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Biovalorization of Olive Mill Waste Water for the Production of Single Cell Protein from *Saccharomyces cerevisiae, Candida utilis* and *Pleurotus ostreatus*

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Authors' contributions

This work was carried out in close collaboration between the authors which designed and executed this study. Author KP designed this research at an initial stage and was responsible for the treatments of olive mill wastewater prior to fermentation, and author IG performed the literature search, the bioprocessing and fermentation experiments, managed the analyses of the study, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The aim of this work was to investigate and optimize the potential of olive mill waste water (OMWW) to be utilized as a substrate for the production of single cell protein (SCP).
Study Design: The study was divided in two phases, a preparatory phase for the removal of olive polyphenols and/or condensation of OMWW, and a bioprocessing-fermentation phase.
Place and Duration of Study: The study was conducted at the TEI of Thessaly from January 2013 to August 2015.

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Methodology: OMWW was used as a fermentation substrate for production of SCP after dephenolization by microfiltration, condensation via reverse osmosis, and addition of minerals/nitrogen sources. *Saccharomyces cerevisiae* (*S. cerevisiae*), *Candida utilis* (*C. utilis*) and *Pleurotus ostreatus* (*P. ostreatus*) were grown in this substrate under controlled process conditions in shake flasks and a 15 lt bioreactor. Biomass, sugars, phenol concentration of OMWW and the protein content of the harvested biomass were evaluated.
Results: Dephenolization of OMWW is essential for sufficient growth of both yeasts, while *P. ostreatus* grows better in the untreated substrate, as it can degrade the polyphenols while the dephenolization step reduced also the sugar content of OMWW. Optimum process conditions for *S. cerevisiae* included a pH 7, 350 rpm agitation rate, addition of $(NH_4)_2SO_4$ to a 3/1 condensed medium, while *C. utilis* grew best at pH 5, 250 rpm, after addition of NH_4NO_3 in a 3/1 condensed medium. Maximum biomass concentration was 13.5 g/l and 14.6 g/l, respectively. 14.8 g/l of *P. ostreatus* biomass were obtained at pH 5, 150 rpm after addition of NH_4NO_3. This mold had the highest yield but lowest productivity due to slow growth.
Conclusion: *P. ostreatus* is a good producer of SCP in OMWW and reduces its organic load, but it has lower protein content. *C. utilis* had the highest SCP productivity, and the two yeasts had the highest protein concentration but require dephenolization of OMWW.

Keywords: Single cell protein; Saccharomyces cerevisiae; Candida utilis; Pleurotus ostreatus; olive mill waste water utilization; olive polyphenols; dephenolization.

1. INTRODUCTION

Sustainable agriculture and the minimization of environmental pollution from agro-industrial waste is one of the challenges and targets of modern agricultural economy. Olive mill waste waters (OMWW) are the effluent waters that derive from the washing and pressing of olive fruits from which olive oil is produced after centrifugation of the crushed and milled olive fruits [1-2]. The residual by-product of olive oil production is a major environmental pollutant (when disposed of without processing) in Mediterranean countries where most of world's olive oil is produced, since it has a high organic load and high BOD and COD values as well toxicity against plants and aquatic organisms, due to the presence of sugars, polyphenols, polyalcohols, etc. The chemical composition of olive mill waste waters is variable according mainly to the type and maturity of the olive fruit and the type of olive oil production system (there are "two-phase" or "three-phase" systems, the latter using more water and producing a larger volume of more dilute waste water), as has been described by several authors [1-4]. Many attempts have been made for the chemical/enzymatic/biological treatment of OMWW which aim at the degradation of the organic compounds (especially sugars and polyphenols) and subsequently the reduction of BOD/COD values, in order to allow the disposal of a less harmful by-product to the environment. However, due to its sugar content the OMWW can not only be discarded more safely, but it can also be utilized as a fermentation substrate for biotechnological production of useful and added-value microbial metabolites, such as single cell protein (SCP), which is the protein contained in microbial biomass, that can be used as animal feed in crude form or in human nutrition after treatment (removal of nucleic acids and/or cell wall) [5-8]. Yeasts such as *S. cerevisiae* and *C. utilis* are two of the most common candidates for SCP production from yeasts, while *P. ostreatus* is an edible fungi (mushroom) which is suitable for SCP production [9-12]. One of the bottlenecks in the full utilization of OMWW as a fermentation substrate is the presence of polyphenols which have antimicrobial activity, as well as the low concentration of fermentable sugars (~5g/l) [1,4,13]. In our approach, the olive polyphenols were removed from OMWW by microfiltration, without use of organic solvents, in order to be purified and then used as a natural antioxidant or antimicrobial ingredient for use in food, cosmetics or in agriculture and the dephenolized waste was condensed via reverse osmosis in order to increase its sugar content and reduce the size of the total OMWW [14-17]. This type of biovalorization of OMWW can effectively lead to the production of a relatively high concentration of microbial biomass (cells) with medium (for *P. ostreatus*) to high (for *S. cerevisiae* and *C. utilis*) protein content, while the residual waste after the harvesting (filtration or centrifugation) of biomass has a much lower sugar and potentially lower polyphenol concentration and thus much lower organic load and toxicity.

2. MATERIALS AND METHODS

2.1 Olive Mill Waste Water (OMWW) as a Fermentation Substrate

The OMWW derived from different olive oil producing companies from the region of Thessaly, Greece, all of which used a three-phase production system. The OMWW from these sources was initially collected and kept refrigerated by the company Polyhealth S.A. (olive polyphenol producer, Larisa, Greece) which then supplied the necessary OMWW for our studies. The OMWW was refrigerated before following further treatments, which included dephenolization (partial but not complete removal of phenols) without use of organic solvent, via microfiltration through macroporous resins (XAD-4 and AMBERLITE FPX66), and also condensation via reverse osmosis (using PCI MEMBRANES-FP 100) up to 4 times the initial volume of the waste. To this substrate, several additional nutrients were added in some cases, especially nitrogen sources which are scarce in this substrate. In all processes, the substrate was also added with 2 g/l of potassium phosphate (1,2 g/l K_2HPO_4 + 0,8 g/l KH_2PO_4) and 2 g/l of magnesium sulphate ($MgSO_4$) in order to have sufficient potassium, phosphorus, sulphur and magnesium concentration in the fermentation broth to support cell growth. All chemicals used were of analytical grade.

2.2 Microorganisms

S. cerevisiae (DSM-70449), *C. utilis* (DSM-2361), and *P. ostreatus* (DSM-1020) were supplied by the German culture collection DSMZ. They were cultivated and maintained in slants and petri dishes with Potato Dextrose agar (LAB-M) and test tubes with Malt Extract Broth (LAB-M). *S. cerevisiae* and *C. utilis* were incubated at 25℃ for 3 days, while *P. ostreatus* was incubated at 25℃ for up to 7 days.

2.3 Production of Inoculum

In the standard process for inoculum preparation, one colony from Potato Dextrose agar (PDA) was used to inoculate a test tube with 10 ml Malt Extract Broth (MEB), and after incubation and adequate growth this was used as the inoculum for shake flasks containing the fermentation substrate. In the production of inoculum that was adapted to OMWW and the presence of polyphenols, the colonies were grown on PDA supplemented with 25% OMWW and subsequently, these colonies were inoculated in test tubes with 10 ml MEB supplemented with 25% OMWW (MEB-OMWW). This partial inclusion of OMWW at 25% was chosen in order to help the cells adapt to the fermentation substrate (OMWW), while at the same time ensuring a dense cell population.

2.4 Fermentation Process and Production of SCP

The production of SCP was carried out mostly in 500 ml shake flasks containing 300 ml substrate using an Innova 40 R shaker (New Brunswick) at a constant temperature and agitation rate. After optimization of culture condition and substrate composition, the optimal conditions were applied in a scaled-up batch fermentation using a 15lt bioreactor (Bioflo 415, New Brunswick) with 10lt working volume. Unless stated otherwise, the standard fermentation conditions were 30℃ temperature, 250 rpm agitation rate, pH 5, 5% inoculum (cells grown on MEB). Where necessary, pH was adapted to 5, 6 or 7, by addition of 2M NaOH. All substrates were sterilized in an autoclave at 121℃ for 15 min before use.

2.5 Analyses

For the measurement of biomass concentration 30 ml of the fermentation broth was either centrifuged at 5000 rpm for 30 min (for *S. cerevisiae* and *C. utilis*) or filtered through 1.2 μm filters (for *P. ostreatus*). The sediment was dried at 105℃ overnight until constant weight and the biomass concentration was counted after the weighing of the dried cell debris. The supernatant of the centrifugation (or the filtrate after filtration) of the fermentation broth was collected separately and used for the estimation of polyphenol and sugars concentration of the fermentation broth. Total sugars, polyphenols and cell protein concentrations were measured spectrophotometrically. Reducing sugars were measured according to the DNS (dinitrosalicylic acid) method [18], while total polyphenols were measured according to the Folin-Ciocalteu method [19] and expressed as gallic acid equivalent. The protein concentration of the microbial biomass was estimated according to the Bradford method [20-21], after the resuspension of the fresh sediment of biomass in 10 ml saline water and the rupture of the cells with a sonicator probe for 10 minutes. The disrupted cells were then centrifuged at 5000 rpm x 30 min and after sedimentation of the

fragmented cell wall the supernatant was used for the estimation of the (intracellular) protein content of the cell biomass. All chemicals used were of analytical grade, and all analyses described above were carried out in triplicate and the mean values are reported here. For the shake of clarity of the figures, the values of standard deviation (in the form of error bars) are depicted only in the optimized scaled-up processes carried out in the bioreactor.

3. RESULTS AND DISCUSSION

3.1 Production of Single Cell Protein by *S. cerevisiae* and *C. utilis*

The production of SCP, based on the ability to effectively produce microbial biomass was comparatively studied for *S. cerevisiae* and *C. utilis* in OMWW. Our aim was to improve this substrate by removing (partly) the polyphenols which restrict cell growth, by supplying additional nitrogen sources which are scarce in this substrate, and by increasing the sugar content by condensing the initial OMWW, as well as to indentify some of the optimal environmental and process conditions (pH, agitation rate, type and volume of inoculum) for biomass production by *S. cerevisiae* and *C. utilis* in this substrate. The results of this experiment are presented below.

3.1.1 Effect of removal of phenols (dephenolization) of OMWW on SCP production

The effect of polyphenol removal via microfiltration through macroporous resins is shown in Figs. 1a, 1b and 1c. We observe that *C. utilis* can grow better than *S. cerevisiae* in the original substrate of OMWW regardless of the dephenolization processing (Figs. 1a,1b). The removal of phenols is not complete (Fig. 1c), and it also leads to some loss (13-15%) in the sugar content of the substrate (Fig. 1b), probably due to partial attachment of sugars to the phenolic compounds and the resin used for dephenolization. However, for both the two yeasts studied here the removal of phenols was clearly beneficial to cell growth and carbohydrate metabolism, showing that the polyphenols exert a restrictive effect on these yeasts. It is also very likely that the absence of necessary nutrients (such as nitrogen sources which are necessary for protein biosynthesis) restricts the utilization of sugars for further growth, since only about half of the sugar content of OMWW was utilized in these bioprocesses. This hypothesis was tested in the following step.

3.1.2 Effect of addition of nitrogen sources

In order to fortify the dephenolized OMWW substrate, which was shown to be preferable than the original untreated OMWW, we added different nitrogen sources, as well as other nutrients. Namely, we added 5 g/l of Yeast Extract (YE), or ammonium nitrate [NH_4NO_3], or ammonium sulphate [$(NH_4)_2SO_4$], or soy peptone. The results (Figs. 2a and 2b) showed that yeast extract was very beneficial for the growth of both yeasts and resulted in the highest growth rate, probably because it also contains many minerals and vitamins. For *C. utilis* YE lead to the highest production of biomass (9.3 g/l) at 70 h of fermentation, followed by ammonium nitrate (7.5 g/l biomass in 70 h). For *S. cerevisiae*, however, the fast growth due to YE addition was abruptly stopped at 63h followed by an equally fast decline in biomass, which was not due to the exhaustion of sugars, since 8.5 g/l of total sugars were available by the end of the fermentation (data not shown). For *S. cerevisiae* ammonium sulphate seems to be the most beneficial nitrogen (as well as sulphur) source, leading to the production 5.7 g/l biomass at 85 h. Peptone was the least preferable nitrogen source for both yeasts, possibly due to the extra energy required for breaking down the peptides to simpler nitrogen sources (e.g. ammonia), but any addition of the above mentioned nitrogen sources in OMWW resulted in an improvement of cell growth of *S. cerevisiae* and *C. utilis*, showing that supplementation with a readily assimilated and preferably cheap nitrogen source is necessary for this fermentation. Taking into account the relatively high cost of YE, especially for producing SCP, it was decided that the addition of 5 g/l of ammonium sulphate and ammonium nitrate is preferable for optimizing SCP production by *S. cerevisiae* and *C. utilis*, respectively.

3.1.3 Effect of the initial pH of the substrate

In order to see whether the initial pH of the OMWW (~5), which remains practically unchanged during the fermentation (data not shown), is another limiting factor for this bioprocess, *S. cerevisiae* and *C. utilis* were grown in OMWW supplemented with nitrogen source (5 g/l of ammonium sulphate and 5 g/l ammonium nitrate, respectively) at an initial pH of 5, 6 and 7. The results (Fig. 3) indicated that although for *C. utilis* pH 5 was optimal for cell growth in OMWW reaching 7.5 g/l in 70 h and the highest growth rate, *S. cerevisiae* grew faster and accumulated more biomass at pH 7 (6.3 g/l

at 85 h) than at pH 5 (5.7 g/l at 85 h). Thus, the optimal pH 5 and pH 7 were applied to all fermentations with *C. utilis* and *S. cerevisiae*, respectively, thereafter.

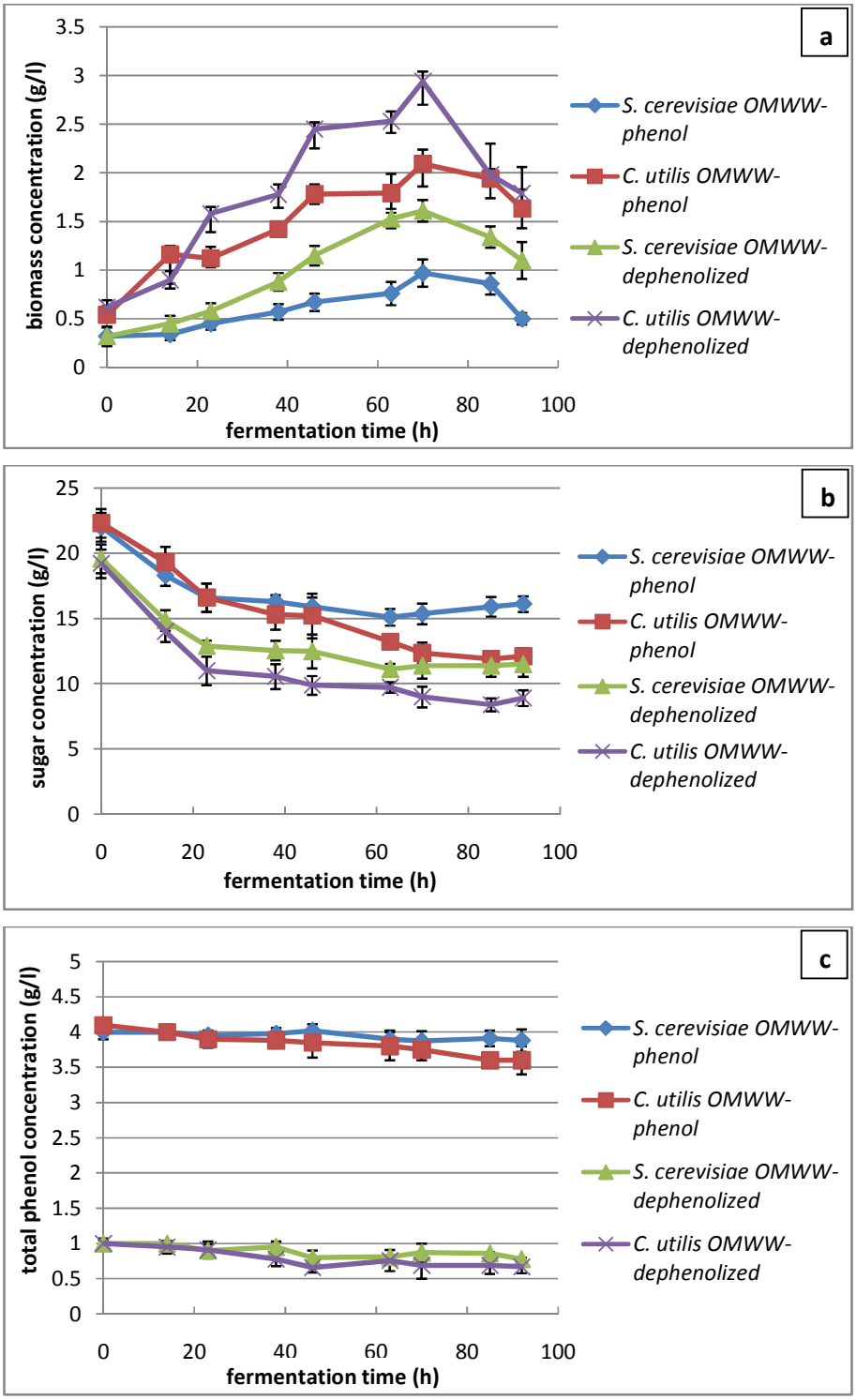

Fig. 1. Biomass production (a), sugar consumption (b) and total phenol concentration (c) during growth of *S. cerevisiae* and *C. utilis* in OMWW with and without removal of phenols (dephenolization)

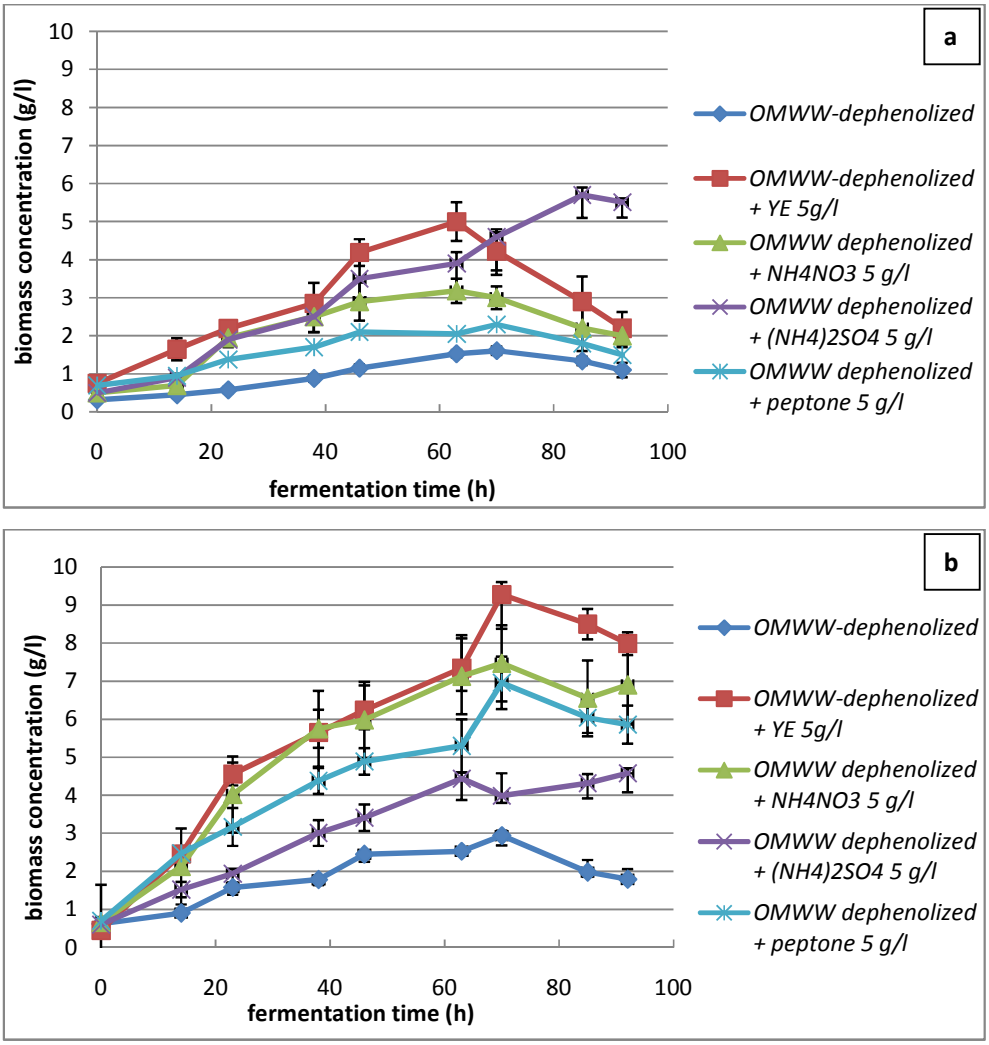

Fig. 2. Effect of the addition of nitrogen sources on biomass growth in OMWW by *S. cerevisiae* (a) and *C. utilis* (b)

3.1.4 Effect of the type and volume of inoculums

Since biomass growth is relatively limited and the cells cannot easily degrade the polyphenols found (even in the "dephenolized" substrate), we tested whether the use of a higher percentage of inoculum (10% instead of 5%) could be beneficial to biomass production. For similar reasons, we also tested whether a previous adaptation of the cells to dephenolized OMWW (i.e. perpetual growth of the pure culture in Malt Extract agar and then Malt Extract broth, both containing 25% dephenolized OMWW) could help the cells adapt better to the environment of the OMWW fermentation substrate. In Figs. 4(a and b) we observe that indeed, the previous step of adaptation of the inoculum to OMWW enhanced the growth of both *S. cerevisiae* and *C. utilis*. For both yeasts a 10% inoculum previously adapted to growing in dephenolized OMWW (MEB-OMWW inoculum) had much better performance compared to the standard inoculum (MEB inoculum), leading to the formation of 8.2 g/l and 9.8 g/l biomass (at 85 h) of *S. cerevisiae* and *C. utilis*, respectively.

3.1.5 Effect of agitation rate

The agitation or stirring rate is another important parameter influencing industrial fermentation as it affects mass and heat transfer, as well as aeration, especially in shake flask experiments. A high agitation rate for an aerobic process such as SCP production from fungi can improve mixing and thus cell growth, however, if it is too high it

may impose a shear stress or oxidative stress that may be detrimental to cell growth [22]. In this experiment we applied 3 different agitation rates (150, 250 and 350 rpm) using the optimum pH and nitrogen source supplementation for each yeast, namely, pH 7 and addition of 5 g/l of ammonium sulphate for *S. cerevisiae* and pH 5 and 5 g/l of ammonium nitrate for *C. utilis*. The results (Fig. 5) demonstrate that the maximum biomass was achieved at 350 rpm for *S. cerevisiae* (7.1 g/l at 70 h), which exhibits very poor growth at 150rpm compared to 250 and 350 rpm, while for *C. utilis* a maximum growth rate and maximum biomass concentration of 7.5 g/l was obtained at 70 h when agitation rate was maintained at 250 rpm.

3.1.6 Effect of condensation and sugar content of the substrate

In order to further improve the production of biomass from OMWW as well as reduce the size of the total OMWW that requires handling and storage, the dephenolized substrate was condensed via reverse osmosis 2,3 and 4 times (2/1, 3/1, 4/1, respectively) so as to increase the amount of fermentable sugars. In each case the initial sugar concentration of the substrate was 19.2, 27.7, 35.1, and 42.7 g/l respectively for the non-condensed, the condensed 2/1, 3/1 and 4/1 dephenolized OMWW, while total phenolics were 1.0, 1.7, 2.3, and 2.9 g/l for the non-condensed, the condensed 2/1, 3/1 and 4/1 dephenolized OMWW, respectively. Apparently, a non-linear relation exists between the degree of condensation (based on reduction of volume) and the sugars and phenol concentration, due to losses of some carbohydrates and phenols during the reverse osmosis (RO) condensation, probably caused by their flushing in the effluent water.

As can be observed in Figs. 6(a and b), the preferable degree of condensation of the OMWW is 3/1 for both yeasts. A 4/1 condensation offers the most sugars and is slightly preferable to the non-condensed medium with regard to biomass production, but these sugars cannot be fully metabolized, possibly due to the higher content of polyphenols in this substrate. An optimal 10.9 g/l of biomass of *C. utilis* was achieved at only 63 h after a 3/1 condensation and at that time residual sugars were 19.1 g/l while initial sugar concentration was 35 g/l. This corresponds to an average growth rate of 0.165 g/l/h up to 63 h and a yield of 0,73 (biomass produced/sugars consumed). For *S. cerevisiae*, the maximum biomass reaches 9.1 at 70 h, at which point residual sugars are 20,4 g/l. This means that average growth rate for *S. cerevisiae* was 0.123 g/l/h up to 70h and the yield was 0.62 (biomass produced/sugars consumed). These findings suggest that although none of the two yeast can fully utilize this substrate, *C. utilis* is a better producer of biomass and SCP in condensed 3/1 OMWW compared to *S. cerevisiae.*

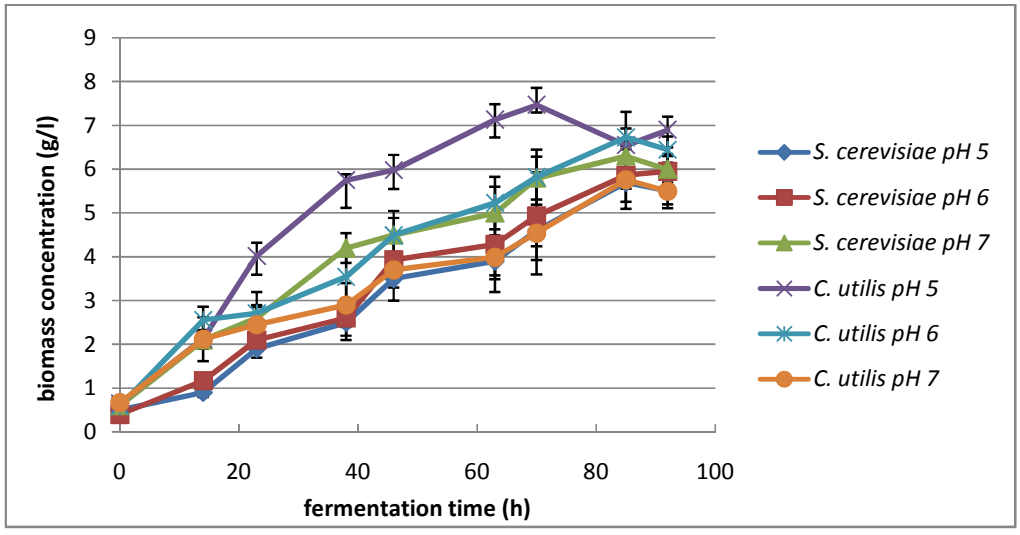

Fig. 3. Biomass of *S. cerevisiae* and *C. utilis* grown in nitrogen-supplemented OMWW at different initial pH values

OMWW substrate was supplemented with 5 g/l ammonium sulphate for S. cerevisiae and 5 g/l ammonium nitrate for C. utilis

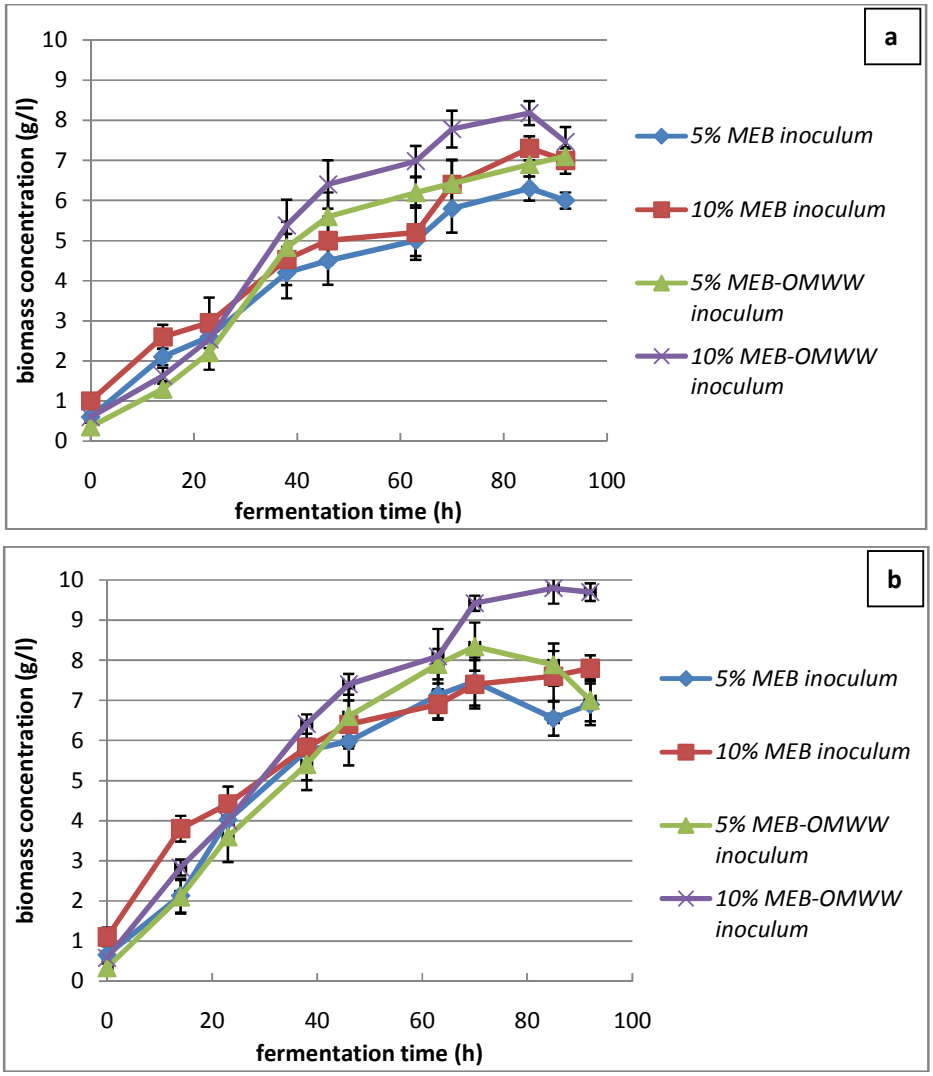

Fig. 4. Effect of type and volume of the inoculum on biomass production by *S. cerevisiae* (a) and *C. utilis* (b) grown in nitrogen-supplemented OMWW

MEB is the standard inoculum grown in Malt Extract broth, MEB-OMWW is the inoculum grown on Malt Extract Broth that contains 25% dephenolized OMWW. The pH of the fermentation substrate was 7 for S. cerevisiae and 5 for C. utilis

3.1.7 Bioprocess optimization in stirrred tank bioreactor

Following the shake flasks experiments where substrate improvement and several fermentation conditions were studied with regard to process optimization, a final optimization step was carried in 15 lt fermentors with 10 lt fermentation broth for both *S. cerevisiae* and *C. utilis*, which combined all the optimal conditions that were previously demonstrated in the shake flask experiments. Therefore, the substrate for *S. cerevisiae* SCP production was a dephenolized, 3/1 condensed OMWW with addition of 5 g/l ammonium sulphate and the process conditions were: pH 7, 10% inoculum previously adapted to OMWW (grown in MEB-OMWW), 350 rpm agitation rate, 30℃ temperature. Aeration was also supplied to the bioreactor via an air compressor and aeration rate was kept stable at 1 vvm (volume of air inserted/volume of fermentor/minute) throughout the fermentation. For *C. utilis* SCP production the substrate was a dephenolized, 3/1 condensed OMWW with addition of 5 g/l ammonium nitrate and the process conditions were: pH 5, 10% inoculum previously adapted to OMWW (grown in MEB-OMWW), 250 rpm agitation rate, 30℃ temperature and 1vvm aeration rate.

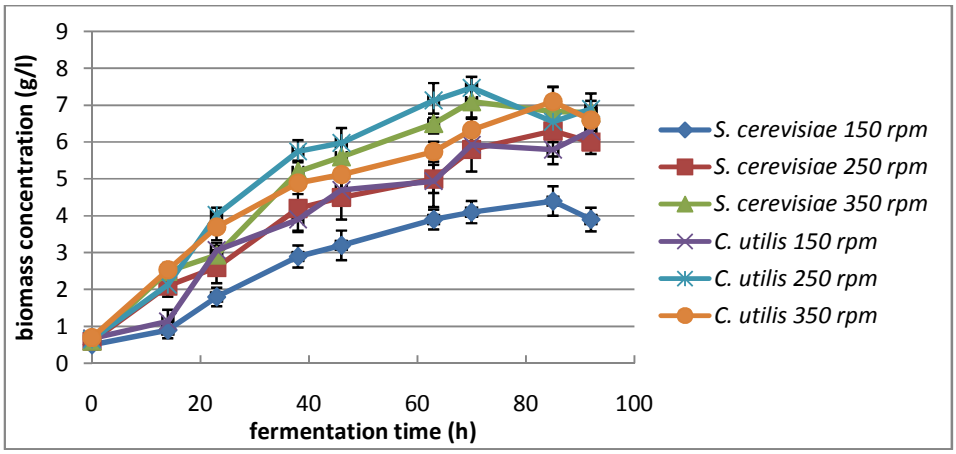

Fig. 5. Effect of agitation rate on biomass production by *S. cerevisiae* and *C. utilis* in OMWW supplemented with 5 g/l of ammonium sulphate for *S. cerevisiae* (pH 7) and 5g/l of ammonium nitrate for *C. utilis* (pH 5)

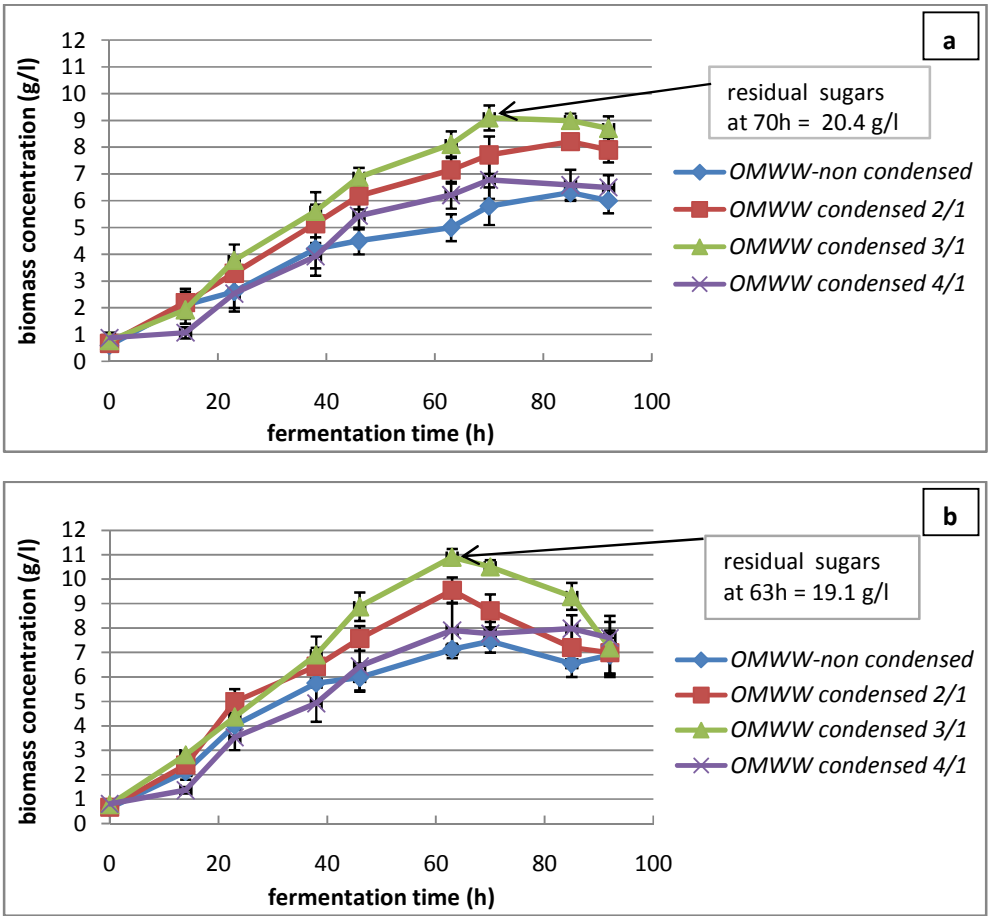

Fig. 6. Production of biomass by *S. cerevisiae* (a) and *C. utilis* (b) in non-condensed and condensed OMWW supplemented with nitrogen source (ammonium sulphate for *S. cerevisiae* and ammonium nitrate for *C. utilis*)

The fermentation broth was adjusted to pH 7 for the growth of S. cerevisiae and pH 5 for the growth of C. utilis. Agitation rate was 250 rpm and a 5% standard inoculum (grown on MEB) was used for both yeasts

As shown in Fig. 7, under the selected optimal condition for growth in OMWW, *C. utilis* grew better at a higher average growth rate (0.23 g/l/h up to 63 h) and produced more biomass (14.6 g/l at 63 h), compared to *S. cerevisiae* which reached a maximum biomass of 13,5 g/l at much later stage (at 83 h), having an average growth rate of 0.16 g/l/h up to 83 h. However, the yield (biomass produced/sugars consumed) was similar for the two yeast, namely 0,59 (at 63 h) for *C. utilis* and 0.57 (at 83 h) for *S. cerevisiae*. It appears that *C. utilis* is a better producer of biomass in OMWW, not only because of the higher concentration it can attain, but also thanks to its higher productivity (higher growth rate) which translates into lower production costs. This aforementioned biomass concentration (~1.5%) maybe much lower to the 4-6% concentration obtained in standard substrates used for SCP production, such as corn/sugar syrups or molasses, however, it is definitely not a negligible concentration if one takes into account the low sugar concentration and the high polyphenol content of OMWW which make it a harsh substrate for single cell protein production [23]. In addition, a significant advantage of the optimized SCP production using OMWW, is the utilization of a costless agricultural byproduct, and the reduction of its organic load, so that environmental pollution can be averted [23-25].

3.2 Production of Single Cell Protein by *P. ostreatus*

In order to study the efficacy of *P. ostreatus* as a producer of SCP, several experiments were carried out in shake flasks and then a final optimization step was conducted in a 15 lt bioreactor. Based on some of the findings of the previous experiments on yeasts (*S. cerevisiae* and *C. utilis*), the inoculum used here was previously adapted to OMWW after sequential growth in Malt extract agar and Malt Extract Broth which both contained 25% OMWW (MEB-OMWW grown inoculum). This inoculum was used at 10% concentration in the fermentation broth. The agitation rate was 350 rpm unless stated otherwise. The fermentation temperature was 25℃ as it was previously observed during inoculum preparation that a higher density (in test tubes with Malt Extract Broth) and a faster expansion of mycelium (in Malt Extract Agar) was obtained at 25 compared to 30℃. We examined the effect of dephenolization, the effect of addition of ammonium nitrate as an extra nitrogen source, the effect of pH, and the effect of agitation rate. The results of this experiment are shown below.

3.2.1 Effect of dephenolization and nitrogen supplementation

The initial OMWW was dephenolized as described above and the efficacy of the untreated and the dephenolized substrate were comparatively examined as concerns biomas production by *P. ostreatus*. Also, the effect of the addition of 5 g/l of ammonium nitrate to the OMWW was studied. Figs. 8 (a,b,c) shows that the removal of phenols via microfiltration is not only unnecessary for achieving efficient cell growth by *P. ostreatus*, but it is also detrimental to the formation of biomass, probably due to the reduced sugar concentration that accompanies the dephenolization step and the fact that *P. ostreatus* evidently degrades olive polyphenols quite efficiently. More specifically, 6.3 g/l of biomass could be harvested at 281 h in the untreated substrate, while the dephenolized medium yielded only 5.0 g/l in 218 h, and apart from the reduced polyphenol concentration, also had a 20% reduction in the carbohydrate content.

As concerns the additional nitrogen source, ammonium nitrate increased the formation of biomass significantly, but this improvement was more pronounced in the untreated OMWW, where 5 g/l additional ammonium nitrate resulted in an increase in biomass from 6.3 (without ammonium nitrate) to 9.8 g/l at 281 h.

As expected [11], olive polyphenols were apparently degraded in this fermentation process and the phenol consumption rate was much higher in the untreated substrate where the initial 3.9 g/l phenol content dropped to 1,7 g/l at 281 h. Interestingly, the addition of ammonium nitrate did not improve phenol degradation by *P. ostreatus*.

3.2.2 Effect of pH and agitation rate

Unlike the two yeasts studied above, it seems that *P. ostreatus* grows better at low agitation rate (150 rpm), possibly because the higher agitation rates may exert a high shear stress to the fungal mycelium which affects its growth [22]. With regard to fungal morphology, it was also observed that at high agitation rates many small pellets (≤ 5 mm) were formed, while at 150 rpm a few large pellets and many dispersed mycelia were formed in the fermentation broth.

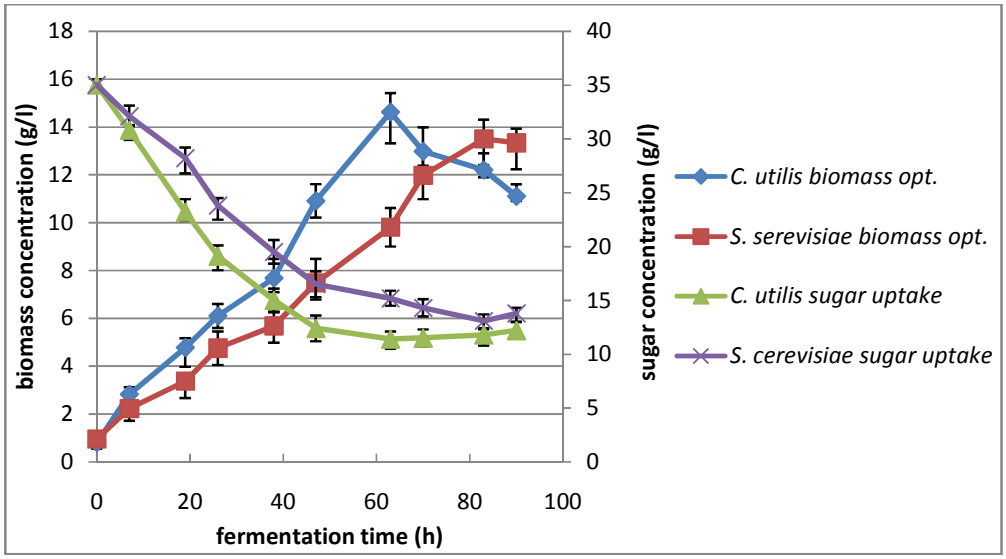

Fig. 7. Optimized biomass and sugar concentration during growth of *S. cerevisiae* and *C. utilis* in dephenolised, 3/1 condensed OMWW supplemented with nitrogen sources (5 g/l ammonium sulphate for *S. cerevisiae* and 5 g/l ammonium nitrate for *C. utilis*), at 30℃, 1 vvm aeration rate, using a 10% inoculum previously adapted to OMWW (grown in MEB-OMWW)

C. utilis was grown at pH 5 and agitated at 250 rpm, S. cerevisiae was grown at pH 7 and agitated at 350 rpm. Error bars represent the standard deviation for each measurement

The initial pH is also important for process optimization. It seems that the most preferable is an initial pH 5 which allows the growth of *P. ostreatus* cells up to 9.8 g/l at 281 h. In this process the average growth rate up to 281 h reaches a maximum value of 0.03 g/l/h. This is of course much lower than the growth rate achieved with the yeasts *S. cerevisiae* and *C. utilis*, but it is normal for a slow-growing higher fungi like *P. ostreatus*.

3.2.3 Process optimization in stirred tank bioreactor

Based on the previous observations, an optimized and scaled up fermentation was carried out in a 15 lt stirred tank bioreactor using 10 lt of fermentation substrate. In this fermentation the following optimal condition were applied: an untreated OMWW was used (without dephenolization), supplemented with 5 g/l ammonium sulphate. *P. ostreatus* was grown at pH 5, temperature 25℃, agitation rate 150 rpm, using 10% of a previously OMWW-adapted inoculum (previously grown in MEB-OMWW). In this fermentation *P. ostreatus* biomass reached 14.8 g/l, while the available sugars were almost fully consumed (there were only 2.9 g/l of residual sugars at 331 h). The average growth rate up to 281 h was 0.047 g/l/h which may be low compared to the two yeasts studied earlier, however it is rather high for a fungi and especially a mushroom such as *P. ostreatus*. The yield at 281 h (biomass produced/sugars consumed) was 0.81, which is significantly higher than that obtained with the two yeast studied above. These results are comparable to results found in other studies regarding agroindustrial waste valorization by *P. ostreatus* [10,26,27].

It can be deduced that, despite its slow growth, *P. ostreatus* is a relatively good producer of biomass and SCP in this kind of substrate, and although the productivity of this process is lower compared to a yeast process for SCP production, *P. ostreatus* can produce more biomass in a non-condensed OMWW. In addition, the fact that no dephenolization is necessary for optimal growth of *P. ostreatus,* is an advantage as it allows us to omit one processing step (dephenolization). The significant reduction of sugars and polyphenol content (75% and 48% reduction respectively, by 281 h which is the optimal harvest point, or 87% and 60% respectively by 381 h) in OMWW by *P. ostreatus* also means that the residual broth after harvesting the biomass will have much lower COD and BOD values, compared to the processes where *S. cerevisiae* and *C. utilis* were used.

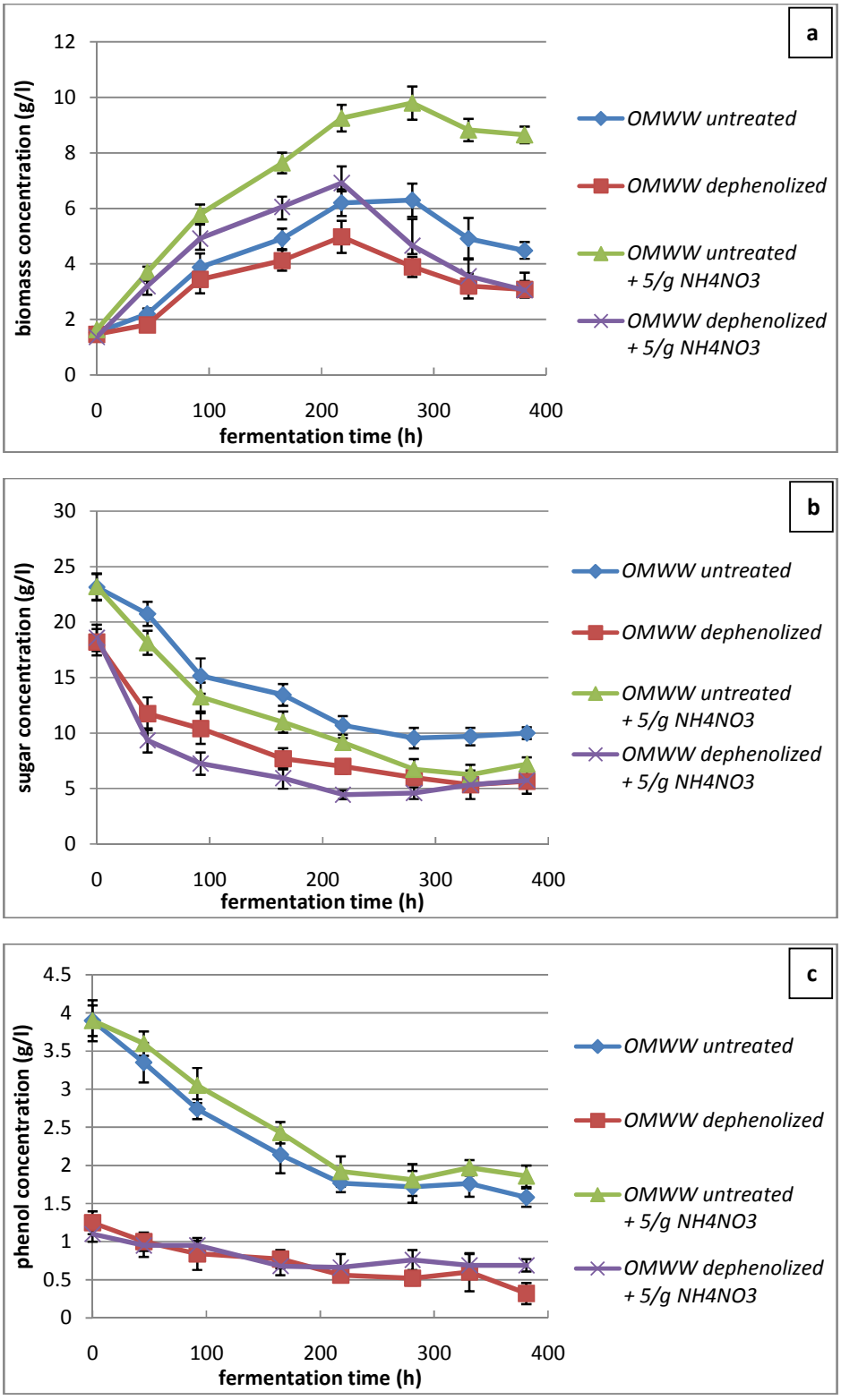

Fig. 8. Biomass (a), sugars (b) and total phenol (c) concentration during growth of *P. ostreatus* in OMWW before and after dephenolization, with or without addition of 5 g/l ammonium nitrate

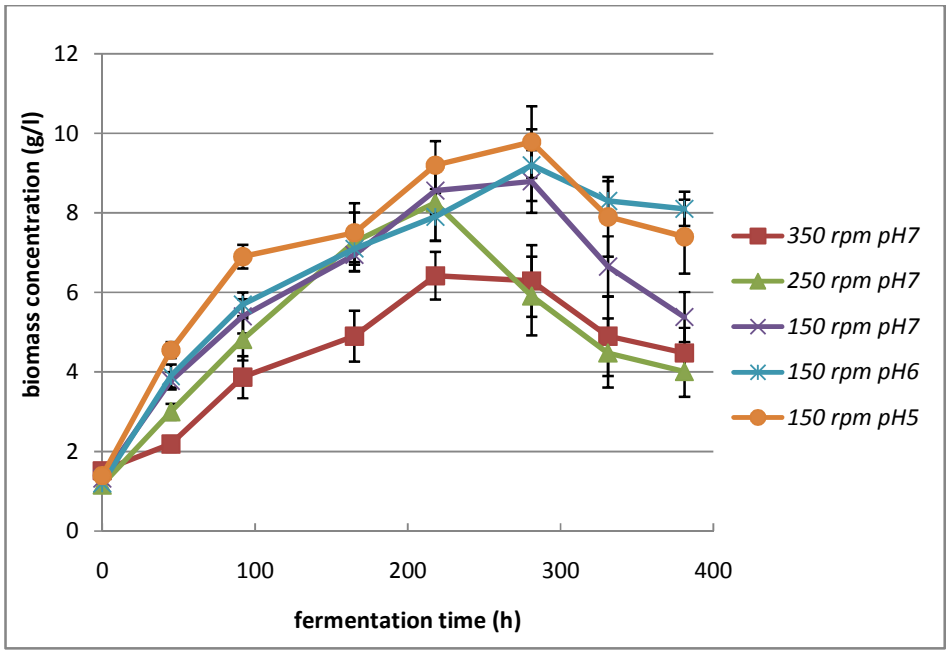

Fig. 9. Biomass concentration of *P. ostreatus* in an untreated (non-dephenolized) OMWW at different agitation rates (150, 250 and 350 rpm) and three different initial pH values (pH 7, 6 and 5)

Process temperature was 25℃ and a 10% inoculum ada pted in OMWW (previously grown in MEB-OMWW) was used

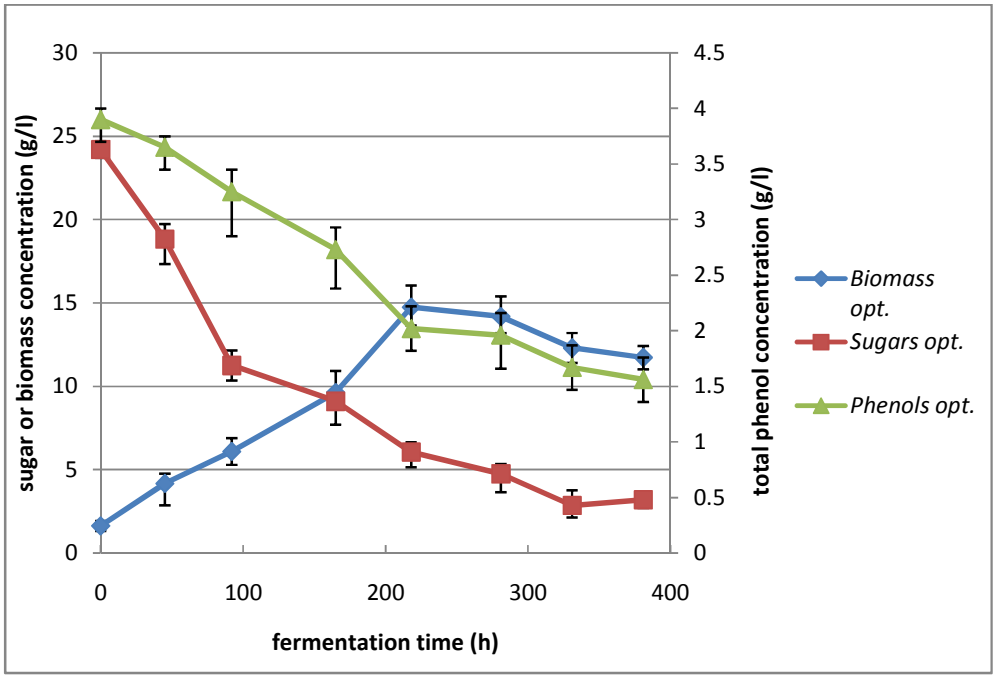

Fig. 10. Biomass, sugars, and phenols concentration during an optimized fermentation of OMWW by *P. ostreatus*

An untreated OMWW was used, supplemented with 5 g/l ammonium sulphate. Process conditions were: pH 5, temperature 25℃, agitation rate 150 rpm, inoculum 10% (previously grown in MEB-OMWW)

It is also probable that if a condensed OMWW substrate is used, the increased carbohydrate concentration may lead to an even higher production of biomass by *P. ostreatus* (as in the case of *S. cerevisiae* and *C. utilis*), which is worth studying in the future.

3.3 Protein and Phenol Concentration of Fungal SCP from OMWW

The fungal biomass derived from *S. cerevisie* and *C. utilis* was harvested via centrifugation of the fermentation medium while mycelium and pellets of *P. ostreatus* were retrieved via filtration. The centrifuged and dried pellets of *C. utilis* biomass harvested from dephenolized and condensed (3/1) OMWW are depicted in Fig. 11. The protein and phenol concentration of this fungal biomass was measured as described in Materials & Methods and the results of these measurements are given in Table 1.

Protein concentration was similar in the two yeasts studied here (slightly higher in *C. utilis* in the dephenolized OMWW) and also much higher in *S. cerevisiae* and *C. utilis* in comparison to *P. ostreatus*, as expected, since molds have a relatively low protein content [5,6,25-26]. An approximately double protein concentration was obtained in the SCP from *C. utilis* using a dephenolized OMWW, compared to previous studies using other yeasts in untreated (non-dephenolized) OMWW [23]. The phenol concentration in the fresh biomass pellet was dependent on the phenol concentration in the fermentation broth and the degree of condensation, and it was highest in *P. ostreatus* biomass. This indicates that *P. ostreatus* can not only degrade polyphenols much more effectively than the yeasts studied here, but it also accumulates more polyphenols in its biomass, resulting in a phenol-rich crude SCP, which could be an advantage when used as animal feed, due to the antioxidant properties of phenols [1,28].

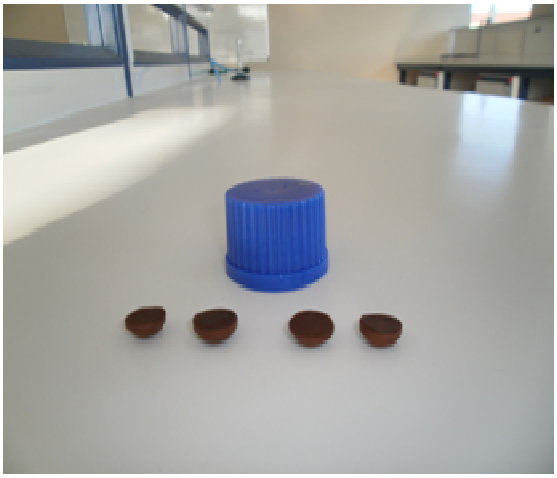

Fig. 11. Picture of dried biomass pellets of *C. utilis* grown in dephenolized and condensed (at a ratio 3/1) OMWW which can be used as crude SCP

Table 1. Protein and total phenol composition of fungal biomass used as SCP. *S. cerevisiae* and *C. utilis* biomass was grown in Dephenolized OMWW with or without condensation (3/1); *P. ostreatus* was grown in untreated (non-dephenolized) OMWW

Type of substrate*	% Protein Concentration in Biomass (g protein /100 g dry biomass)			% Phenol Concentration in Biomass (g phenol /g dry biomass)		
	S. cerevisiae	***C. utilis***	***P. ostreatus***	***S. cerevisiae***	***C. utilis***	***P. ostreatus***
Untreated OMWW (with ~4% phenol)	ND**	ND	35.6±2.4	ND	ND	0.068±0.011
Dephenolized OMWW	51.8±1.1	52.1±2.9	ND	0.016±0.03	0.013±0.002	ND
Dephenolized OMWW Condensed 3/1	49.0±1.3	48.9±1.7	ND	0.039±0.08	0.034±0.005	ND

**All substrates were supplemented with additional nitrogen sourse (5 g/l ammonium sulphate for S. cerevisiae or 5 g/l ammonium nitrate for C. utilis and P. ostreatus).*
***ND: Not determined*

4. CONCLUSION

Based on the findings of this research, we can conclude that OMWW can be utilized and valorized as a substrate for single cell protein production by yeasts such as *S. cerevisiae* and *C. utilis* or by molds such as *P. ostreatus*, after the addition of some minerals and especially nitrogen sources, and preferably after condensation of the substrate (3/1) in order to improve its sugar concentration. For the sufficient growth of *S. cerevisiae* and *C. utilis* removal (or significant reduction) of the olive polyphenols of the substrate is essential, while *P. ostreatus* is advantageous in that it does not require dephenolization of OMWW.

C. utilis is a better and faster producer of SCP from OMWW compared to *S. cerevisiae*, and both yeasts result in a SCP with high protein concentration. *P. ostreatus* is a slow-growing mushroom, but it is readily adapted in the environment of this substrate, as it can degrade olive polyphenols quite efficiently.

Overall, *P. ostreatus* has the highest yield of produced biomass per consumed sugars, but the lowest productivity. Its SCP has a relatively low protein content but is also rich in beneficial olive phenols. Based on these findings, the use of *P. ostreatus* could be proposed for the production of crude SCP for use in animal feed (as well as in human nutrition) and the simultaneous drastic reduction of the polyphenol and sugar content and the organic load of the OMWW, in order to produce a more environmentally-friendly waste. *C. utilis* could be preferable as a producer microorganism when the high protein content is the main objective, especially for use in human nutrition, but it will require dephenolization of OMWW prior to fermentation in order to be able to exhibit a high productivity (high biomass growth rate), and the residual waste after biomass harvesting may require further biodegradation before disposal.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Tsagaraki E, Lazarides HN, Petrotos KB. Olive mill wastewater treatment. In Utilization of by-products and treatment of waste in the food industry. Springer US. 2007;133-157.
2. Fiorentino A, Gentili A, Isidori M, Monaco P, Nardelli A, Parrella A. Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components. J Agr Food Chem. 2003;51:1005-1009.
3. Ryan D, Robards K, Lavee S. Changes in phenolic content of olive during maturation. Int J Food Technol. 1999;34(3):265.
4. *Paraskeva* P, *Diamadopoulos* E. Technologies for olive mill wastewater (OMW) treatment: A review. J Chem Technol Biotechnol. 2006;81:1475-1485.
5. Nasseri AT, Rasoul-Amini S, Morowvat MH and Ghasemi Y. Single cell protein: Production and process. Amer J Food Technol. 2011;6:103-116.
6. Anupama, Ravindra P. Value added food: Single cell protein. Biotechnol Advan. 2000;18:459-479.
7. Garsallah N. Production of single cell protein from olive mill wastewaters by yeasts. Environ Technol. 1993;14:393-395.
8. Mondal AK, Sengupta S, Bhowal J, Bhattacharya DK. Utilization of fruit wastes in producing single cell protein. Intern J Sci Environ Technol. 2012;1(5):430-438.
9. Chanda S, Chakrabarti S. Plant origin liquid waste, a source for single cell protein production by yeast. Biores Technol. 1996; 57:51–4.
10. Villas-Boas SG, Esposito E, de Mendonca MM. Bioconversion of apple pomace into a nutritionally enriched substrate by *Candida utilis* and *Pleurotus ostreatus*. World J Microbiol Biotechnol. 2003;19:461-467.
11. Aggelis G, Iconomou D, Christou M, Bokas D, Kotzailias S, Christou G, Tsagou V, Papanikolaou S. Phenolic removal in a model olive oil mill wastewater using

Pleurotus ostreatus in bioreactor cultures and biological evaluation of the process. Water Res. 2003;37:3897–3904.

12. Rajoka MI, Kiani MAT, Khan S, Awan MS, Hashmi AS. Production of single cell protein from rice polishings using *Candida utilis*. World J Microbiol Biotechnol. 2004; 20:297–301.
13. Dermeche S, Nadour M, Larroche C, Moulti-Mati F, Michaud P. Olive mill wastes: Biochemical characterizations and valorization strategies. Process Biochem. 2013;48:1532–1552.
14. Petrotos KB, Lellis T, Kokkora MI, Gkoutsidis PE. Purification of olive mill wastewater using microfiltration membrane technology. J Memb Separ Tech. 2014;3: 50-55.
15. Petrotos KB, Gkoutsidis PE, Kokkora MI, Giankidou KG, Tsagkarelis AG. A study on the kinetics of olive mill wastewater (OMWW) polyphenols adsorption on the commercial XAD4 macroporous resin. Desalin Water Treat. 2013;51(10-12): 2021-2029.
16. Leontopoulos SV, Giavasis I, Petrotos K, Kokkora M, Makridis C. Effect of different formulations of polyphenolic compounds obtained from omww on the growth of several fungal plant and food borne pathogens. Studies *in vitro* and *in vivo*. Agric Agricult Sci Proced. 2015;4:327-337.
17. Petrotos KB, Karkanta FK, Gkoutsidis PE, Giavasis I, Papatheodorou KN, Ntontos AC. Production of novel bioactive yogurt enriched with olive fruit polyphenols. World Acad Sci, Engineer Technol. 2012;64:867-872.
18. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal Chem. 1959;31:426.
19. Singleton, VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin–ciocalteu reagent. Method Enzymol. 1999;299:152-178.
20. Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.
21. Noble JE, Bailey MJA. Quantitation of protein. Methods Enzymol. 2009;463:73-95.
22. Fazenda ML, Seviour R, McNeil B, Harvey LM. Submerged culture fermentation of "higher fungi": The Macrofungi. Advanc Appl Microbiol. 2008;63:33-103.
23. Gharsallah N. Production of single cell protein from olive mill waste water by yeasts. Environ Technol. 1993;14(4):391-395.
24. Halasz A, Lasztity R. use of yeast biomass in food production. CRC Press; 1990.
25. Begea M, Sirbu A, Kourkoutas Y, Dima R. Single-cell protein production of Candida strains in culture media based on vegetal oils. Roman Biotechnol Lett. 2012;17(6): 2012.
26. Kahlon SS, Arora M. Utilization of potato peels by fungi for protein production. J Food Sci Technol. 1986;23(5):264-267.
27. Bisaria R, Bisaria VS, Hobson PN. An integrated approach to utilization of agro-residues through *Pleurotus* Cultivation. Crit Rev Biotechnol. 1987;7(1):17-41.
28. El-Abbassi A, Kiai H, Hafidi A. Phenolic profile and antioxidant activities of olive mill wastewater. Food Chem. 2012;132(1): 406-412.

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