View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Ulster University's Research Portal

Bioresource Technology 205 (2016) 1–6

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Hydrolysis of olive mill waste to enhance rhamnolipids and surfactin production

Ignacio Moya Ramírez [a,*], Deisi Altmajer Vaz [a], Ibrahim M. Banat [b], Roger Marchant [b], Encarnación Jurado Alameda [a], Miguel García Román [a]

[a] Chemical Engineering Department, Faculty of Sciences, University of Granada, Spain
[b] School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, N. Ireland, UK

HIGHLIGHTS

- Olive mill waste has high potential as carbon source for biosurfactant production.
- Hydrolysis enhanced bioavailability of sugars present in olive mill waste.
- *P. aeruginosa* and *B. subtilis* can use hydrolysed olive mill waste as carbon source.
- Hydrolysis of olive mill waste enhanced biosurfactant yield.
- Hydrolysed olive mill waste showed lower inhibitory effects that non-hydrolysed.

ARTICLE INFO

Article history:
Received 2 December 2015
Received in revised form 7 January 2016
Accepted 8 January 2016
Available online 13 January 2016

Keywords:
Biosurfactant
Surfactin
Rhamnolipids
Olive mill waste
Hydrolysis

ABSTRACT

The aim of this work was to demonstrate the effectiveness of hydrolysis pretreatment of olive mill (OMW) waste before use as a carbon source in biosurfactant production by fermentation. Three hydrolysis methods were assessed: enzymatic hydrolysis, acid pretreatment plus enzymatic hydrolysis, and acid hydrolysis. Fermentation was carried out using two bacterial species: *Pseudomonas aeruginosa* and *Bacillus subtilis*. Our results showed that the enzymatic hydrolysis was the best pretreatment, yielding up to 29.5 and 13.7 mg/L of rhamnolipids and surfactins respectively. Glucose did not show significant differences in comparison to enzymatically hydrolysed OMW. At the best conditions found rhamnolipids and surfactins reached concentrations of 299 and 26.5 mg/L; values considerably higher than those obtained with non-hydrolysed OMW. In addition, enzymatic pretreatment seemed to partially reduce the inhibitory effects of OMW on surfactin production. Therefore, enzymatic hydrolysis proved to effectively increase the productivity of these biosurfactants using OMW as the sole carbon source.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Biosurfactants (BS) are amphiphilic surface active molecules of biological origin which are attracting great interest from both the scientific community and industry in the last few years (Marchant and Banat, 2012a; Reis et al., 2013). This is due to several attractive advantages over synthetic surfactants, including the possibility of production from renewable resources through fermentation. Furthermore they have other favourable characteristics such as better biocompatibility and biodegradability, and good performance under extreme conditions of salinity, temperature or pH (Lima et al., 2011; Lotfabad et al., 2009; Marchant and Banat, 2012b; Mulligan, 2009).

Currently the main problem inhibiting large scale production of biosurfactants is the high production costs (Geys et al., 2014). Substantial improvements are needed in downstream processing in order to find an economically viable process (Banat et al., 2014). Another approach to reduce costs is to use waste products as the fermentation carbon source, which adds value to the waste while reducing production costs (Helmy et al., 2011). The suitability of several waste materials as carbon source for biosurfactant production has been assessed in previous research works (Makkar et al., 2011). Typically, these wastes are produced by the agriculture and food industries, and in general they can be classified as oils,

Abbreviations: OMW, olive mill waste; EH-OMW, enzymatically hydrolysed olive mill waste; PEH-OMW, pretreated and enzymatically hydrolysed olive mill waste; AH-OMW, acid hydrolysed olive mill waste.

* Corresponding author.
E-mail address: ignaciomr@ugr.es (I. Moya Ramírez).

http://dx.doi.org/10.1016/j.biortech.2016.01.016
0960-8524/© 2016 Elsevier Ltd. All rights reserved.

glycerol, sugars and lignocellulosic-containing residues (Henkel et al., 2012).

Olive mill waste (OMW), commonly known as "alpeorujo" or "alperujo" in Spain, is a waste produced after the first extraction of olive oil in the two-phase process (Tortosa et al., 2012). It is a semisolid product, mainly composed of lignocellulosic material, some residual oil, salts and minerals (Dermeche et al., 2013). Nowadays it represents a severe environmental problem, particularly in Mediterranean countries (McNamara et al., 2008). In addition, the high concentration of phenols and polyphenols in this waste are problematic for biological processing. However, the presence of residual oil and free sugars suggest that this waste could be used as carbon source for microbial growth. In two previous papers we have shown that OMW can be used as a carbon source for rhamnolipid and surfactin production, using strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively (Maass et al., 2015; Moya Ramírez et al., 2015). However, our results suggested that an optimisation of the production process is needed.

A prier hydrolysis step to increase the bioavailability of sugars present in the cellulose and hemicellulose fractions of OMW can be a beneficial step. Actually, this kind of pretreatment has been used in previous studies with several waste materials (Taherzadeh and Karimi, 2008), particularly for bioethanol production (Abu Tayeh et al., 2014). However, its use to enhance biosurfactant production has been described in only a few recent reports, and, as far as we know, never with OMW. For example, Ma et al. (2014) and Konishi et al. (2015) used enzymatically and chemically hydrolysed corncob residue to produce up to 42.1 and 49.2 g/L of sophorolipids, respectively. Marin et al. (2015) obtained surfactin from hydrolysed sisal pulp, while Faria et al. (2014) produced up to 2.5 g/L of mannosylerythritol lipids by using enzymatically hydrolysed wheat straw.

In this work we have evaluated the effectiveness of the hydrolysis of OMW, prior to the fermentation step, for enhancing the bioavailability of the cellulosic and hemicellulosic material present in it. To do that, three different hydrolysis processes namely, (i) acid, (ii) enzymatic, and (iii) a combined acid–enzymatic treatment have been tested, and two bacterial strains, *P. aeruginosa* and *B. subtilis*, were used. To the best of our knowledge this is not only the first time that hydrolysed OMW is used as carbon source for biosurfactant production, but that rhamnolipids are produced from a hydrolysed agroindustrial waste.

2. Methods

2.1. Materials

Agar, glucose, peptone, phenol, Folin Ciocalteu reagent and salts for culture media were purchased from Panreac-Applichem (Barcelona, Spain). Ethyl acetate, methanol, cellulose, $MgSO_4$, gallic acid, rhamnolipid and surfactin standards, as well as the enzymes Cellulase from *Trichoderma reesei* ATCC 26921 (700 FPU/g), Viscozyme® L (hemicellulose, 100 FPU/g) and Xylanase from *Termomyces lanuginosus* (2500 FPU/g) were purchased from Sigma Aldrich (St. Louis-MO, USA). OMW was generously supplied by a local olive oil producer (Cooperativa LA UNIÓN, Montilla, Spain), and used as received. Its composition was as follows: dry matter 35.6%, lipids 3.9%, protein 7.1%, and free sugars 9.5%. An elemental analysis, carried out in a Flash 2000 analyser (Thermo Scientific, Waltham-MA, USA) yielded the following results: carbon 48.2%, nitrogen 1.2%, and hydrogen 7.1%.

2.2. Hydrolysis of olive mill waste

Three methods were used to hydrolyse the hemicellulose fraction of OMW: (i) acid hydrolysis, (ii) enzymatic hydrolysis, and (iii) acid pretreatment followed by enzymatic hydrolysis. For the acid hydrolysis the method described by Sluiter et al. (2011) was followed. Briefly, 2 g of OMW were mixed with 1.92 mL of H_2SO_4 (97% purity), and incubated at 30 °C for 1 h. Subsequently, the mixture was diluted to a final volume of 85 mL, autoclaved for 1 h and finally neutralized with concentrated NaOH. Enzymatic hydrolysis was carried out with a mixture of cellulases, hemicellulases and xylanase. The selected amount of OMW (2, 5 or 10 g) was placed in a flask with 50 ml acetate buffer (50 mM, pH 5). Enzymes were added in the following concentrations: 2000 FPU/L of Cellulase, 285 FPU/L of Viscozyme® and 1000 FPU/L of Xylanase. The mixture was maintained at 50 °C and agitated at180 rpm for 72 h. For the acid pretreatment 50 mL of H_2SO_4 0.5% v/v was added to 2 g of OMW which was then autoclaved at 125 °C for 30 min and finally neutralized with NaOH. Afterwards, enzymatic hydrolysis was carried out as described above.

In each case, after the hydrolysis pretreatment, culture medium salts were added and the final volume was adjusted to 0.1 L.

2.3. Fermentations

Bacteria were first inoculated in a Petri dish and incubated at 37 °C for 24 h. To start the batch culture two seed cultures were consecutively prepared: Seed culture 1 was a PPGAS medium with Tris–HCl (19 g/L), protease peptone (10 g/L), glucose (5 g/L), KCl (1.5 g/L), NH_4Cl (1 g/L) and $MgSO_4$ (0.4 g/L) in distilled water. Seed culture 2 was a mineral salt medium composed of glucose (20 g/L), $NaNO_3$ (2 g/L), Na_2HPO_4 (0.9 g/L), KH_2PO_4 (0.7 g/L), $MgSO_4{\cdot}7H_2O$ (0.4 g/L), $CaCl_2{\cdot}2H_2O$ (0.1 g/L), $FeSO_4{\cdot}7H_2O$ (0.001 g/L) and the following trace elements $ZnSO_4{\cdot}7H_2O$ (0.7 mg/L), $CuSO_4{\cdot}5H_2O$ (0.5 mg/L), $MnSO_4{\cdot}H_2O$ (0.5 mg/L), H_3BO_3 (0.26 mg/L) and $Na_2MoO_4{\cdot}2H_2O$ (0.06 mg/L). Seed culture 1 was inoculated with one loop from the Petri dish and seed culture 2 with 5% v/v from culture 1, both grown at 37 °C and 160 rpm for 24 h.

Batch fermentation experiments were conducted with the three forms of hydrolysed OMW described above. The culture medium was the same as that for seed culture 2, fixing glucose concentration to the desired value or substituting it for hydrolysed OMW. One litre Erlenmeyer flasks were used with a final culture volume of 100 mL. Cultures were inoculated with 5% v/v of seed culture 2 and maintained at 37 °C and 160 rpm. All the experiments were carried out in triplicate.

The identities of the two microorganisms used were confirmed through sequencing the 16S rRNA gene as *B. subtilis* N1 (GenBank accession number KT595698) and *P. aeruginosa* PAO1. Both strains are available at University of Ulster's culture collection.

2.4. Analytical procedures

Dry weight (DW), and phenol and sugar concentrations of the culture medium were measured. Cells were separated by centrifugation at 10^5 g for 15 min at 4 °C. Cell growth was monitored by dry weight (DW) of pellets obtained from 1 mL of culture medium. Because of the solid fraction of OMW, these results were not accurate, and therefore they were only used as indicative results. The supernatant was used for subsequent measurements. The phenol–sulfuric method was used to quantify total sugars (Albalasmeh et al., 2013), while Folin Ciocalteu reagent was used to find the total phenol concentration (Magina et al., 2010).

For the biosurfactant extraction (rhamnolipids or surfactin) 50 mL of supernatant was adjusted to pH 2. Afterwards it was gently mixed in a funnel with the same volume of ethyl acetate and left at rest until phase separation. The organic phase was collected. These steps were repeated three times. The three organic fractions were combined, dried with $MgSO_4$ and rotatory evaporated. The crude extract was dissolved in a small amount of

methanol and dried. Finally the produced biosurfactants were identified and quantified by UPLC-MS as described previously (Moya Ramírez et al., 2015).

3. Results and discussion

3.1. Hydrolysis pretreatment and biosurfactant production

The objective of this work was to study the effectiveness of a hydrolysis pretreatment of OMW for a subsequent fermentation and biosurfactant production. All the hydrolysis methods used, i.e., acid hydrolysis (AH), enzymatic hydrolysis (EH) and acid pretreatment followed by enzymatic hydrolysis and acid hydrolysis (PEH), considerably increased the total soluble sugar concentration, yielding up to 3.1, 4.0 and 4.3 times the initial amount of soluble sugars for EH, PEH and AH respectively (Table 1). These values were similar to those reported by Haagensen et al. (2009) for PEH of OMW. In spite of being the least aggressive hydrolysis procedure, EH liberated up to 71.8% of the sugars achievable by acid hydrolysis. As observed, the three hydrolysis procedures yielded a considerable increase in bioavailable sugars for fermentation, something which could lead to increased biosurfactant production. Indeed, the two bacterial strains effectively metabolized the hydrolysed sugars, although there was always a fraction of unconsumed sugars for both microorganisms (Table 1). This fraction probably corresponds to non-fermentable sugars. Considering that AH completely hydrolysed all the cellulose and hemicellulose, results suggest that the fraction of fermentable sugars was similar for both *Pseudomonas* and *Bacillus*. However *Bacillus* could metabolize a higher fraction of the sugars released by the two pretreatments which involved enzymatic hydrolysis.

Hydrolysis pretreatments strongly influenced biosurfactant production for both bacterial species, although they did not significantly affect the cell growth (Fig. 1). Despite the different soluble sugar concentration, both strains showed similar cell DW for the three pretreatments, with the exception of *Pseudomonas* grown in AH-OMW, for which the increase in bioavailable sugars was also reflected in DW. Likewise, BS production showed identical patterns for both strains, i.e., EH yielded the highest BS amounts, whereas AH-OMW generated the lowest one. Rhamnolipid concentration reached values of 29.5 mg/L with EH-OMW and only 6.0 mg/L were obtained with AH-OMW, while surfactins values were 13.7 and 5.1 g/L respectively. Therefore, although EH yielded the lowest amount of soluble sugars available for fermentation, it maximised biosurfactant productivity. The lower BS production with PEH and AH could be due to the release of inhibitory substances during the acid hydrolysis step, which hinder cellular growth and BS production. Marin et al. reported the same effect for sisal pulp hydrolysed chemically and enzymatically, and suggested that furfurals and hydroxymethylfurfurals liberated during acid hydrolysis could be responsible of the decrease in surfactin production (Marin et al., 2015). Furthermore salts generated in the neutralization of the acid could also have had an inhibitory effect. Consequently, PEH and AH, in spite of releasing more soluble sugars from the OMW, showed significant disadvantages for the production of biosurfactant.

Table 1
Total soluble sugars per gram of dry OMW (S/OMW) after hydrolysis, initial (S_0) and final sugar concentration after fermentation with *P. aeruginosa* and *B. subtilis* cultures after each hydrolysis pretreatment of OMW. Cultures were carried out at 37 °C and 160 rpm during 6 days.

			Soluble sugars after fermentation (g/L)	
	S/OMW (g/g)	S_0 (g/L)	*P. aeruginosa*	*B. subtilis*
HE-OMW	0.30 ± 0.01	2.11 ± 0.08	0.65 ± 0.08	0.47 ± 0.03
PEH-OMW	0.38 ± 0.02	2.72 ± 0.11	0.60 ± 0.03	0.51 ± 0.01
AH-OMW	0.41 ± 0.01	2.94 ± 0.10	0.59 ± 0.02	0.64 ± 0.05

However, all the hydrolysis pretreatments noticeably enhanced BS productivity in comparison to the fermentations conducted with non-hydrolysed OMW of our previous work (Moya Ramírez et al., 2015). For example, for EH pretreatment, the produced surfactin increased from 3.1 to 13.7 mg/L and the rhamnolipid production augmented from 8.8 to 29.5 mg/L in the culture media. The high titres reached after EH confirmed that an appropriate hydrolysis pretreatment is a key factor for a potential industrial production of biosurfactant from OMW. Furthermore, this assumption could be valid for other agroindustrial wastes with a considerable cellulose and hemicellulose fractions (Marin et al., 2015). In addition, it is worth noting that while many agroindustrial residues demand some kind of physical pretreatment before the hydrolysis stage (Sun and Cheng, 2002), OMW is already ground, so its use will avoid this energy-intensive step. This imparts a great advantage over other lignocellulosic agroindustrial wastes in a future development of a cost effective BS production process.

3.2. Kinetics

A kinetic study was performed to compare a commonly used carbon source in fermentation, glucose, with OMW hydrolysed by the best method found in the previous section, i.e., enzymatic hydrolysis. With this comparison in mind the glucose concentration was fixed at the same concentration as the soluble sugar found after EH.

In general terms, the obtained results for DW and biosurfactant production, were quite similar for both carbon sources, see Fig. 2. *Pseudomonas* uptake of sugars took place within the first 24 h for both, glucose and EH-OMW. Therefore, the results suggest that the remaining soluble sugars detected after 24 h for EH-OMW correspond to a fraction of non-fermentable sugars (Fig. 2a and b). This residual fraction was also observed for *Bacillus* cultures, although it was slightly lower as mentioned above. Again, with this strain all the fermentable sugars were consumed in the first 24 h for EH-OMW, while glucose consumption lasted 144 h (Fig. 2c and d). Concerning cellular growth, *Pseudomonas* reached its maximum biomass concentration after 24 and 48 h for glucose and EH-OMW respectively. This difference was probably because EH-OMW did not provide a culture medium as favourable as that with glucose, and therefore cellular growth was faster with glucose. With *Bacillus*, results were quite similar for both carbon sources, DW slightly increased after the first 24 h, reaching the maximum after 144 h, and again glucose yielded slightly more biomass than EH-OMW.

Finally, we did not detect significant differences in biosurfactant production between both carbon sources. When *P. aeruginosa* grew in glucose, rhamnolipid concentration rose during the first 48 h up to a value of 44.5 mg/L followed by a decrease during the next 48 h to values around 30 mg/L, and remained constant until the end of the experiment. However, EH-OME did not show the aforementioned maximum. Instead, it reached a concentration close to 30 mg/L after the first 24 h of culture, a value which remained constant during all the experiment. *B. subtilis* showed the same profile for both carbon sources, surfactin concentration rose during the first 96 h of culture reaching values of 26.1 and 19.0 mg/L with glucose and EH-OMW respectively. After that, surfactin concentration started to decrease in both cases. A maximum in surfactin concentration was also observed by Maass et al. (2015).

In view of these results we can confirm that HE-OMW is a carbon source comparable to glucose in terms of biosurfactant production, especially if we take into account the benefits of employing a cheap agroindustrial waste to obtain a high added

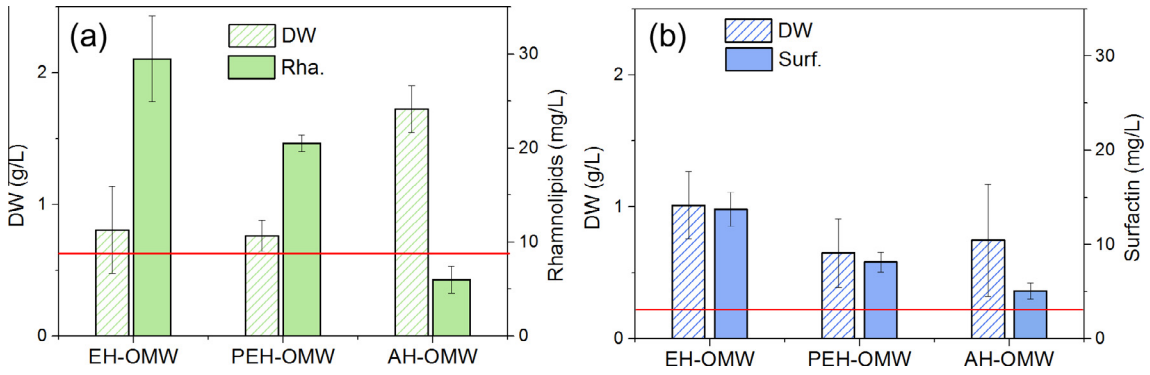

Fig. 1. Effect of OMW hydrolysis procedure (enzymatic, EH-OMW, pretreatment before enzymatic hydrolysis, PEH-OMW, or acid, AH-OMW) on cell dry weight (DW) and biosurfactant production for *P. aeruginosa* (a) and *B. subtilis* (b) cultures. The values were obtained after 6 days of fermentation at 37 °C and 160 rpm, with 2% w/v of hydrolysed OMW. Red lines correspond to biosurfactant concentration obtained with non-hydrolysed OMW.

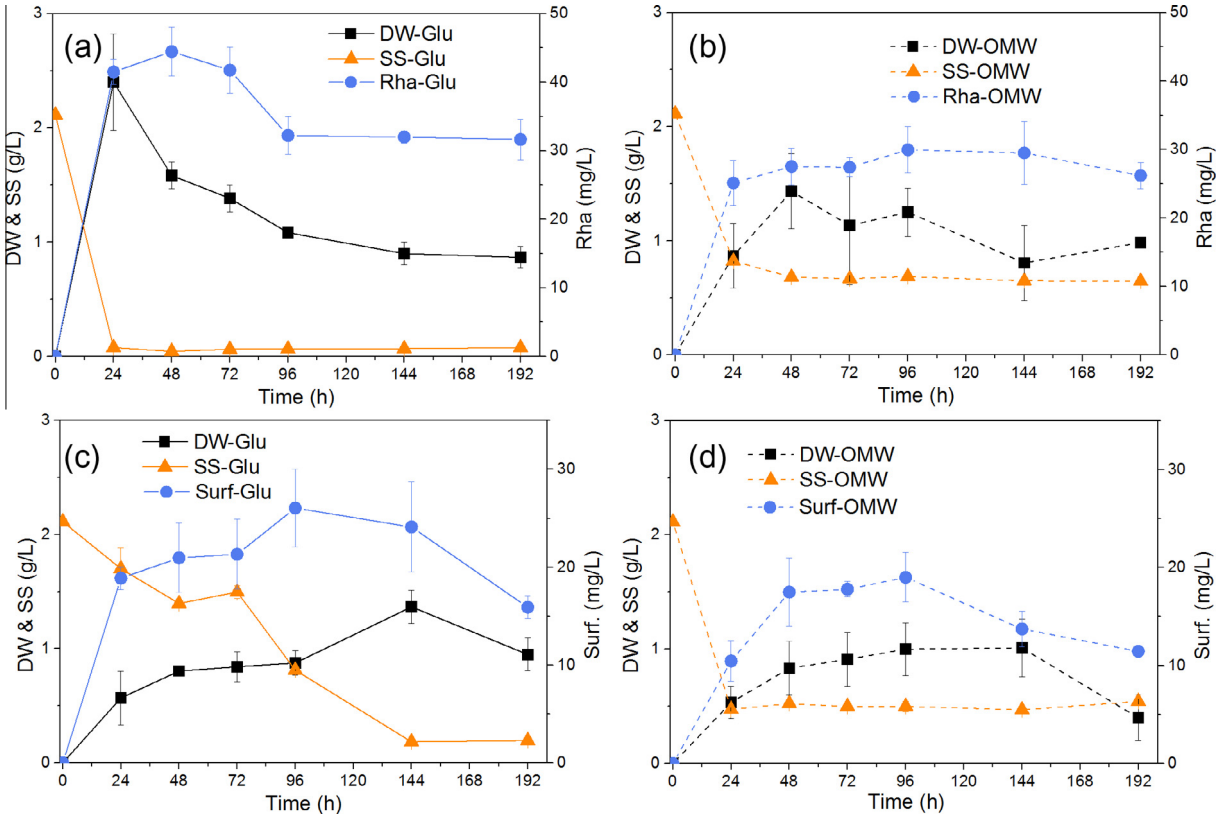

Fig. 2. DW (■, g/L), soluble sugar (SS) concentration (▲, g/L) and rhamnolipid or surfactin concentration (●, right axis, mg/L) in the culture media vs culture time, using glucose (continuous line) or HE-OMW (dashed line) as carbon source. Graphs (a) and (b) correspond to *P. aeruginosa*, (c) and (d) to *B. subtilis* cultures. HE-OMW concentration was 2% w/v. Initial glucose concentration was fixed as the equivalent to the amount of soluble sugars after EH of OMW, i.e., 2.11 g/L. All the experiments were carried out at 37 °C and 160 rpm.

value substance, and the avoidance of some of the environmental problems that OMW generates. In addition to the higher values obtained with HE-OMW compared with non-hydrolysed OMW referred to in the previous section, BS production was faster when using the pretreated waste (Moya Ramírez et al., 2015). This fact supports the proposition that a large scale production process might be feasible.

3.3. Oil mill waste concentration effect

In our first work with OMW we concluded that the concentration of this waste has a considerable effect on the BS produced by the two bacterial species. While *P. aeruginosa* showed increased rhamnolipid production with increments of OMW, surfactin production by *B. subtilis* was strongly inhibited. Comparing these results with those obtained with EH-OMW for the same three concentrations, a meaningful improvement in the biosurfactant production was observed under all the conditions and for both bacterial species (Fig. 3). In relative terms, EH supported a greater improvement in surfactin production, although it enhanced biosurfactant production for both microorganisms. In the case of rhamnolipid and in relative terms, the biggest increment was at 2% of EH-OMW, BS production being almost 3.4 times higher after EH pretreatment of OMW. As well as for untreated OMW, the maximum yield was observed at 10%, reaching a value of 298.9 mg/L in the culture medium. Interestingly, B. *subtilis* showed a different

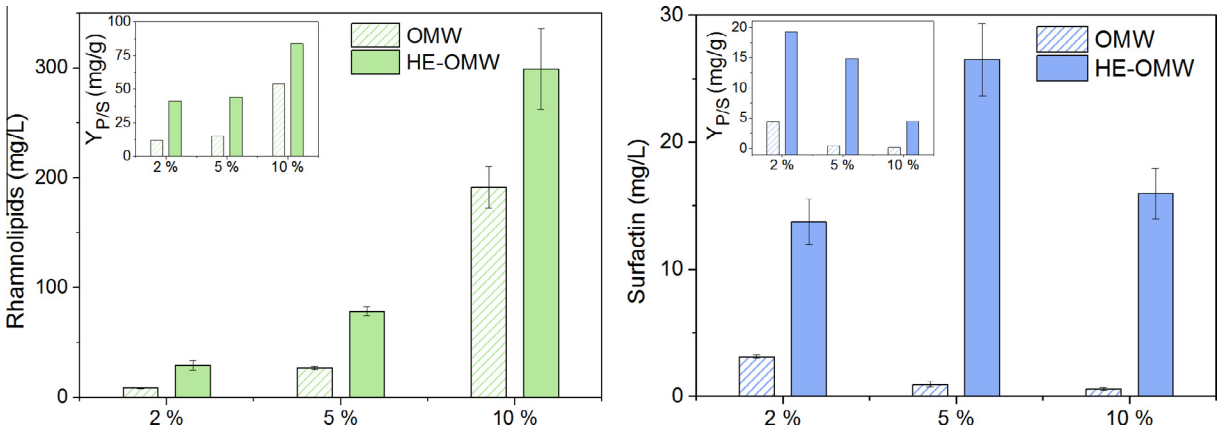

Fig. 3. Effect of concentration (% w/v) of EH-OMW (coloured bars) and non-hydrolysed OMW (pattern bars) on rhamnolipid and surfactin production (mg/L in the culture media) by *P. aeruginosa* and *B. subtilis* after 6 days of culture at 37 °C and 160 rpm. Insets figures show $Y_{P/S}$ (mg/g) for the same experiments referred to dry OMW. Data of non-hydrolysed OMW correspond to our previous work (Moya Ramírez et al., 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pattern with the increase in concentration of EH-OMW compared to the use of non-hydrolysed OMW. By using the pretreated waste, the highest surfactin yield was achieved at 5%, instead of 2%, and reached a concentration of 26.5 mg/L, almost 28.4 times the value obtained with non-hydrolysed OMW at 5%, and 8.5 times the best yield obtained at 2% with non-hydrolysed OMW. The observed maximum in surfactin production suggested that the EH pretreatment and the subsequent increase in the bioavailable sugar concentration reduced the inhibitory effects of OMW on surfactin production by *B. subtilis*. Nevertheless at 10% the inhibitory effect seemed to be stronger than the positive influence of the increase of soluble sugar concentration in the culture medium, leading to a decrease in surfactin concentration. In view of all these results we can state that the increase in bioavailable sugars of OMW produces a considerable increase in surfactin and rhamnolipid production, and that OMW concentration is a key factor which needs to be optimised.

In terms of productivity, $Y_{P/S}$ (in mg biosurfactant/g dry OMW) showed the same trend for EH and non-hydrolysed OMW for both microorganisms (insets of Fig. 3). Increasing the substrate concentration resulted in an increase of $Y_{P/S}$ for *P. aeruginosa* and a decrease for *B. subtilis*. These data clearly showed the inhibitory effect of OMW over surfactin production, probably due to the phenol composition of this waste (Gursoy-Haksevenler and Arslan-Alaton, 2014). On the other hand, *P. aeruginosa* showed a better performance at higher OMW concentration. Furthermore, the increase in carbon source concentration was also reflected in the $Y_{P/S}$ value for both (hydrolysed and non-hydrolysed OMW) and in the overall rhamnolipid production. However, given the negligible price of OMW and the high added value of biosurfactants, the best conditions for BS production would be those yielding the highest BS concentration in absolute terms (and not the optimal ones in terms of productivity).

3.4. Study of phenolic compounds development

In the previous section we have discussed the inhibitory effect of OMW, which is probably due to the presence of phenolic compounds in this waste. The behaviour of both strains at high OMW concentration suggests that *Pseudomonas* is more resistant to the presence of these substances than *Bacillus*. This is probably because *Pseudomonas* is a Gram-negative organism, less sensitive to phenols than the Gram-positive *Bacillus* strain (Ramos-Cormenzana et al., 1996). In order to gain more information about this issue, phenol concentration was measured after culture growth.

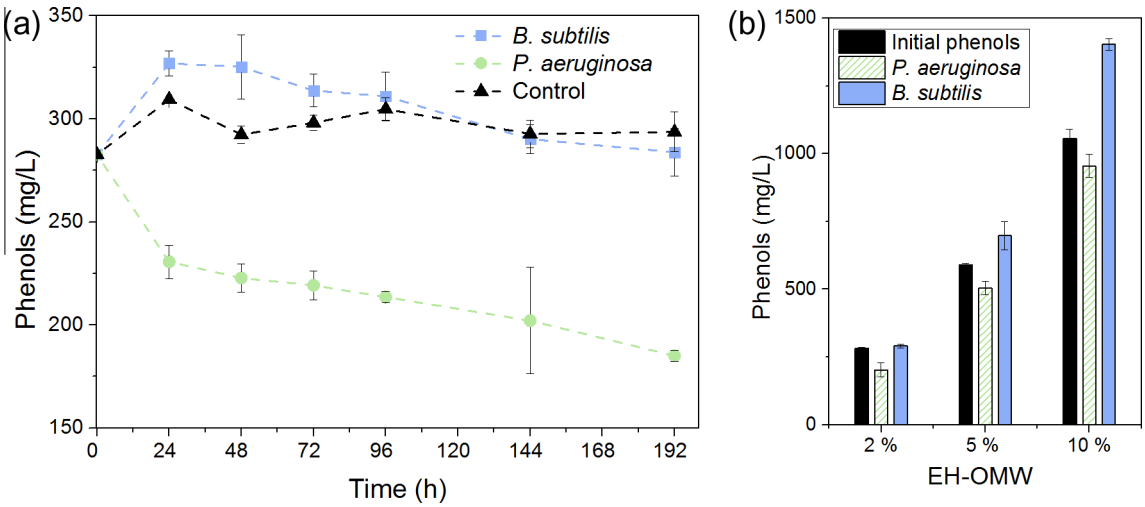

Fig. 4. Phenol concentration for (a) kinetic assays of *P. aeruginosa* and *B. subtilis* cultures with HE-OMW 2% w/v (control-cultures without inoculum), and (b) after 6 days of culture at different concentrations of EH-OMW. All the cultures where carried out at 37 °C and 160 rpm.

Fig. 4(a) shows the changes in phenolic compounds concentration during the kinetic assays. In *B. subtilis* cultures, phenol concentration remained almost constant and quite similar to that in the control (experiment without inoculum). On the other hand, the amount of phenols in *Pseudomonas* cultures gradually dropped down up to 71.5% of the initial concentration. This reduction is in agreement with previous works which reported that *Pseudomonas* could utilise phenolic substances for growth (Mercadé et al., 1993; Venieri et al., 2010).

Considering the phenol concentration for different amounts of OMW in the culture media, approximately the same amount of phenols disappeared in *Pseudomonas* cultures for each of the three assayed concentrations (Fig. 4b). Therefore, in spite of being able to use phenols, the ability of *Pseudomonas* to metabolize phenolic compounds is limited. In the case of *Bacillus* cultures the final phenol concentrations were higher than the initial ones and clearly increased with the OMW concentration. This could be due to the fact that *Bacillus* has the ability to hydrolyse lignocellulosic materials, liberating phenolic compounds into the medium (Chang et al., 2014; Sheikhi et al., 2012). However, this hypothesis needs further investigation.

Therefore the reduction in phenol concentration in *Pseudomonas* cultures and the increase in *Bacillus* is in agreement with the results obtained above for BS production and inhibition. These results confirm that inhibition in surfactin production is related to phenol concentration and that the resistance of *Pseudomonas* to phenols, allows it to take advantage to the higher amounts of nutrients (soluble sugars and oil) with increasing OMW concentration.

4. Conclusions

A hydrolysis pretreatment step of olive mill waste (OMW) is a suitable method to enhance biosurfactant production by fermentation with *B. subtilis* and *P. aeruginosa*. Enzymatic hydrolysis (EH) produced the greatest increase in biosurfactant production as compared to acid pretreatment and full hydrolysis with acid. Additionally, the use of glucose as sole carbon source did not show any meaningful differences with respect to EH-OMW. Furthermore biosurfactant production improved considerably when the OMW concentration was increased. Finally, EH-OMW showed lower inhibitory effects in comparison to non-hydrolysed OMW.

Acknowledgements

The authors acknowledge financial support provided by the Andalusian Regional Government (Junta de Andalucía) through the Project P10-TEP-6550. I.M.R. also acknowledges a research fellowship Granted by the Andalusian Regional Government.

References

Abu Tayeh, H., Najami, N., Dosoretz, C., Tafesh, A., Azaizeh, H., 2014. Potential of bioethanol production from olive mill solid wastes. Bioresour. Technol. 152, 24–30.

Albalasmeh, A.A., Berhe, A.A., Ghezzehei, T.A., 2013. A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. Carbohydr. Polym. 97, 253–261.

Banat, I.M., Satpute, S.K., Cameotra, S.S., Patil, R., Nyayanit, N.V., 2014. Cost effective technologies and renewable substrates for biosurfactants' production. Front. Microbiol. 5, 1–18.

Chang, Y.C., Choi, D., Takamizawa, K., Kikuchi, S., 2014. Isolation of *Bacillus* sp. strains capable of decomposing alkali lignin and their application in combination with lactic acid bacteria for enhancing cellulase performance. Bioresour. Technol. 152, 429–436.

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., Michaud, P., 2013. Olive mill wastes: biochemical characterizations and valorization strategies. Process Biochem. 48, 1532–1552.

Faria, N.T., Santos, M., Ferreira, C., Marques, S., Ferreira, F.C., Fonseca, C., 2014. Conversion of cellulosic materials into glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma* spp. under SHF and SSF processes. Microb. Cell Fact. 13, 1–13.

Geys, R., Soetaert, W., Van Bogaert, I., 2014. Biotechnological opportunities in biosurfactant production. Curr. Opin. Biotechnol. 30C, 66–72.

Gursoy-Haksevenler, B.H., Arslan-Alaton, I., 2014. UV fluorescence, FTIR, and GC–MS analyses and resin fractionation procedures as indicators of the chemical treatability of olive mill wastewater. Desalin. Water Treat., 1–11

Haagensen, F., Skiadas, I.V., Gavala, H.N., Ahring, B.K., 2009. Pre-treatment and ethanol fermentation potential of olive pulp at different dry matter concentrations. Biomass Bioenergy 33, 1643–1651.

Helmy, Q., Kardena, E., Funamizu, N., Wisjnuprapto, 2011. Strategies toward commercial scale of biosurfactant production as potential substitute for it's chemically counterparts. Int. J. Biotechnol. 12, 66–86.

Henkel, M., Müller, M.M., Kügler, J.H., Lovaglio, R.B., Contiero, J., Syldatk, C., Hausmann, R., 2012. Rhamnolipids as biosurfactants from renewable resources: concepts for next-generation rhamnolipid production. Process Biochem. 47, 1207–1219.

Konishi, M., Yoshida, Y., Horiuchi, J., 2015. Efficient production of sophorolipids by *Starmerella bombicola* using a corncob hydrolysate medium. J. Biosci. Bioeng. 119, 317–322.

Lima, T.M.S., Procópio, L.C., Brandão, F.D., Leão, B.A., TótolaBorges, M.R., Borges, A.C., 2011. Evaluation of bacterial surfactant toxicity towards petroleum degrading microorganisms. Bioresour. Technol. 102, 2957–2964.

Lotfabad, T.B., Shourian, M., Roostaazad, R., Najafabadi, A.R., Adelzadeh, M.R., Noghabi, K.A., 2009. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran. Colloids Surf. B Biointerfaces 69, 183–193.

Ma, X.J., Li, H., Wang, D.X., Song, X., 2014. Sophorolipid production from delignined corncob residue by Wickerhamiella domercqiae var. sophorolipid CGMCC 1576 and *Cryptococcus curvatus* ATCC 96219. Appl. Microbiol. Biotechnol. 98, 475–483.

Maass, D., Moya Ramírez, I., García Román, M., Jurado Alameda, E., Ulson de Souza, A.A., Borges Valle, J.A., Altmajer Vaz, D., 2015. Two-phase olive mill waste (alpeorujo) as carbon source for biosurfactant production. J. Chem. Technol. Biotechnol. Article in press.

Magina, M.A., Gilioli, A., Moresco, H.H., Colla, G., Pizzolatti, M.G., Brighente, I.M.C., 2010. Atividade antioxidante de três espécies de Eugenia (Myrtaceae). Lat. Am. J. Pharm. 29.

Makkar, R.S., Cameotra, S.S., Banat, I.M., 2011. Advances in utilization of renewable substrates for biosurfactant production. AMB Express 1, 5.

Marchant, R., Banat, I.M., 2012a. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol. Lett. 34, 1597–1605.

Marchant, R., Banat, I.M., 2012b. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol.

Marin, C.P., Kaschuk, J.J., Frollini, E., Nitschke, M., 2015. Potential use of the liquor from sisal pulp hydrolysis as substrate for surfactin production. Ind. Crops Prod. 66, 239–245.

McNamara, C.J., Anastasiou, C.C., O'Flaherty, V., Mitchell, R., 2008. Bioremediation of olive mill wastewater. Int. Biodeterior. Biodegradation 61, 127–134.

Mercadé, M.E., Manresa, M.A., Robert, M., Espuny, M.J., de Andrés, C., Guinea, J., 1993. Olive oil mill effluent (OOME). New substrate for biosurfactant production. Bioresour. Technol. 43, 1–6.

Moya Ramírez, I., Tsaousi, K., Rudden, M., Marchant, R., Jurado Alameda, E., García Román, M., Banat, I.M., 2015. Rhamnolipid and surfactin production from olive oil mill waste as sole carbon source. Bioresour. Technol. 198, 231–236.

Mulligan, C.N., 2009. Recent advances in the environmental applications of biosurfactants. Curr. Opin. Colloid Interface Sci. 14, 372–378.

Ramos-Cormenzana, A., Juárez-Jiménez, B., Garcia-Pareja, M.P., 1996. Antimicrobial activity of olive mill wastewaters (alpechin) and biotransformed olive oil mill wastewater. Int. Biodeterior. Biodegradation 38, 283–290.

Reis, R., Pacheco, G., Pereira, A., Freire, D., 2013. Biosurfactants: Production and Applications, In: Chamy, R. (Ed.), Biodegradation-Life of Science, InTech, pp. 31–61. http://dx.doi.org/10.5772/56144.

Sheikhi, F., Roayaei Ardakani, M., Enayatizamir, N., Rodriguez-Couto, S., 2012. The determination of assay for laccase of *Bacillus* subtilis WPI with two classes of chemical compounds as substrates. Indian J. Microbiol. 52, 701–707.

Sluiter, A., Hames, B., Ruiz, R.O., Scarlata, C., Sluiter, J., Templeton, D., Energy, D., 2011. Determination of structural carbohydrates and lignin in biomass. Lab. Anal. Proced.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int. J. Mol. Sci. 9, 1621–1651.

Tortosa, G., Alburquerque, J.A., Ait-Baddi, G., Cegarra, J., 2012. The production of commercial organic amendments and fertilisers by composting of two-phase olive mill waste ("alperujo"). J. Clean. Prod. 26, 48–55.

Venieri, D., Rouvalis, A., Iliopoulou-Georgudaki, J., 2010. Microbial and toxic evaluation of raw and treated olive oil mill wastewaters. J. Chem. Technol. Biotechnol. 85, 1380–1388.