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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.

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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.

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).

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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