¹⁴C-labeled glucose uptake (GUR) by the cells of the microorganisms was determined.

2. Experimental

2.1. Culture and media

The mixed bacterial anaerobic culture (sludge) was obtained from an UASB reactor and was grown under anaerobic conditions without any stirring at 37 °C in glucose or sucrose nutrient media containing: 50 g L⁻¹ glucose or sucrose, 2.9 mL from an NH₃ solution 25% w/w and 0.6 mL from a H₃PO₄ solution 50% v/v at a COD:N:P ratio of 100:5:1, 4 g L⁻¹ NaHCO₃ and 4 g L⁻¹ yeast extract, without pH adjustment. Every time about 12 g L⁻¹ of prepared wet cells were separated by centrifugation.

The OMWs used for all fermentation experiments were obtained from a centrifugal olive mill of the agricultural association in Asopos (Lakonia, Greece). It contained about 15 g L⁻¹ sugars (glucose, sucrose, fructose etc.), water, organic acids, fatty acids, phenolic compounds, etc. and had a pH value about 5.4. The wastes were centrifuged before the use for solids removal.

Molasses was obtained from the local Spiliopoulos Distillery S.A. (Patras, Achaia, Greece) and consists (% w/w) of water (17-25), sucrose (30-40), glucose (4-9), fructose (5-12), polysaccharides—dextrin, pentosans, polyuronic acids (2-5), and inorganics. Before its use molasses was diluted with water to Baume density (°Be) 3. All media were sterilized by autoclaving at 120 °C for 15 min.

2.2. Batch fermentation of OMWs mixtures

The effect of OMWs concentration on ethanol and OAs production was studied by a series of fermentations conducted using sterilized mixtures (250 mL) composed of synthetic glucose medium (5% w/v) or diluted molasses (3 °Be) and OMWs in various concentrations (20, 30, 45, 55 and 65% v/v). About 3 g of free biomass cells were added along with every mixture into an Erlenmeyer flask of 500 mL and allowed to ferment without air supply, stirring and pH adjustment at 37 °C. Fermentation kinetics was monitored by Baume density and pH value measurements. Samples of the fermented broths were collected and stored at -20 °C until further analysis. The produced ethanol and OAs were expressed as grams per 1 L of mixture. The recorded results were the mean value of three repeats.

At the beginning of some runs 1 mL of diluted labelled glucose [ARC 147 Glucose, D (2-¹⁴C), 50 μCi mL⁻¹] was added, as had been done in previous investigations (Soupioni *et al.*, 1998) in order the GUR by cells of UASB mixed culture to be estimated.

2.3. Residual sugars determination

At the beginning and at the end of fermentation, sugars (i.e. glucose, sucrose, fructose etc.) were determined in the samples of OMWs mixtures. An HPLC system (LC-9A, Shimadzu Corporation, Kyoto, Japan) was used consisting of pump, column oven (CTO-10A), refractive index detector (RID-6A), and degassing unit (DGU-2A), and equipped with a Nucleogel ION-300 OA column. A solution 0.008 N H₂SO₄ was used as mobile phase at a flow rate of 0.5 mL min⁻¹ and 1-propanol (1% v/v) was used as internal

standard. Column temperature was 30 °C. Sample dilution was 0.4% v/v, and the injection volume was 60 μL.

Sugar conversion was calculated by the following equation: (initial sugar – residual sugar)/initial sugar×100.

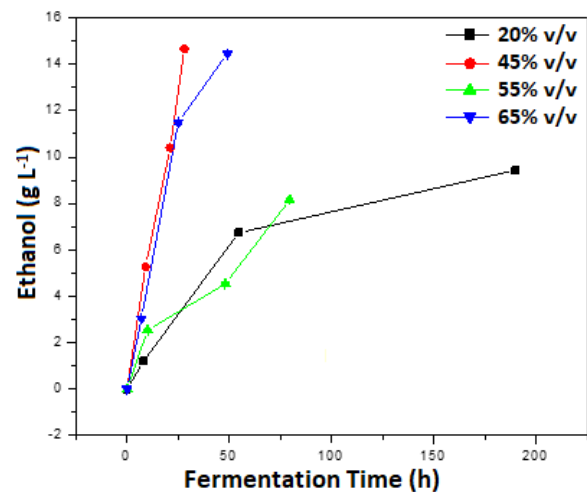


Figure 1. Ethanol concentration, during fermentation of OMWs mixtures with synthetic glucose medium (5% w/v) by free cells of an UASB mixed culture at 37 °C

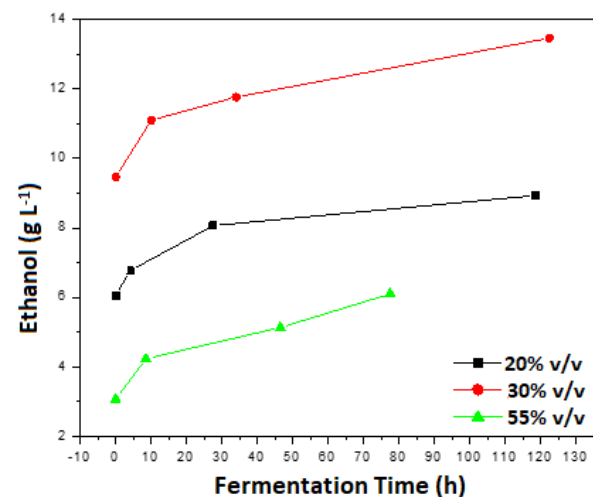


Figure 2. Ethanol concentration, during fermentation of OMWs mixtures with molasses solution (3 °Be) by free cells of an UASB mixed culture at 37 °C

2.4. Ethanol determination

Ethanol was determined by Gas Chromatography on a proper GC system (8A, Shimadzu) equipped with a FID detector, a HayeSep Q 80/100 mesh column (2 m × 1/8") (Teknokroma, Barcelona, Spain), and an integrator (C-R6A Chromatopack, Shimadzu). Helium was used as carrier gas at 20 mL min⁻¹. The injection port and detector temperatures were 200 and 220 °C. The column temperature was programmed from 80 to 180 °C at a rate of 16 °C min⁻¹. Samples of 1 μL were injected directly onto the column. Determinations were done by means of standard curves. 1-Butanol was used as internal standard at a concentration of 0.5% (v/v).

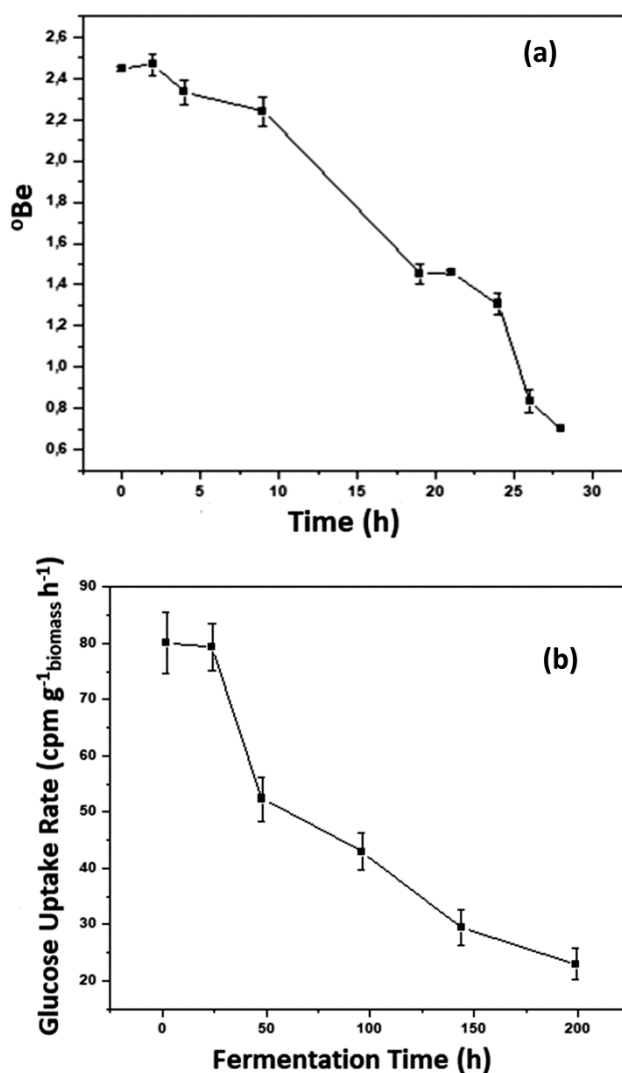


Figure 3. (a) Fermentation kinetics of sugar in 45% v/v OMWs mixed with synthetic glucose medium (5% w/v) (b) Glucose uptake rate by free cells of an UASB mixed culture, observed at 37 °C

Table 1. Effect of OMWs concentration on kinetic parameters and average (N=3) concentrations (\pm SD) of residual sugar, ethanol and OAs produced during fermentation of OMWs mixtures with synthetic glucose medium (5% w/v) or molasses solution ($^{\circ}$ Be 3) by free cells of an UASB mixed culture at 37 °C

	OMWs (% v/v)	Initial sugar (g L ⁻¹)	Initial pH value	Ferment. time (h)	Residual sugar (g L ⁻¹)	Ethanol (g L ⁻¹)	Ethanol yield* (g g ⁻¹)	Sugar conversion (%)	Final pH value	Organic acids (g L ⁻¹) <u>Succinic</u>
Glucose	20	18.3 \pm 0.5	8.7 \pm 0.04	190	0.4 \pm 0.2	9.4 \pm 0.05	0.81	95.08	4.11 \pm 0.05	4.00 \pm 0.20
	45	16.1 \pm 0.7	7.71 \pm 0.03	28	2.3 \pm 1.1	14.7 \pm 0.05	1.09	85.71	3.37 \pm 0.03	12.5 \pm 0.20
	55	18.3 \pm 1.5	8.00 \pm 0.03	92	4.9 \pm 0.8	8.15 \pm 0.08	0.60	74.32	3.15 \pm 0.10	13.7 \pm 0.10
	65	15.5 \pm 0.5	6.64 \pm 0.03	49	2.1 \pm 1.3	14.5 \pm 0.05	1.08	86.45	3.23 \pm 0.03	16.2 \pm 0.40
Total										
Molasses	20	26.1 \pm 1.05	8.82 \pm 0.03	118.5	15.8 \pm 2.2	8.93 \pm 0.05	0.74	89.24	9.07 \pm 0.01	2.05 \pm 0.1
	30	18.1 \pm 0.7	8.91 \pm 0.06	112.5	15.7 \pm 1.3	13.4 \pm 0.05	0.47	80.14	9.16 \pm 0.04	2.50 \pm 0.3
	55	8.6 \pm 1.3	9.01 \pm 0.03	77.5	6.5 \pm 0.2	6.10 \pm 0.1	0.69	73.28	9.13 \pm 0.01	6.65 \pm 0.1

* g of ethanol per g of converted sugar

2.5. OAs determination

Organic acids were determined by High Performance Liquid Chromatography (HPLC) on a Jasco LC-2000 Plus chromatograph equipped with an Aminex column (300 \times 7.8 mm i.d., 9 μ m particle size; HPX-87H, Bio-Rad, Hercules, CA, USA) column thermostat (CO-2060 Plus

Intelligent, Jasco), quaternary gradient pump (PU-2089 Plus), autosampler (AS-2050 Plus Intelligent), photodiode array detector (MD-2018 Plus) operated at 210 nm, and a hardware interface (LC-Net II/ADC Chromatography Data Solutions). Isocratic separation at 22 °C was performed with a solution 0.005 N H₂SO₄ as mobile phase at a flow

rate of 0.5 mL min⁻¹. Before analysis the samples were diluted 1/5 and filtered by a membrane filter of 0.22 µm pore size.

2.6. ¹⁴C labelled glucose determination

The labelled glucose was fermented in the same way as the non active one. At various time intervals samples of 5 mL of the fermented broth were centrifuged, and the biomass was kept in plastic vials with 5 mL of liquid scintillation cocktail (Opti-Fluor, Perkin Elmer, Waltham, MA, USA). The ¹⁴C within cells was measured by a Liquid Scintillation Analyzer TRI-CARB 1500 PACKARD connected to PC and recorder (DOT MATRIX PRINTER CITIZEN Swift 24). The resulted GUR were expressed in [cpm/(g_{biomass}·h)].

2.7. Statistical methods and data analysis

During OMWs mixtures fermentations the standard deviations (SD) of each of three recorded values for sugars, ethanol and OAs concentrations as well as GUR were calculated (Origin 8, Microcal Software Inc., Northampton, MA, USA). The data were analyzed using the analysis of variance technique. Significant differences between means were identified by one-way ANOVA. Statistical analyses were carried out using the computer software SPSS version 21.0 for Windows (SPSS Inc., Chicago, IL).

3. Results and discussion

The effect of OMWs concentration on the ethanol and the OAs amount produced during anaerobic fermentation of OMWs mixtures with synthetic glucose medium (5% w/v) or molasses solution (3 °Be) by free cells of mixed culture from an *UASB bioreactor* at 37 °C is shown in Figures 1 and 2 and Tables 1 and 2.

Table 2. Effect of OMWs concentration on average (N=3) concentrations (±SD) of OAs produced during fermentation of OMWs mixtures with molasses solution (3 °Be) by free cells of an *UASB* mixed culture at 37 °C (Table 2)

OMWs (% v/v)	Organic acids (g L ⁻¹)			
	Succinic	Malic	Acetic	Butyric
20	-	0.3±0.01	-	1.8±0.1
30	2.2±0.3	0.3±0.01	-	-
55	-	-	0.35±0.02	6.3±0.1

The highest ethanol concentration (14.7 g L⁻¹) was observed during fermentation of 45% v/v OMWs mixed with synthetic glucose medium (5% w/v) in only 28 h (Figure 1, Table 1). Specifically, the ethanol concentration was significantly higher ($P < 0.05$) in the case of the 45% v/v OMWs glucose mixtures compared to that from fermentations of 20 or 55% v/v ones.

However, during fermentation of OMWs blends with molasses solution (3 °Be) significantly higher ($P < 0.05$) bioethanol amount (13.4 g L⁻¹) produced when 30% v/v OMWs were used, compared with the 20 or 55% v/v OMWs (Figure 2, Table 1).

Concerning the OAs production is remarkable that only succinic acid was produced during fermentation of OMWs

blends with synthetic glucose medium. Indeed, the highest succinic acid concentration (16.2 g L⁻¹) was observed in 49 h, when 65% v/v OMWs mixed with synthetic glucose medium (5% w/v) (Table 1). This was significantly higher ($P < 0.05$) compared to the succinic acid concentration from fermentation of 20% v/v but near that in the case of the 45% v/v (12.5 g L⁻¹) and 55% v/v (13.7 g L⁻¹) OMWs glucose mixtures. As it is known, in 2004, the succinate was placed on the US Department of Energy's list of top 12 platform chemicals from biomass [<https://www.nrel.gov/docs/fyo4osti/35523.pdf>]. Also, succinic acid is a precursor to some biodegradable polymers, resins or chemical solvents, used as food additive and dietary supplement etc.

Moreover, low concentrations of varied OAs (succinic, malic, butyric and acetic) were observed in mixtures of 20, 30 and 55% v/v OMWs with molasses (Table 2).

Figures 3a and b show the fermentation kinetics of sugar and glucose uptake rate by free cells of an *UASB* mixed culture in 45% v/v OMWs mixed with synthetic glucose medium (5% w/v) observed at 37 °C, respectively. It is obvious that GUR was strongly related to fermentation rate as previous reported for the lactose uptake rate by kefir (Golfinopoulos *et al.*, 2009; 2011; 2012; Soupioni *et al.*, 2013) and the produced ethanol did not affect the glucose uptake by mixed culture.

4. Conclusions

The low cost anaerobic mixed bacterial culture from an *UASB bioreactor* contains suitable microorganisms of producing ethanol simultaneously with OAs during fermentation of OMWs blends with synthetic glucose medium (5% w/v) or molasses solution (3 °Be) at 37 °C. The highest ethanol concentration was recorded for 45% v/v OMWs mixed with glucose medium in 28 h fermentation time and in that case the GUR was strongly correlated to fermentation rate. Valuable succinic acid was the unique organic acid produced during fermentation of OMWs blends with glucose medium and its highest amount was observed for 65% v/v OMWs in 49 h. Lower ethanol and OAs concentrations (succinic, malic, butyric and acetic) were obtained using molasses solution in about the same volume for the blends preparation.

Therefore, bioconversion of OMWs sugars by free cells of *UASB* culture during the aforementioned processes could be used by olive mills to produce fast saleable products, whilst simultaneously reduce the organic load of their wastes.

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