Olive oil by-products as potential alternative substrates for xylooligosaccharides production

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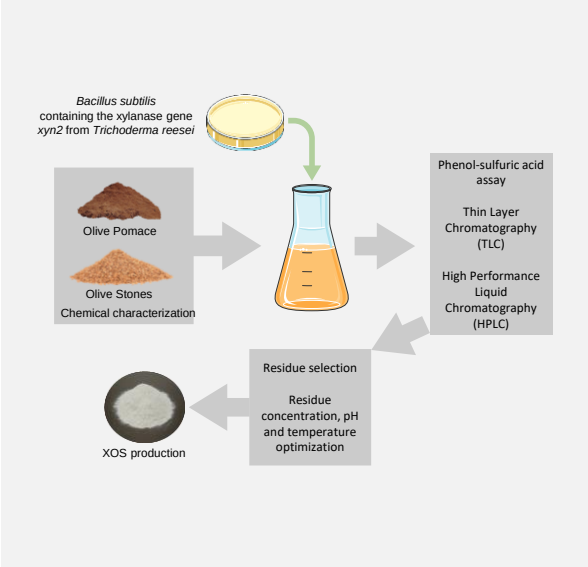

Olive pomace (OP) and olive stones (OS) are industrial by-products from the olive oil production. These residues have a high percentage of cellulose, hemicellulose, and lignin, which makes them a good source of fermentable sugars and xylan, as well as a potential alternative substrate for xylooligosaccharides (XOS) production. In this work, OP and OS were chemically characterized and used for the first time as a xylan source to produce XOS through direct fermentation by *Bacillus subtilis* 3610 containing the xylanase gene *xyn2* from *Trichoderma reesei*. OS presented the highest potential for XOS production. The fermentation process was further optimized for this residue in terms of residue concentration (5, 10, 20, 40 and 60 g L^{-1}), pH (5.0, 6.0, 7.0 and 8.0), and temperature (30, 37, 45 and 50°C). The highest total sugars yield (27 ± 2 mg g^{-1}) was achieved after 12 h, using 20 g L^{-1} of OS at pH 7.0 and 45°C.

Introduction

Mediterranean countries, including Portugal, are the main producers of olive oil. According to the International Olive Oil Council, these countries produce 93.3% of the total olive oil in the world (www.internationaloliveoil.org). The olive oil extraction is done by mechanical processes, which originate large amounts of different residues that the producers have difficulties to discard, *e.g.*, olive pomace (OP). Depending on the extraction methods, OP can have different percentages of moisture, being rich in polyphenols, tocopherols, proteins, and squalene [1]. The olive stones (OS) are another residue obtained from the olive oil industry, which can be separated from the olive pulp by centrifugation and used as fuel for heating dryers [2].

Both residues have a significant percentage of hemicellulose, particularly xylan, which makes them a potential alternative substrate to produce prebiotic xylooligosaccharides (XOS), by direct fermentation [3].

XOS have been reported to have prebiotic effects, and beneficial health effects such as anti-cancer, anti-inflammatory, and anti-diabetic properties. Additionally, these compounds can also improve the organoleptic properties of foods, being interesting its incorporation into functional foods [4].

XOS are short chain oligosaccharides composed of 2 to 10 xylose units and are conventionally produced through enzymatic hydrolysis or autohydrolysis methodologies, or a combination thereof. However, an emerging production approach based on direct fermentation has the potential to compete with the stablished processes by reducing the operational costs [5].

In this work, we evaluated the potential of OP and OS for XOS production by direct fermentation, using *Bacillus subtilis* 3610 containing the xylanase gene *xyn2* from *Trichoderma reesei* previously reported as a successful microbial XOS produced with other residues [6].

Materials and Methods

Chemical characterization of olive pomace and stones

The OP and OS were provided by Cooperativa Agrícola de Macedo de Cavaleiros (Portugal). The lignin, cellulose and hemicellulose were quantified according to the protocols of the National Renewable Energy Laboratory (NREL) (www.nrel.gov/docs/gen/fy13/42618.pdf).

Microorganism and culture conditions

Engineered *Bacillus subtilis* 3610, containing the xylanase gene *xyn2* from *Trichoderma reesei* [7], was grown on LB agar, overnight, at 37°C. The pre-inoculum was prepared by picking one colony into 4 mL of LB medium and cultivated at 40°C and 250 rpm during approximately 2 h until reaching an OD_{600nm}~1.0. This culture was then diluted to an OD_{600nm}~0.020 with the fermentation medium, being grown at 150 rpm [7]. The fermentation medium consisted in a mixture of grinded residue in 2% (v/v) of basal Vogel medium, autoclaved at 121°C for 15 min. Different residue concentrations (5, 10, 20, 40 and 60 g L^{-1}), pH values (5.0, 6.0, 7.0 and 8.0) and temperatures (30, 37, 45 and 50°C) were tested to optimize the process. Samples were collected over time and evaluated using different analytical methods [8].

Analytical methods

Phenol - sulphuric acid assay was used to analyse the total sugar content [9]. Thin Layer Chromatography (TLC) was performed using TLC plates (Macherey-Nagel, Duren, Germany) and butanol, acetic acid, and water (2:1:1 v/v/v) as mobile phase. The bands were detected by spraying with a staining solution containing 1% (w/v) diphenylamine and 1% (v/v) aniline in acetone, followed by heating at 120°C during 10 min [8]. For monosaccharides quantification, a High Performance Liquid Chromatography (HPLC) (Agilent Technologies, USA) fitted an Aminex HPX 87H column (300 mm × 7.8; Biorad, USA) was used. A 5mM H_2SO_4 solution was used as the mobile phase at a

flow rate of 0.7 mL min^{-1} and 60°C. For the oligosaccharides' quantification, a HPLC (JASCO, Japan) fitted with a Shodex HILICpak VG-50 4E column (4.6 mm × 250 mm; Shodex, Tokyo, Japan), was used. A 75:20:5 (v/v) acetonitrile/methanol/water mixture was used at a flow rate of 0.9 mL min^{-1}, at 40°C.

Results

The chemical composition of OP and OS, particularly their content in xylan, provides an important indication of their potential as alternative substrate for XOS production by direct fermentation.

OP presented 31.4 ± 0.7% of lignin (7.0 ± 0.5% of soluble lignin and 27.6 ± 0.6% of Klason lignin), 18.5 ± 0.5% of cellulose and 25.5 ± 0.5% of hemicellulose, of which 12.9 ± 0.4% correspond to the xilan, 0.68 ± 0.03% of arabinan and 8.3 ± 0.3% of acetic groups. While OS was composed by 27.6 ± 0.9% of lignin (3.8 ± 0.2% of soluble lignin and 23.1 ± 0.9% of Klason lignin), 21.1 ± 0.8% of cellulose and 26.8 ± 0.9% of hemicellulose, of which 14.8 ± 0.4% correspond to the xylan and 10.1 ± 0.6% of acetic groups. The values were expressed in % of dry weight and are in accordance with the literature [1],[2]. When compared with brewers' spent grain composition, previously reported as XOS fermentative substrate [6], OP and OS have comparable xylan contents, thus presenting potential for XOS production.

OP and OS potential for XOS production was then compared between each other by performing a fermentation with 20 g L^{-1} of residue, at pH 7.0, 45°C and 150 rpm. OS was the residue with the highest potential, presenting an increase of the total sugars yield from 0 h (15 ± 1 mg g^{-1}) until 12 h (27 ± 2 mg g^{-1}) and low amounts of undesired free sugars (0.02 ± 0.01 g L^{-1}), thus being selected for further optimization studies. The TLC and HPLC results suggested that XOS with a degree of polymerization superior to 6 were produced with OS (Figure 1). After optimization, the highest total sugars yield (27 ± 2 mg g^{-1}) was achieved at 12 h with low amounts of free xylose (0.02 ± 0.01 g L^{-1}), using 20 g L^{-1} of OS at pH 7.0 and 45°C.

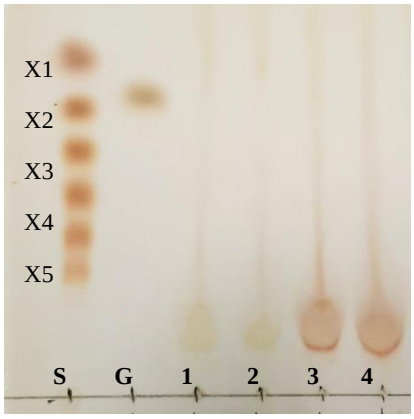

Figure 1. TLC of the supernatants obtained from the direct fermentation of OS under optimal conditions at 0 h (1 and 2) and 12 h (3 and 4) (optimal time). A mixture (S) containing 2 g L^{-1} of xylose (X1) and XOS with a degree of polymerization between 2 and 6 (X2-X6) and a solution of 1 g L^{-1} of glucose (G) were used as standard.

Conclusions

OS presented the highest potential for XOS production by direct fermentation. The optimization of the fermentation process allows to increase 1.8-fold the yield in total sugars, achieving its maximum value at 12 h (27 ± 2 mg g^{-1}). Direct fermentation of OS revealed to be a promising and competitive strategy to produce XOS likely to significantly reduce their production cost.

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