Production of single cell protein by *Saccharomyces cerevisiae* and *Candida utilis* from treated (dephenolized) and untreated olive mill waste

I. Giavasis [*1], P. Zara[1], P. Andriopoulos[1], C. Mpouros[1], P. Goutsidis[2], A. Manouras[1], K. Petrotos [2]

[1*] Technological Educational Institute of Larisa, Department of Food Technology, Karditsa, Greece;
[2]Technological Educational Institute of Larisa, Department of Biosystems Engineering, Larissa, Greece
igiavasis@teilar.gr

Abstract

Olive mill waste (OMW) is a large by-product of olive oil processing with high BOD and COD value, and high phenolic content,with phytoxic properties and a problematic biodegradation process. Our main objective is the utilization of OMW for the production of single cell protein (SCP-supplement for human/animal nutrition), and concomitant reduction of BOD and COD. In our study, OMW was dephonolized using macroporous absorbing resins and utilized as fermentation medium for the cultivation of *Saccharomyces cerevisiae* and *Candida utilis* (sources of SCP). Dephenolized and untreated OMW with or without addition of nitrogen sources were as used as bioprocess medium. Also, cheese whey protein concentrate (WPC) was used as an additional nitrogen/nutrient source. The effects of total sugar concentration (condensed/ uncondensed OMW, addition of WPC), phenol content, type and concentration of nitrogen sources, process pH and temperature, inoculum concentration, agitation rate, and type of yeast on SCP production were examined. The results showed that *Candida utilis* exhibited somewhat better performance than *Saccharomyces cerevisiae* in most experiments (higher biomass). However, for each strain, different optimal conditions for growth were observed. Yeast extract and ammonium sulphate seemed to enhance biomass production more than other nitrogen sources, and DCW stimulated yeast growth. Condensing OMW at a 3:1 rate improved SCP production, as did a high agitation rate and 10% inoculum. Dephenolizing increased the production of SCP. Optimal biomass concentration reached or exceeded 10 g/l (pure protein concentration ~80%). Moreover, BOD and COD values decreased after dephonolization (~3 to 5-time reduction), and dropped further after fermentation and removal of biomass. Overall, OMW could be successfully utilized for SCP production and offer high added value. BOD and COD levels decreased significantly, and phenol content was almost zeroed, facilitating its biodegradation or potential use for irrigation.

Introduction

Olive mill waste (OMW) is a large by-product of olive oil processing with high BOD (~40.000 ppm) and COD (~50.000 ppm) value, and high phenolic content (~0,5%),with phytoxic properties and a problematic biodegradation process [1-2]. The aerobic degradation of OMW is partly blocked by the polyphenols found in OMW, and thus phenol-degrading microorganisms, mainly yeasts and fungi have been used in order to reduce phenol content of OMW and facilitate further biodegradation of the waste [2-5]. Also, another process involves the removal of polyphenols from OMW by alkaline treatment [5], which is however costly. In our approach, an ultrafiltration method was employed for concentrating and removing most of the polyphenols found in OMW (which can be used as a polyphenol concentrate/powder in food and pharmaceutical applications), in order to pave the way not only for the degradation ond reduction of BOD and COD of the waste, but also for the utilization of this substrate for the production of single cell protein. The latter has been produced from several yeast and fungi, such as *Candida*, *Schizosaccharomyces*, *Saccharomyces*, *Pleurotus*, *Aspergillus*, etc, but only on phenol-containing, untreated OMW [5-7]. In this study we examine the production of SCP from GRASS organisms, *Saccharomyces cerevisiae* and *Candida utilis* using dephenolized OMW.

Materials and Methods

Substrate and organisms: Olive mill waste was used as the basic substrate for the comparative production of single cell protein by *Candida utilis* and *Saccharomyces cerevisiae* (bakers' yeast), with or without the addition of organic and inorganic nitrogen sources, namely yeast extract, peptone, ammonium nitrate and

ammonium sulphate, and in some cases with the supplementation of whey protein concentrate (where whey proteins were isolated by ultrafiltration followed by thermal condensation) to increase nitrogen (and sugar-mineral) content of the fermentation medium. In most cases, dephenolized OMW was used. This was dephenolized (i.e. polyphenols were largely removed) by ultrafiltration using macroporous absorbing resins, and in some cases OMW was condensed by thermal treatment at 80°C to produce media concentrated by 2:1, 3:1, and 4:1 ratios.

Fermentation conditions: Agitation speed was either 250rpm, or 350 rpm, temperature was either 25°C or 30°C, initial process pH was either 5, 6, or 7, and inoculum was added at 5%, 10% and 15%. All experiments were carried out in 500ml shake flasks containing 250ml substrate, using an incubating rotary shaker. The pH was manually controlled by adding a buffer solution of 1 g/l of K2HPO4 and KH2PO4 solutions, and by manual adjustment (when/if necessary) after each sampling, by adding aseptically appropriate volumes of 1N NaOH or 1N HCl.

Analytical methods: Total biomass was assessed gravimetrically, by centrifugation of the fermentation broth at 5000rpm for 30 min, followed by drying of the precipitate at 105°C for at least 10h to constant weight.Total sugar content of the centrifugate of the process medium (after removal of biomass debri) was analysed by the dinitrosalycilic acid method. Ttotal proteins in the dry cell precipitate were assessed by the Bradford method, total phenols in the process medium by the Folin-Ciocalteu method. Respiratory Quotient (CO2/O2) was determined by measuring the CO2 produced by the culture and the available O2 in the flasks using an IR-gas analyser whose probe was inserted into the headspace of the fermentation flasks. The pH was measured by a bench pH-meter.

Results and Discussion

Effect of dephenolization

Figure 1 shows the effect of dephenolization on the production of biomass and sugar consumption by *S. cerevisiae*. Fermentation were carried out at 30°C, pH 5, and 250rpm agitation rate using a 5% inoculum. Biomass concentration increased from 0.45 g/l to 1.05 g/l in the dephenolized medium, while sugar consumption was higher by approximately 5g/l in the dephonolized OMW, although sugar utilization was incomplete in both treated (dephonlized) and untreated media. From an initial sugar concentration of approximately 22 g/l, untreated medium contained 14 g/l by the end of the fermentation, while dephenolized medium contained 10,4 g/l of residual sugars, showing that a limiting factor existed in both cases, preventing complete utilization of carbon sources. RW values were near zero for untreated OMW, showing very slow metabolism, while RQ was distinctively higher for the better-growing cultures in dephenolized OMW. Similar effects were observed with *Candida utilis.*

Effect of nitrogen sources

As OMW is poor in nitrogen, which is essential for biomass growth and SCP accumulation, and probably the limiting factor for sugar utilization, the effect of adding different organic and inorganic sources to OMW was studied. Namely, 5g/l of either yeast extract, peptone, ammonium nitrate and ammonium sulphate were added to the dephenolized process medium. Fermentation were carried out at 30°C, pH 5 and 250rpm agitation rate using a 5% inoculums. The effects on *S. cerevisiae* biomass concentration compared to dephenolized medium without any additives are shown in Fig. 2a. Ammonium sulphate seems to enhance biomass growth of *S. cerevisiae* significantly, much more than any other nitrogen source studied here, as it reached nearly 6,5 g/l after 80h. In contrast, *C. utilis* biomass reached maximum level (about 9 g/l at 70 h) after addition of yeast extract, which also offers sugars, minerals and vitamins to the yeast cells, apart from organic nitrogen (Fig.2b). The maximum average growth rate (until the time of peak biomass concentration) for *S. cerevisiae* was 0.0677 g/l/h with ammonium sulphate added, while for *C. utilis* the maximum average growth rate was 0.1325, thus showing a significantly faster growth rate for *C. utilis*. Sugar utilization was indeed improved by the addition of nitrogen sources, leading e.g. to a reduced 7 g/l of residual sugars in the process of *C. utilis* with added yeast extract (data not shown).

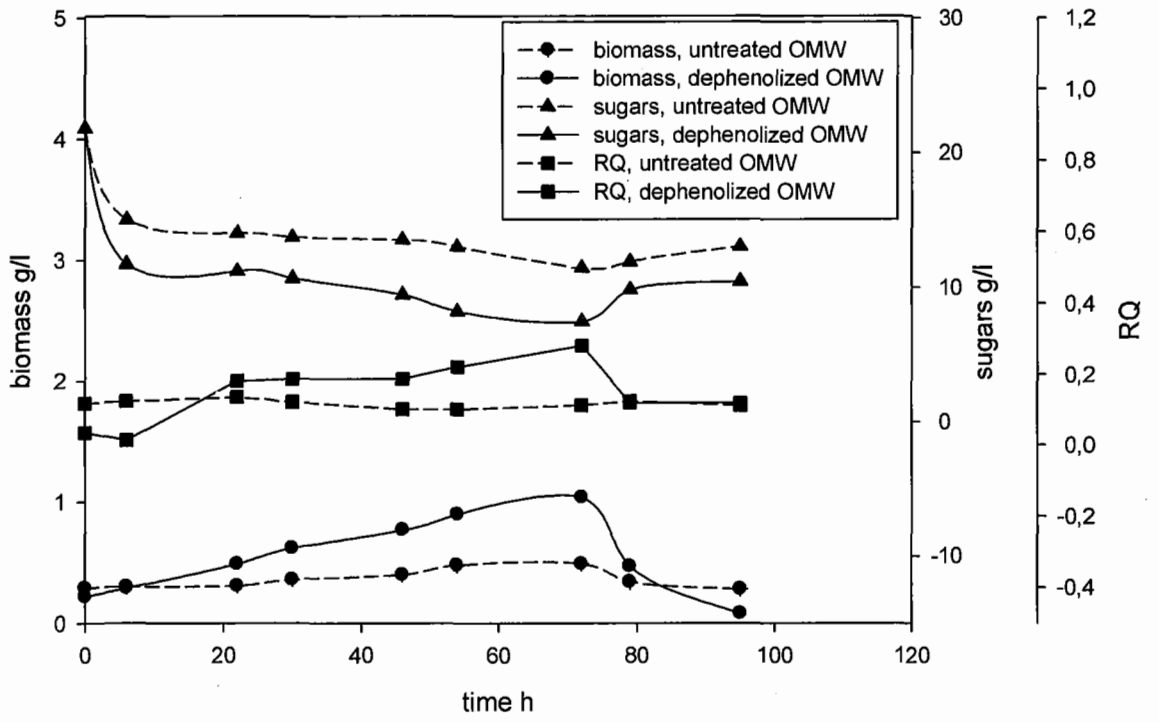
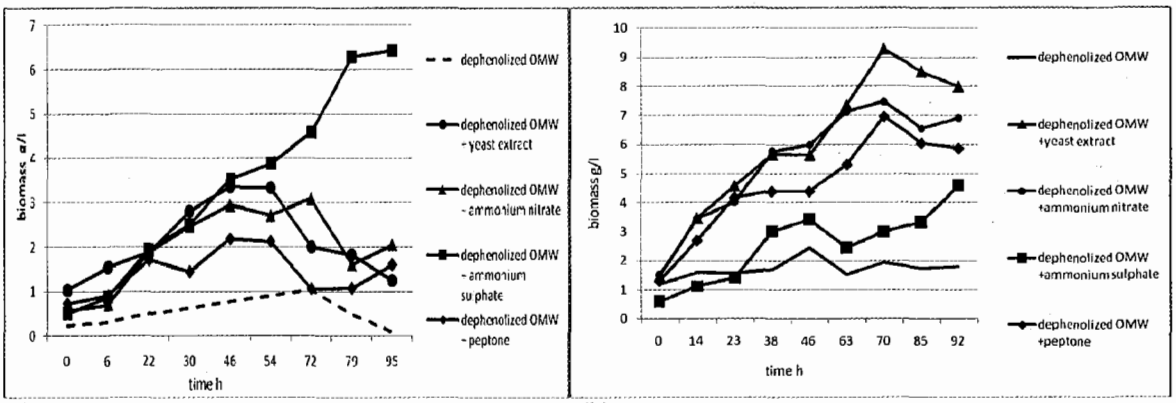


Figure1. Time profiles of biomass and sugar concentration during cultivation of *S. cerevisiae* in crude (untreated) and dephenolized (treated) OMW. Process conditions: temperature 30°C, 250rpm, 5% inoculum.

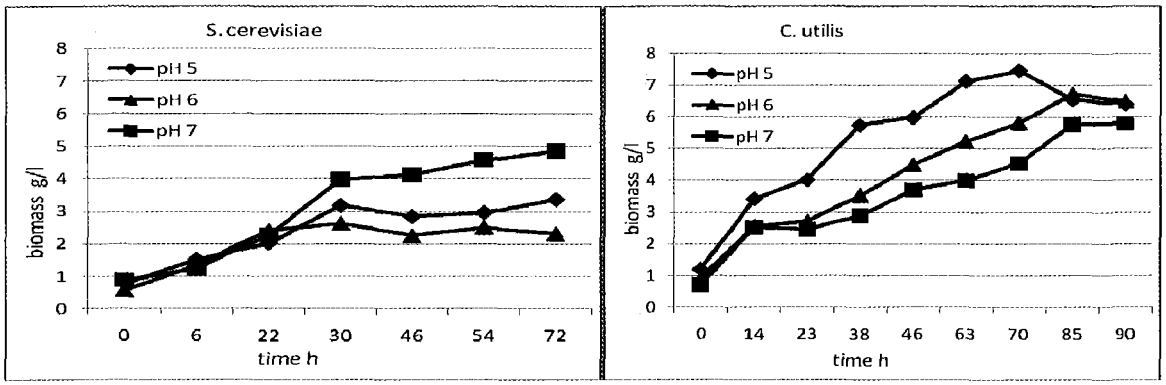


(a) (b)

Figure 2. Time profiles of (a) *S. cerevisiae* and (b) *C. utilis* biomass concentration in dephenolized OMW supplemented with organic and inorganic nitrogen sources at 5g/l concentration (compared to control without additional nitrogen process conditions: temperature 30°C, 250rpm, 5% inoculum.

Effect of pH

The pH during fermentation is a crucial parameter in yeast growth, and several optimal pH values exist for different yeasts. Here, optimal pH for growth of *S. cerevisiae* was pH 7, while *C. utilis* grew best at pH 5, where peak biomass concentration was 7,5 g/l (Fig. 3). Again, *C. utilis* reached a higher biomass level and had a higher growth rate compared to *S. cerevisiae*.



(a) (b)

Figure 3. Time profiles of (a) *S. cerevisiae* and (b) *C. utilis* biomass concentration at different process pH values in dephenolized OMW supplemented 5 g/l ammonium nitrate. Process conditions: temperature 30°C, 250rpm, 5% inoculum.

Effect of inoculum

The percentage of inoculum used in most cases was 5%, as it was found that it was optimal for *C. utilis* and that there was little difference (as to the final biomass levels) among an inoculum of 5%, 10% or 15% for *S. cerevisiae*. Also, the prior adaptation of both cultures to an OMW-based inoculum was essential for ensuring suffient growth of the cultures in the OMW-based process medium (data not shown).

Table 1. Phenol content of untreated, dephenolised, and condensed OMW (mean values of triplicate samples).

Medium	Phenol content (g/l)	Sugar content (g/l)
Untreated (crude) OMW	6,00	22,1
Dephenolized OMW	0,33	19,2
Dephenolized OMW, concentrated 2 times (2:1 concentration)	0,83	27,8
Dephenolized OMW, concentrated 4 times (3:1 concentration)	1,10	35,1
Dephenolized OMW, concentrated 4 times (4:1 concentration)	1,42	41,7

Effect of medium concentration condensation

The dephenolized OMW based medium was thermally condensed (evaporated) at 80°C in order to produce media with 2,3 and 4 times the sugar concentration of the initial medium and make available more carbohydrates to the yeast studied. At the same time all media were supplemented with 5 g/l yeast extract to cover any shortage of nitrogen or minerals sources. However, this condensation also increased the phenol content of the condensed media, as shown in Table 1 and may also impose an osmotic stress at high solid concentration. These resulted in an adverse effect on biomass growth, at a 4:1 concentration ratio for both organisms. Both had an optimum biomass concentration at 3:1 concentration ratio. For *C. utilis* biomass there was little difference between the 3:1 concentrated and the non-concentrated medium (only a increase from 6,5 to 7,1 g/l biomass), while for *S. cerevisiae* biomass there was an increase from 3,3 g/l in the control medium (non-condensed) to 4,7 g/l in the 3:1 condensed medium (data not shown). However, sugar utilisation was not improved compared to the control (dephenolized medium without condensation), and maximum consumption of sugar could not exceed 15 g/l in all cases (data not shown). Thus, despite the addition of 5 g/l yeast extract there were limiting factors other than sugar concentration, which hindered biomass accumulation.

Effect of agitation rate

Three different processes were run at 150, 250 and 350 rpm to investigate the impact of agitation and mixing on biomass growth. For *S. cerevisiae* optimal stirring was at 350 rpm (aproximately 1 g/l increase in biomass compared to 250 rpm, and 1,8g/l increase compared to 150 rpm), while *C. utilis* biomass was

highest at 250 rpm with only a 0,5 g/l difference from the process at 150 rpm (data not shown). At 350 rpm C. utilis biomass dropped by 2 g/l, indicating an adverse effect of high stirring rate, possibly due to mechanical damage of cells (shear stresss), or oxygen-induced streess.

Addition of whey protein concentrate

In order to substitute the lacking nitrogen sources in OMW with a relatively cheap alternative originating from another organic by-product of the food industry, whey protein concentrate (WPC) from bovine milk (produced by ultrafiltration and condensation) was added to process medium at 6,25 g/l concentration. WPC contained approximately 80% protein (including low molecular weight proteinaceous compounds) and 15% lactose, thus the 6,25 g/l of WPC yielded 5 g/l protein in the process medium, equal to the amount of other nitrogen sources used previously, as well an extra 0,94 g/l of sugars (mainly lactose). The lactose content of the WPC is not fermentable by *S. cerevisiae* and *C. utilis*, however, residual glucose (and galactose) molecules present in WPC could be readily utilized. Its addition also increased the concentration of useful minerals, vitamins, etc in the process medium. S. cerevisiae was able to accumulate slightly higher biomass under these conditions (13,6 g/l), in comparison with *C. utilis* (12,15 g/l), possibly due to the process pH of 7, which was optimal for *S. cerevisiae*, and/or the activity and availability of galactose-hydrolysing enzymes in S. cerevisiae, which are absent in *C. utilis*, and can utilize the galactosidic moiety of formerly hydrolysed lactose molecules. For both organisms the use of WPC improved biomass growth significantly, compared to other nitrogen sources used previously. Sugar utilization was also slightly improved leading to a reduced 3,3 g/l of residual sugars at 79h in the *S. cerevisiae* process compared to previous processes.

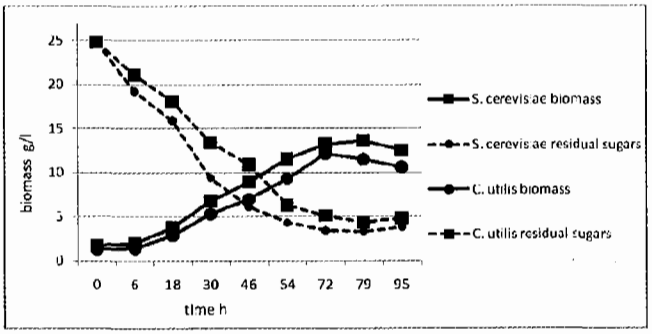


Figure 4. Time profiles of *S. cerevisiae* and *C. utilis* biomass and residual sugar concentration after addition of 15% deproteinized whey in dephenolized OMW. Process conditions: temperature 30°C, 250rpm, pH 7, 10% inoculum.

Protein content of the produced SCP

The crude biomass produced by edible yeasts can be used directly as animal feed, but for use in food, final protein concentration of the SCP is of great significance. Table 2 shows the mean values of protein concentration in different OMW based media. It exhibits that the addition of WPC increased the total protein content of the dry precipitate of SCP, whereas the condensation of the OMW lead to a decrease of the protein content of the SCP, possibly due to the accumulation of more solids in the medium, some of which maybe precipitated along with cell biomass, and thus reduce the percentage of pure protein.

Table 2. Protein concentration in SCP of *S. cerevisiae* and *C. utilis*, produced from three different OMW-based media (mean values of triplicate samples)

Type of substrate	Pure protein content in SCP (%)	
	S. cerevisiae	*C. utilis*
Dephenolized OMW	77,7	78,1
Dephenolized condensed OMW (3:1)	73,5	73,4
Dephenolized OMW with 6,25 g/l WPC	81,7	80,8

BOD-COD values

BOD and COD values decreased after dephenolization (~3 to 5-time reduction), and dropped further after fermentation and removal of biomass (data not shown).

References

1. Paraskeva P. and Diamadopoulos E. 2006. Technologies for olive mill wastewater (OMW) treatment: A review. J. Chem. Technol. Biotechnol., 81, 1475-1485.
2. Tsagaraki E, Lazarides H and Petrotos K., 2007. "Olive Mill Wastewater Treatment". In Utilization of By-Products and Treatment of Waste in the Food Industry, Edited by Vasso Oreopoulou and Winfried Russ, Springer Publishing, New York.
3. Crognale S, D'Annibale A, Federici F, Fenice M, Quaratino D, and Petruccioli M. 2006. Olive oil mill wastewater valorisation by fungi, Journal of Chemical Technology and Biotechnology 81:1547–1555.
4. Ben Sassi Z N., Ouazzani Z G. M., Walker Z S., Ibnsouda Z M., El Mzibri ZA., Boussaid, 2008. Detoxification of olive mill wastewaters by Moroccan yeast Isolates. Biodegradation, 19, 337–346.
5. Laconi S.Z., Molle G.Z., Cabiddu A.Z., Pompei R. 2007. Bioremediation of olive oil mill wastewater and production of microbial biomass. Biodegradation (2007) 18:559–566.
6. Garsallah, N., 1993. Production of single cell protein from olive mill wastewaters by yeasts. Environmental Technology 14, 393–395.
7. Rajoka I.M., Kiani T.M.A., Khan S., Awan M.S. and Hashmi A-S. 2004. Production of single cell protein from rice polishings using Candida utilis, 2004. World Journal of Microbiology & Biotechnology 20: 297–301, 2004.