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Valorisation of olive oil by-products into pectic- and glucuronoxylo-oligosaccharides via one-step fermentation

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ABSTRACT

Olive pomace (OLP) and stones (OLS) are key by-products of olive oil production, rich in lignocellulose and pectin, making them viable substrates for prebiotic oligosaccharide (OS) production. This study evaluated the chemical composition of OLP and OLS powders (OLPp and OLSp) and their potential for OS production through one-step fermentation using recombinant *Bacillus subtilis* 3610. Both substrates had comparable xylan and pectin levels, but OLSp showed greater potential, achieving a maximum total sugar yield of 60 ± 3 mg.g^{-1} after 12 h under optimal conditions (20 g.L^{-1} OLSp, pH 7.0, 45 °C). The resulting OS mixture from OLSp was predominantly composed of pectic oligosaccharides (72.1 %mol) and glucurono-xylooligosaccharides (11.6 %mol). This innovative process, competitive with commercial enzymes, highlights the potential of by-product valorisation for producing value-added food compounds. The findings provide insights into low-cost bioprocesses and underscore the importance of sustainable approaches in the industry of functional food ingredients.

1. Introduction

The increasing health consciousness in recent years prompts consumer preferences for specific food ingredients that can provide health benefits (Narisetty et al., 2022), including prebiotic compounds (Alongi & Anese, 2021; Gur et al., 2018) Consequently, the global market for prebiotics is expected to grow at a CAGR of 13 % from 2023 to 2028, reaching $14.5 billion by 2028 (Reportlinker, 2023).

A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Prebiotic oligosaccharides (OS), such as xylooligosaccharides (XOS) and pectic oligosaccharides (POS), are a type of carbohydrate composed of 2 to 10 monosaccharides (You et al., 2022). Conventional methodologies for XOS and POS production include autohydrolysis, chemical and enzymatic hydrolysis (Gütsch et al., 2012; Xiao et al., 2013). However, chemical and autohydrolysis methods are associated with high production of several by-products, including toxic compounds (Zhang et al., 2016), and the enzymatic methods require the prior production/purification or purchase of adequate enzymes.

XOS are comprised by xylose units linked through β(1–4) linkages, and they can be produced through the hydrolysis of xylan, the main component of hemicellulose present in lignocellulosic biomass (Álvarez et al., 2017). Due to their average selling price per dose (USD 25 to 50 $.kg^{-1}) and minimum daily dose required (1.4–2.8 g), XOS are more economically compared to other well-established prebiotics (Finegold et al., 2014; Valladares-Diestra et al., 2023).

POS are considered emerging prebiotics, generally products of partial depolymerization of pectin present in several agricultural by-products. POS can be defined as an OS with a degree of polymerization (DP) of 2–10 and consisting of the units of →4)-α-GalpA-(1 → 4)-α-GalpA-(1 → or →4)-α-GalpA-(1 → 2)-α-Rhap-(1 → (Tang et al., 2024), however their structure and composition depend on the pectin source and the production process (Combo et al., 2013).

Both XOS and POS have been reported to have several health benefits as they improve digestion and gut function, potentially providing protection against colon cancer and an anti-inflammatory effect, reducing

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symptoms of irritable bowel syndrome and body weight gain, or regulating the immune system (Mazzucotelli & Goñi, 2022; Gonçalves et al., 2023; Jia et al., 2018). Besides their health benefits, XOS can also improve the organoleptic properties of food products, presenting high stability at acidic pH and high temperatures, while POS can be used as stabilizers in food formulations, enhancing the texture and consistency, which makes them attractive compounds for the food industry (Pattarapisitporn et al., 2024).

Considering the OS market potential, the development of alternative cost-effective production approaches is of utmost importance. The production of XOS by one-step fermentation of lignocellulosic materials has been studied using different by-products and microorganisms, as soybean fibre by *Aspergillus nidulans* or rice hush by *Aspergillus brasiliensis* BLf1 (Menezes et al., 2017; Pereira et al., 2018) These studies demonstrate that this process holds the potential to reduce the number of steps involved in the process, overcoming the costs associated with the enzymes production or purchase (de Capetti et al., 2021; Kallel et al., 2015). The primary methods employed in the production of POS are enzymatic and autohydrolysis using lignocellulose as orange peel waste, onion skins, or sugar beet pulp (Babbar, Baldassarre, et al., 2016; Babbar et al., 2017; Gonçalves et al., 2023; Sabajanes et al., 2012). Additionally, the pectic polysaccharides of olive pomace (OLP) are considered unique substrates for OS production due to the presence of arabinan (Babbar, Dejonghe, et al., 2016). Lama-Muñoz et al. (2012a, 2012b) reported the hydrothermal treatment of OLP to produce a mixture of POS, neutral and acidic XOS.

OLP is the main by-product originated by the olive oil industry, being difficult to discard due to their content in phenolic compounds which are toxic to the soil and waters (Cruz et al., 2017; Roig et al., 2006). Other significant generated by-products are the crushed olive stones (OLS) that can be separated from the OLP pulp by centrifugation or by a screening system (Khwaldia et al., 2022). OLP and OLS have been traditionally utilized in low-value applications such as animal feed, compost, or bioenergy generation (Miranda et al., 2019; Valvez et al., 2021). However, recent research has increasingly focused on their valorization as fermentation substrates due to their rich content in lignocellulosic components, including cellulose, xylan, and pectin. Several studies have explored enzymatic hydrolysis or microbial fermentation of OLP and OLS to produce biofuels, organic acids, and enzymes (Contreras et al., 2020; Gómez-Cruz et al., 2024). Despite this progress, the direct use of untreated or minimally processed OLP and OLS as substrates to produce high-value prebiotic oligosaccharides remains underexplored.

The main aim of this work was to develop an alternative bioprocess based on one-step fermentation to produce a mixture of OS from OLP and OLS powders (OLPp and OLSp, respectively) in a sustainable and competitive way, using a genetically modified *Bacillus subtilis* 3610 harbouring the xylanase gene *xyn2* from *Trichoderma reesei.* This microorganism was previously reported to be a successful producer of arabino-xylooligosaccharides (AXOS) by one-step fermentation of brewer's spent grains (BSG) (Amorim et al., 2018). However, the broader application of this one-step fermentation strategy had not yet been explored for substrates with different chemical compositions and structural complexities, such as OLP and OLS. These by-products present unique bioconversion challenges but also offer the potential to yield different high-value prebiotic compounds. To the best of our knowledge, this is the first study to demonstrate the direct microbial production of a novel OS mixture, rich in both pectic- and glucoronoxylo-oligosaccharides (POS and GXOS), from olive oil by-products through a simplified and competitive process. The complexity of the produced mixture points to its potential for functional food applications, as diverse OS profiles are linked to an enhanced prebiotic effect (Dong et al., 2023).

2. Methods

2.1. Materials

Unless otherwise stated, all chemicals, media and media components were of analytical grade obtained from Sigma-Aldrich Chemical Ltd. (Germany). The standard XOS (xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6)) were purchased from Megazyme (Ireland). The OLP and the OLS were provided by the olive oil mill of the Cooperativa Agrícola de Macedo de Cavaleiros (Portugal) (Fig. S1). For this study, the OLP was lyophilized (SCANVAC Coolsafe 110–4, Bjarkesvej, Denmark) for 4 to 5 days. The lyophilized OLP and the OLS were then individually grounded into powder (OLPp and OLSp, respectively) using a mechanical milling (IKA, Model: M20, 230 V 50/60 Hz 620 W IP 21, Germany). The relative humidity content of the OLPp and OLSp was determined with a moisture analyser MAC 50/1/NH apparatus, being <5 % (*w*/w). The powders were further stored at room temperature until being used directly as fermentative substrate.

2.2. Chemical characterisation of olive pomace and stones powders

The OLPp and OLSp were chemically characterised according to AOAC standards. Moisture (AOAC 934.6), ash (AOAC 942.05), and total lipidic (AOAC 920.39) contents were determined. The total protein content was determined using a Micro Kjeldahl block digestion system behr K 24 (ExpotechUSA). The composition in lignin, cellulose and hemicellulose were determined through acid hydrolysis of the extractive-free OLPp and OLSp, using 72 % (w/w) of sulfuric acid, according to the National Renewable Energy Laboratory protocols (Sluiter et al., 2008). The previous extraction was done in Soxhlet system with petroleum ether (8 h), ethanol (16 h), and water (16 h), to remove lipids, phenolic compounds and free sugars that can interfere with the hydrolysis (Ribeiro et al., 2020). The sugars and their degradation products present in the hydrolysates were analysed by High Performance Liquid Chromatography (HPLC) (section 2.6.2.) and used to calculate the cellulose and xylan fraction. The acid soluble lignin was obtained by the analysis of the hydrolysate by UV-VIS/UV-1280 spectrophotometry (Shimadzu, Kyoto, Japan) at 206 nm. Klason lignin was determined by gravimetry (Lu et al., 2021). The pure pectin content was quantified by stirring the by-products in a mixture of 5 mL of ethanol (96 %), 1 g of sodium chloride, and 100 mL of deionised water until the dissolution of the pectin. Following dissolution, a titration was performed using 0.1 M NaOH with phenol red as the indicator. (Grassino et al., 2018).

2.3. Microorganism and culture conditions

The recombinant *B. subtilis* 3610 used in this work was previously engineered, as described by Amorim et al. (2018) to contain *xyn2* gene from *T. reesei* and a secretion tag endogenous to *B. subtilis* (> tr| A0A0S2II12| Uniprot) coupled to xylanase N-terminus site. To prepare the pre-inoculum, the microorganism was grown on LB agar overnight at 37 °C. One colony was picked into 4 mL of LB medium (Difco, New Jersey, USA). The cells were then cultivated at 40 °C and 250 rpm during approximately 2 h until reaching an $OD_{600nm} \sim 1.0$. This starter culture was then diluted to $OD_{600nm} \sim 0.020$ with the fermentation media (Amorim et al., 2018).

2.4. Screening of olive pomace and stones powders for oligosaccharides production by one-step fermentation

To investigate the potential of OLPp and OLSp as alternative substrates for OS production by one-step fermentation, 1 g of by-product powder (section 2.1), were added to 50 mL of the minimal medium Vogel's 50× salts at 2 %(*v*/v) and the pH was adjusted to 7.0. Erlenmeyer flasks (250 mL) with the OLPp or OLSp–liquid mixture were

sterilised at 121 °C during 15 min (Amorim et al., 2018). The fermentation was performed at 45 °C, 150 rpm, during 30 h after inoculum addition as described in section 2.3. Samples of the fermentation broth were collected and centrifuged (7000 rpm during 7 min), for further analysis as described in section 2.6.

2.5. One-step fermentation of olive stones powder: Sterilisation effect and one factor-at-a-time optimisation

The impact of the sterilisation method on the one-step fermentation of OLSp was evaluated comparing autoclave and UV sterilisation. The autoclave sterilisation was performed at 121 °C for 15 min as described in section 2.4. For the UV sterilisation, the OLSp was irradiated with a UV lamp at 254 nm, placed at 30 cm, during 1 h, and added to the minimal medium, Vogel (2 %(v/v), pH 7.0), previously filtered using a 0.2 μm cellulose membrane, at sterile conditions. The comparative assay was then carried at 45 °C and 150 rpm for 30 h and the inoculum was prepared as described in section 2.3. An assay without the inoculum was also carried out as a control.

The fermentation process was optimised for OLSp using the one-factor-at-a-time method. The individual effects of OLSp concentration (5, 10, 20, 40 and 60 $g.L^{-1}$), initial pH (5.0, 6.0, 7.0 and 8.0) and temperature (30, 37, 45 and 50 °C) were evaluated sequentially and for a 30 h fermentation time-period. The starter culture was prepared as described in section 2.3. Samples of the supernatant were collected and centrifuged (7000 rpm during 7 min) for sugar analysis as described in section 2.6.

2.6. Assessment of oligosaccharides production by one-step fermentation

2.6.1. Quantification of total and reducing sugars

The total sugars (TS) quantification was performed by the phenol-sulfuric acid method, using xylose or galacturonic acid as standard for the calibration curves (DuBois et al., 1956). The DNS (3,5-dinitrosalicylic acid) method was used as a qualitative screening technique to assess total reducing sugars (RS) using xylose as standard. The total or reducing sugar production yields, Y_{TS} or Y_{RS} ($mg.g^{-1}$), were calculated as the ratio between TS or RS (mg) and the mass of substrate (g) used in the assay. The optimal time corresponds to the time at which the highest value of Y_{TS} was achieved, Y_{TS_max} ($mg.g^{-1}$), while the maximum fermentation productivity, $P_{\Delta Y_max}$ ($mg.g^{-1}.h^{-1}$), was calculated by dividing the difference between the Y_{TS} at 0 h and Y_{TS} by the optimal time. The free monosaccharides yield, Y_{FM} (Y_{Glc} for glucose and Y_{xyl} for xylose) ($mg.g^{-1}$) were calculated as the ratio between the concentration of monosaccharides obtained by HPLC (section 2.6.2.) and the mass of substrate used.

2.6.2. Monosaccharides analysis by HPLC

A HPLC was used to quantify monosaccharides and their degradation products. To analyse the monosaccharides, a volume of 10 μL of sample were eluted using 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} and a temperature of 60 °C in an HPLC (Smartline KANUER, Spark Holland) with a RI detector (KNAUER K2300, Spark Holland) and an Aminex HPX 87H column (300 mm × 7.8 mm × 9 μm, Biorad, USA). To quantify the degradation products of xylan and cellulose, the UV detector (KANUER K-2501, Spark Holland) was used to determine the concentrations of furfural (280 nm) and hydroxymethylfurfural (HMF) (284 nm), respectively.

2.7. Chemical characterisation of the oligosaccharides produced from olive stones powder

To reduce the salt interference, a partial purification of the OS produced by one-step fermentation of OLSp under optimal conditions, was performed with activated charcoal as described by (Amorim et al., 2019b).

2.7.1. Sugar analysis

Neutral sugars were released from the sample using a treatment with 72 %(*w*/w) H_2SO_4 during 3 h at room temperature with occasional stirring followed by hydrolysis with 1 M H_2SO_4 for 2.5 h at 100 °C. The neutral sugars were determined as their alditol acetates by gas chromatography with flame ionization detector (GC-FID, Perkin Elmer Clarus 400, USA), following the chromatographic conditions described by Lopes et al. (2016). Uronic acids (UA) content was quantified using the colorimetric *m*-phenylphenol method with a calibration curve of D-galacturonic acid (10–100 $\mu g.mL^{-1}$).

2.7.2. Glycosidic-linked analysis

Glycosidic-linked analysis was determined by gas chromatography-quadrupole mass spectrometry (GC-qMS, Shimadzu GC–MS QP2010) of the partially methylated alditol acetates based on Ferreira et al. (2021). The dried sample was dissolved in anhydrous dimethylsulfoxide and powdered NaOH was dissolved in anhydrous dimethylsulfoxide and powdered NaOH was added under argon atmosphere. The sample was methylated with CH_3I for 30 min with stirring (this step was repeated twice). A volume of 3 mL of 50 %(*v*/v) ethanol was added, and the solution was dialyzed using 0.5 kDa cutoff membranes against 50 %(v/v) ethanol. The same sample was also methylated in the previous described conditions and 3 mL of $CHCl_3$/methanol (1:1 v/v) was added. The solution was dialyzed against 50 %(v/v) ethanol using a 1 kDa cut off membrane. After dialysis, the retentates were evaporated to dryness. The remethylated material was hydrolysed with 2 M trifluoroacetic acid at 120 °C for 1 h, followed by a reduction with $NaBD_4$, and acetylation with acetic anhydride in the presence of 1-methylimidazole as a catalyst.

Glycosidic-linked composition of the OLSp fermented sample was also analysed without using the dialysis step. Instead of the dialysis step, 2 mL of water was added, the solution was neutralised with 1 M HCl, and sequential liquid-liquid extractions were performed by the addition of CH_2Cl_2 and water. The aqueous phase was discarded and the CH_2Cl_2 was evaporated until dryness. The remethylated samples were hydrolysed, reduced and acetylated, as described above.

The partially methylated alditol acetates from the different methylation procedures were separated and analysed by GC-qMS (Shimadzu GC–MS QP2010). The GC was equipped with a ZB-5HT column (J&W Scientific, Folsom, CA, USA), with 30 m length, 0.25 mm of internal diameter, and 0.25 μm of film thickness. The samples were injected using the chromatographic conditions described by Martin-Pastor et al. (2019).

2.7.3. Oligosaccharides's profile determined by GC-qMS

To determine the neutral OS profile, the fermented OLSp samples were analysed after reduction and acetylation using 2-deoxyglucose (1.0 $mg.mL^{-1}$), as internal standard. The alditol acetates were dissolved in anhydrous acetone and analysed by GC-qMS (Shimadzu GC–MS QP2010) equipped with a ZB-5HT (J&W Scientific, Folsom, CA, USA), column (30 m length, 0.25 mm of internal diameter, and 0.25 μm of film thickness), according to the chromatographic conditions described by Imperio et al. (2021), with minor modifications. The samples were injected in "split" mode with the injector temperature at 400 °C. The temperature program used was as follows: an initial temperature of 140 °C; an increase of 5 $°C.min^{-1}$ until 180 °C, and hold for 1 min; an increase of 5 $°C.min^{-1}$ until 250 and a hold time of 10 min; an increase of 5 $°C.min^{-1}$ until 390 °C and a hold time of 9 min. The carrier gas has helium with a flow of 8.1 $mL.min^{-1}$.

The peaks identification was achieved by comparing with standard mass spectra injection. To quantify the OS, relative response factors of xylose, glucose, arabinobiose, sucrose, cellobiose, maltotriose, kestotetraose, and maltotetraose to 2-deoxyglucose were determined based on 6-point calibration curves. The pentoses and hexoses monosaccharides were, respectively, quantified using the response factors of xylose (limit of detection (LoD) 0.06 $m_{sugar}/m_{standard}$; limit of quantification (LoQ) 0.19 $m_{sugar}/m_{standard}$) and glucose (LoD 0.07; LoQ 0.21). The amount of

di- were quantified using the response factors of arabinobiose (LoD 0.12; LoQ 0.37), sucrose (LoD 0.13; LoQ 0.38), and cellobiose (LoD 0.13; LoQ 0.40). The amount of tri- were quantified using the response factors of maltotriose (LoD 0.26; LoQ 0.80). The amount of tetra- were quantified using the response factors of kestotetraose (LoD 0.20; LoQ 0.62) and maltotetraose (LoD 0.32; LoQ 0.97). The LoD and LoQ were defined as the lowest mass of sugar/ mass standard with estimated peak height greater than 3.3 and 10 times of the noise levels (S/N > 3.3 and 10), respectively. Total carbohydrate content was calculated by the sum of the results from the sugars measured individually.

2.7.4. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

In order to determine the OS profile, including charged OS, the sample was diluted in MilliQ water 1 %(*w/v*) filtered through a nylon filter measuring 0.22 μm (WhatmanTM, Buckinghamshire, UK), and analysed via high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using a Dionex ICS-600 system equipped with a DC oven and SP, controlled by Chromeleon 7.3 software (Thermoscientific Dionex – Waltham, MA, USA. Carbohydrates were detected using an electrochemical detector in integrated amperometry mode with an AgCl reference electrode and a conventional electrode. Sugars separation was achieved using a Dionex CarboPac PA100 pre-column (50 mm × 4 mm) and a Dionex CarboPac PA100 analytical column (250 mm × 4 mm). As a reference, polysaccharide of galacturonic acid was hydrolysed with H_2SO_4 for 2.5 h at 100 °C and analysed as referred above.

3. Results and discussion

3.1. Chemical characterisation of olive pomace and stones powders

OLP typically contains olive oil, pulp, skin, crushed OLS and water. OLS can be further separated from OLP during the extraction of the remaining olive oil by centrifugation and sieving (Martin et al., 2020). The chemical composition of these by-products varies depending on factors like the olive tree variety, agro-climatic cultivation conditions, fruit ripeness (Contreras et al., 2020), or the extraction process used in olive oil production (Abbattista et al., 2021). OLP is mainly composed by cellulose, hemicellulose and lignin (Miranda et al., 2019; Ribeiro et al., 2020), thus holding potential as alternative substrates for OS production (Negro et al., 2017). The chemical composition (expressed in dry weight) of the OLP and OLS herein used was determined by acid hydrolysis, and the results are presented in Table 1.

The chemical characterisation of the by-products (Table 1) showed variations in their compositions, consistent with findings reported in the literature (Khwaldia et al., 2022). The content in cellulose (18.5 ± 0.5 and 21.1 ± 0.8 %(*w*/w)) and xylan (23.2 ± 0.4 and 26.8 ± 0.9 %(w/w)) was similar for OLPp and OLSp. Regarding lignin content, a higher percentage was anticipated in OLSp. However, the measured lignin content in OLPp is 1.7 times greater. This discrepancy likely arises from the presence of fragments of OLS and olive pulp in OLPp, both of which are rich in phenolic compounds. These compounds can undergo oxidation and polymerization, potentially being quantified as lignin during analysis (Cardoso et al., 2005).

Table 1
Chemical characterisation of olive pomace powder (OLPp) and olive stones powder (OLSp) in dry weight (% (*w*/w)).

% (w/w)	OLPp	OLSp
Lipids	11.1 ± 0.3	0.8 ± 0.1
Ashes	4.3 ± 0.6	0.46 ± 0.02
Pectin	1.38 ± 0.03	1.7 ± 0.1
Protein	7.4 ± 0.2	1.5 ± 0.2
Xylan	23.2 ± 0.4	26.8 ± 0.9
Cellulose	18.5 ± 0.5	21.1 ± 0.8
Lignin	46.2 ± 0.8	27.6 ± 0.9
Acid soluble lignin	8.6 ± 0.4	3.8 ± 0.2
Insoluble lignin	36.5 ± 0.5	23 ± 1

The proportion of olive pectin in these by-products is generally regarded as relatively insignificant, with its level tending to diminish as olive maturity increases (Millan-Linares et al., 2021). In the present study, a pectin content below 2 % was obtained (1.3 % (OLPp) and 1.7 % (OLSp)). Millan-Linares et al. (2021) studied the pectin's derived from OLP at different ripening stages and from various olive varieties as an "alcohol-insoluble residue" (AIR), which contents varied from 3.5 % to 15.2 %. However, it is important to note that AIR may also result in the extraction of cellulose, hemicellulose, or protein (Millan-Linares et al., 2021).

Regarding the lignocellulosic components, the results obtained are within the ranges reported in the literature. Namely, OLPp can be composed of 30.0–41.6 % (*w*/w) of lignin, 13.8–30.0 % (w/w) of cellulose, and 18.5 %-32.2 % (w/w) of hemicellulose (Freitas et al., 2022), while OLSP may present 20.6–25.1 % (w/w) of lignin, 29.8–34.4 % (w/w) of cellulose and 21.5–27.6 % (w/w) of hemicellulose (Rodríguez et al., 2008).

The xylan content is an important indicator of the by-product potential for XOS production by one-step bioprocess. Several by-products from different industries have been reported for XOS production as soybean fibre with 22.1 % (w/w) of xylan or wheat bran (30 % (w/w) of xylan) (Liu et al., 2022; Menezes et al., 2017). Interestingly, the composition in xylan of both OLPp and OLSp is approximately 1.6-fold higher than the one reported for BSG, 16.5 % (w/w), which was successfully used as fermentative substrate to produce AXOS (Amorim et al., 2019b). Thus, based on the chemical characterisation results, these by-products present potential to be used as promising substrates for OS production.

3.2. Screening of olive pomace and stones powders as fermentation substrates

The production of AXOS through one-step fermentation was firstly reported by Amorim et al. (2019b) who constructed a recombinant *B. subtilis* to ferment BSG. This approach showed promise as a competitive alternative to conventional XOS production methods, however, its applicability was suggested to be dependent on the substrate nature. Hence, to test the substrate-dependency of this bioprocess approach and to explore the potential of expanding it to other alternative substrates, OLPp and OLSp were screened for xylo based OS production, as fermentative substrates, using the same recombinant *B. subtilis*. The Y_{TS} and Y_{RS} were used as qualitative indicators of the total and reducing sugars, respectively (Fig. 1).

Although the two by-products exhibited a similar Y_{RS} at 0 h (9 ± 1 $mg.g^{-1}_{OLSp}$ and 9.1 ± 0.4 $mg.g^{-1}_{OLPp}$), OLPp presented a downward trend in the Y_{RS} profile, while OLSp showed an initial increase at 4 h (13.8 ± 0.3 $mg.g^{-1}_{OLSp}$) followed by a subsequent decrease and stabilisation of the reducing sugars, as shown in Fig. 1.

Comparing the Y_{TS} results, two significantly distinct profiles were obtained probably due to the different impact of the sterilisation method on the initial sugar extraction (0 h) from OLPp or OLSp into the fermentation medium (Fig. 1). OLPp presented a 10-fold higher Y_{TS} at 0 h (121 ± 5 $mg.g^{-1}_{OLPp}$), compared to OLSp (11.8 ± 0.1 $mg.g^{-1}_{OLSp}$). The free monosaccharides analysis obtained by HPLC (Table S1) corroborated the results from Fig. 1, revealing that at 0 h OLPp showed an Y_{FM} of 108.3 ± 0.9 $mg.g^{-1}$ (81.5 % of glucose and 18.5 % xylose), that decreased to 17.839 ± 0.004 $g.L^{-1}$ after 12 h of fermentation, when the initial glucose was totally consumed together with 10 % of the initial xylose.

On the other hand, no monosaccharides were quantified for OLSp either at 0 or 12 h. As a highly lignified seed, no free sugars were expected to remain after sterilisation. However, minimal monosaccharides may have formed after 12 h of fermentation due to microbial activity.

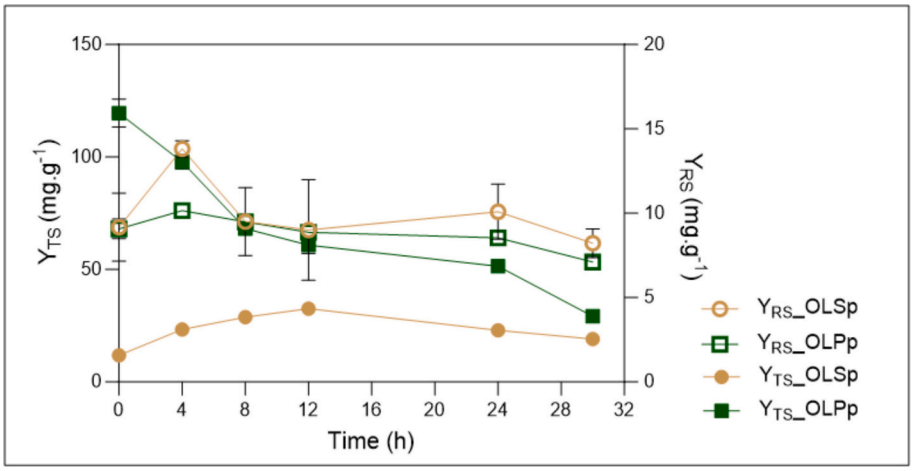

Fig. 1. Total and reducing sugar production yield for the olive pomace powder (OLPp), (Y_{TS_OLPp}(□), Y_{RS_OLPp}(□)) and olive stones powder (OLSp), (Y_{TS_OLSp} (●), Y_{RS_OLSp} (○)) obtained from the one-step fermentation of 20 g.L^{-1} of OLSp or OLPp by recombinant *Bacillus subtilis* containing the xylanase gene *xyn2* from *Trichoderma reesei* in 2 % (*v*/v) Vogel medium at pH 7.0, 45 °C, and 150 rpm. Results represent the average of two independent assays ± standard deviation. Xylose-based calibration curves were used.

These sugars might not have been detected, potentially due to the sensitivity limits of the analytical method used (Table S2). The low amount of available free monosaccharides in the medium of OLSp fermentations may have driven the microorganism to use its enzymatic machinery to hydrolyse the by-product to obtain a viable carbon source, and consequently, an increase on Y_{TS} was observed until 12 h when its maximum was achieved (32.4 ± 0.6 mg.g^{-1}, Fig. 1). These results were also in accordance with the TLC analysis (Fig. S2), where it was possible to observe the consumption of the initial sugars after 12 h of OLPp fermentation, while OLSp showed the production of complex sugars at 12 h with a probable DP higher than 6. These results suggest that fermentation inhibition by phenolic compounds in OLPp may have a greater impact than the lignification of OLSp, as reported by Cuevas et al. (2009). Therefore, OLSp presented the highest potential for OS production by one-step fermentation, being selected for further studies. The approach adopted in this study is suitable for proof-of-concept research, intended to demonstrate the technical feasibility of the bioprocess for producing a novel oligosaccharide mixture under controlled laboratory conditions.

3.3. Oligosaccharides production by one-step fermentation of olive stones powder

The OS production has previously been reported using by-products from the olive oil industry. Lama-Muñoz et al. (2012a, 2012b) developed a hydrothermal process to obtain mainly a mixture of tetra-, tri- and di-galacturonic acids from OLP, along with a range of neutral and acidic XOS. Aligned with this study, it is suggested that in the present work a mixture of OS was produced from OLSp, probably composed mainly of POS and glucurono-xylooligosaccharides (GXOs) (Reis et al., 2003).

Although the xylan content, 16.8 ± 0.6 %(*w*/w), was considerably higher than the pectin content, 1.7 ± 0.1 % (w/w), the partial hydrolysis of pectin-rich regions during thermal sterilisation (121 °C, 15 min), coupled with the higher enzymatic accessibility of solubilized pectic polysaccharides, may likely contribute to the predominance of pectic OS in the final product. *B. subtilis* is known to produce a broad spectrum of carbohydrate-active enzymes, including xylanases, cellulases, and particularly a robust set of pectinolytic enzymes, such as polygalacturonases and pectin lyases, which are more active when pectin fragments are already solubilized in the medium (Alqahtani et al., 2022; Su et al., 2020) This enzymatic versatility, combined with the discrepancy previously observed between the Y_{RS} and Y_{TS} values (Fig. 1, section 3.2), suggests the *co*-production of sugars with varying degrees of polymerization, including reducing sugars like GXOs and non-reducing sugars such as POS (Grassino et al., 2018).

The by-products screening was done using a calibration curve based on xylose, from which it was concluded that OLS holds the greatest potential for OS production. However, since this by-product appears to have greater potential for POS production, a calibration curve using galacturonic acid equivalents was used for further studies, given that this is the primary component of the backbone of POS (Gómez-Cruz et al., 2024).

3.3.1. Effect of sterilisation

Although medium sterilisation is an integral part of any fermentation process, it shares certain similarities with the thermal pretreatment of lignocellulosic by-products. Nevertheless, the conditions required are significantly milder than those necessary for the afore mentioned pretreatments (Cuevas et al., 2015; Freitas et al., 2022; Miranda et al., 2019). In this sense, to investigate the role of the sterilisation on the production of OS from OLSp via one-step fermentation, two different conditions were studied: OLSp sterilised by autoclave and by UV. Plus, to understand the importance of the microbial metabolism on the OS production and the effect of the process conditions, a control was performed with autoclave-sterilised medium but without inoculum. Fig. 2A shows the Y_{TS} profiles obtained for the tested conditions.

As expected, the Y_{TS} obtained at 0 h for the autoclave sterilisation (28.1 ± 0.3 mg.g^{-1}) was higher compared with the UV sterilisation (16.7 ± 0.2 mg.g^{-1}). The higher content of sugars released due to the autoclave sterilisation may have caused a favourable initial biomass boost, that allowed to achieve the highest Y_{TSmax} (73 ± 1 mg.g^{-1} at 12 h) sooner than with UV sterilisation (70 ± 2 mg.g^{-1} at 30 h) (Fig. 2). Therefore, these results suggest the importance of autoclave sterilisation on the improvement of the microbial metabolism for OS production, increasing Y_{TS} and reducing the optimal production time. Amorim et al. (2018) reported a similar effect of autoclave/UV sterilisation effect on AXOS production from BSG, with the autoclave sterilisation resulting in the lowest optimal production time (12*h*), compared with UV (28 h).

Furthermore, the control without microorganism demonstrates that the microbial role is key for OS production, and the operational conditions do not contribute for sugar release (Fig. 2A).

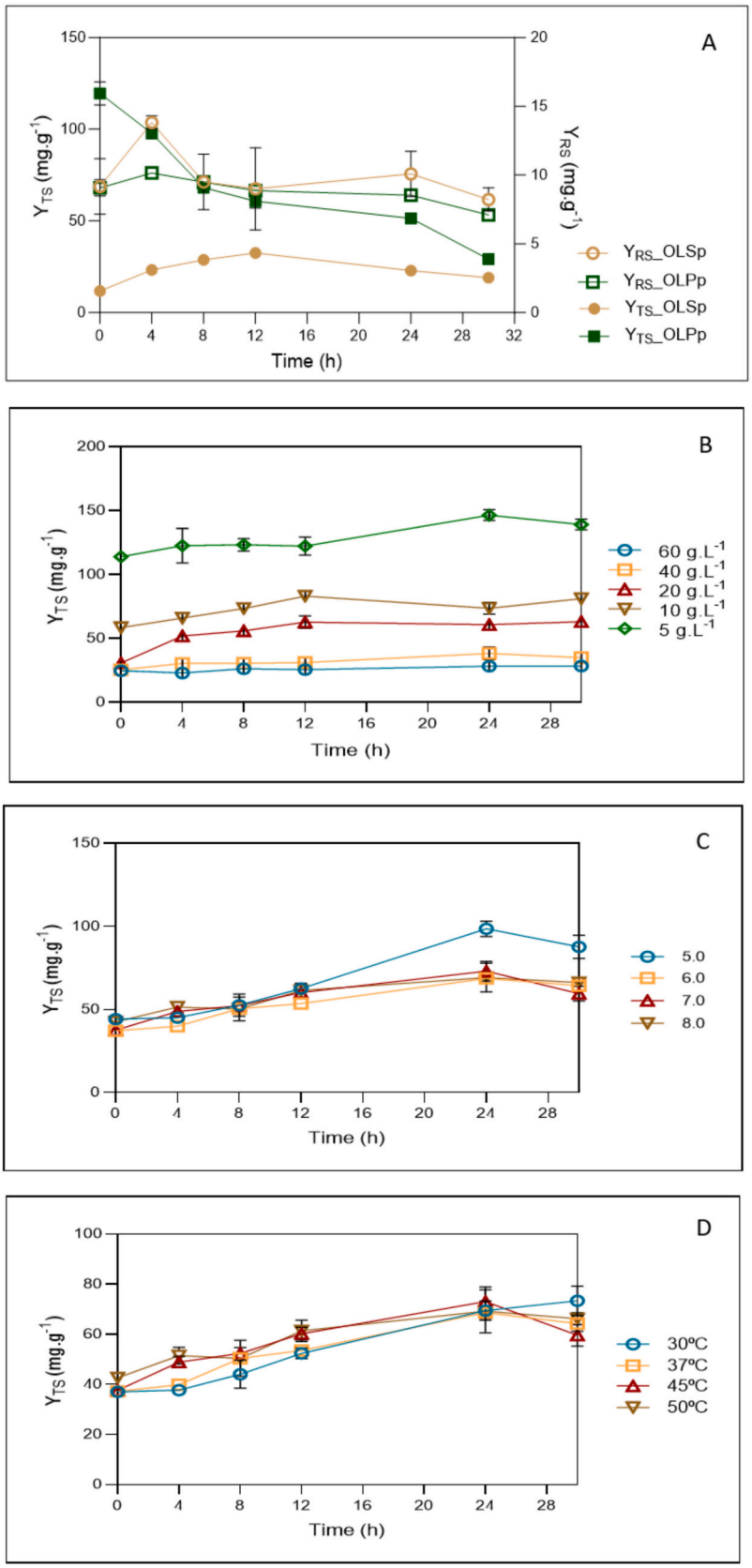

Fig. 2. Sugar production yield (Y_{TS}) obtained from the one-step fermentation of olive stones powder (OLSp) by *Bacillus subtilis* with the xylanase gene *xyn2* from *Trichoderma reesei* in 2 %(v/v) Vogel medium at 150 rpm and (A) at pH 7.0, 45 °C, using 20 g.L^{-1} of OLSp sterilised by: UV (○) or autoclave (□). A control without inoculum was performed with autoclave-sterilised medium (Δ); (B) at pH 7.0, 45 °C, using 5 (◊), 10 (▽), 20 (Δ), 40 (□) and 60 g.L^{-1} (◊) of OLSp; (C) with 20 g.L^{-1} of OLSp at 45 °C, and was tested 5.0 (○), 6.0 (□), 7.0 (Δ), and 8.0 (▽) pH values; (D) with 20 g.L^{-1} of OLSp, pH 7.0 and 150 rpm, at 30 (○), 37 (□), 45 (Δ), and 50 °C (▽). Results represent the average of two independent assays ± standard deviation.

3.3.2. One factor-at-a-time optimisation of the one-step fermentation of olive stones powder: Olive stones powder concentration, initial pH and temperature

A one factor-at-a-time optimisation was performed to evaluate the individual effect of three process variables on the OS production by one-step fermentation of OLSp. The optimal time, Y_{TSmax}, Y_{FM}, concentration of TS, and $P_{\Delta Ymax}$, were used as selection criteria. The Y_{TS} obtained during the optimisation process for the different variables are presented in Fig. 2.

Fig. 2B presents the Y_{TS} obtained during the one-step fermentation of OLSp at different concentrations, 45 °C and pH 7.0. The Table 2 summarizes the results obtained at optimal time (45 °C and pH 7.0), for each concentration tested.

Although lower concentrations of OLSp (5 and 10 g.L^{-1}) allowed to achieve the highest Y_{TSmax} (149 ± 4 and 83 ± 2 mg.g^{-1}, respectively), the use of 20 g.L^{-1} led to the highest $P_{\Delta Ymax}$ (2.6 ± 0.5 mg.g^{-1}.h^{-1}), as shown in Fig. 2B and Table 2. Furthermore, the lower concentrations of OLSp generated low concentration of TS_{max} which is a significant disadvantage.

On the other hand, with 60 and 40 g.L^{-1} of OLSp, higher concentration of TS_{max} (1.68 ± 0.01 and 1.5 ± 0.1 g.L^{-1}, respectively) were obtained, nevertheless, in these cases, the optimal time increased significantly (24 h) which resulted in the lowest $P_{\Delta Ymax}$ values (0.1 ± 0.1 and 0.5 ± 0.2 mg.g^{-1}.h^{-1}, respectively). These results may indicate a potential inhibition by the substrate for concentrations greater than 20 g.L^{-1} (Hidayatullah et al., 2020). Additionally, issues related to mass transfer and aeration, potentially stemming from the increased viscosity and density of the mixture may potentially occur at higher substrate concentrations (Figueiredo et al., 2017; Moteshafi et al., 2016).

Concerning the free monosaccharides analysis, only concentrations of 20 to 60 g.L^{-1} generated low values of Y_{FM} (≤ 0.5 mg.g^{-1}, Table 2), suggesting the high potential of the one-step fermentation to produce OS mixtures free of undesired monosaccharides.

Considering the above evidence, it was concluded that 20 g.L^{-1} was the most suitable concentration for OS production by one-step fermentation, being selected for subsequent optimization studies, regarding temperature and pH conditions.

Indeed, environmental factors as the pH or the temperature of incubation can influence bacterial growth and enzyme production and activity (Amorim et al., 2018; Heng & H. L. H., 2014; Kallel et al., 2015). Thus, different pH values were tested for the optimal concentration previously found and at fixed temperature of 45 °C. Fig. 2C presents the Y_{TS} values obtained from the one-step fermentation of 20 g.L^{-1} of OLSp, at 45 °C, and different pH values (5.0, 6.0, 7.0 and 8.0). Table 3 provides a summary of the primary process performance indicators observed for each tested pH.

Table 2
Optimal time (h), maximum total sugar concentration, TS_{max} (g.L^{-1}), maximum total sugar production yield, Y_{TSmax} (mg.g^{-1}), maximum fermentation productivity, $P_{\Delta Y_max}$ (mg.g^{-1}.h^{-1}), and yield of free monosaccharides, Y_{FM} (mg.g^{-1}), obtained with the one-step fermentation of different concentrations of olive stones powder (OLSp) by *Bacillus subtilis* containing the xylanase gene *xyn2* from *Trichoderma reesei* in 2 %(*v*/v) of Vogel medium, pH 7.0 and 45 °C. Results represent the average of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p < 0.05$) according to one-way ANOVA followed by the Tukey's test.

OLSp (g.L^{-1})	Optimal time (h)	TS_{max} (g.L^{-1})	Y_{TSmax} (mg.g^{-1})	$P_{\Delta Ymax}$ (mg.g^{-1}.h^{-1})	Y_{FM} (mg.g^{-1})
5	24	0.73 ± 0.02^{a}	146 ± 4^{a}	1.4 ± 0.3^{a}	n.d.
10	12	0.83 ± 0.01^{b}	83 ± 2^{b}	2.1 ± 0.2^{b}	n.d.
20	12	1.3 ± 0.1^{c}	63 ± 5^{c}	2.6 ± 0.5^{c}	0.5 ± 0.3^{a}
40	24	1.5 ± 0.2^{d}	38 ± 4^{d}	0.5 ± 0.2^{d}	0.5 ± 0.1^{a}
60	24	1.68 ± 0.01^{d}	28.3 ± 0.1^{e}	0.1 ± 0.1^{e}	0.43 ± 0.02^{a}

n.d.: not detected.

Table 3
Optimal time (h), maximum total sugar concentration, TS_{max} (g.L^{-1}), maximum total sugar production yield, Y_{TSmax} (mg.g^{-1}), maximum fermentation productivity, $P_{\Delta Ymax}$ (mg.g^{-1}.h^{-1}), and yield of free monosaccharides, Y_{FM} (mg.g^{-1}), obtained from one-step fermentation of 20 g.L^{-1} of olive stones powder by *Bacillus subtilis* with the xylanase gene *xyn2* from *Trichoderma reesei* in 2 % (*v*/v) of vogel medium, 45 °C and pH 5.0, 6.0, 7.0, and 8.0. Results represent the average of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p < 0.05$) according to one-way ANOVA followed by the Tukey's test.

pH	Optimal time (h)	TS_{max} (g.L^{-1})	Y_{TSmax} (mg.g^{-1})	$P_{\Delta Ymax}$ (mg.g^{-1}.h^{-1})	Y_{FM} (mg.g^{-1})
5.0	24	1.04 ± 0.04^{a}	52 ± 2^{a}	0.4 ± 0.2^{a}	n.d.
6.0	12	1.1 ± 0.1^{b}	56 ± 3^{b}	1.3 ± 0.2^{b}	1.17 ± 0.02^{a}
7.0	12	1.3 ± 0.1^{c}	64 ± 5^{c}	2 ± 1^{c}	1.4 ± 0.1^{b}
8.0	24	1.40 ± 0.04^{d}	70 ± 2^{d}	1.4 ± 0.2^{b}	1.54 ± 0.01^{b}

n.d.: not detected.

The values presented in Fig. 2C and Table 3 suggest that increasing the initial acidity (pH 5.0 and 6.0) does not enhance the Y_{TSmax}. These pH values achieved similar Y_{TS} at 0 h (41 ± 4 and 40.1 ± 0.4 mg.g^{-1}.h^{-1}, respectively) and lower Y_{TSmax}, however, the optimal time of pH 5.0 was delayed until 24 h while the pH 6.0 optimal time was at 12 h, consequently, pH 5.0 had the lowest $P_{\Delta Ymax}$ (0.4 ± 0.2 mg.g^{-1}.h^{-1}). Interestingly, the pH values tested in this assay appeared to not have a significant influence on Y_{TS} at 0 h (Fig. 2B), thus not impacting the sugar release linked to the sterilisation step.

The highest alkaline pH studied led to the highest concentration of TS_{max} (1.40 ± 0.04 g.L^{-1}), yet also increased the optimal time in 12 h when compared to pH 6.0 and 7.0, which, when compared with pH 7.0 (2 ± 1 mg.g^{-1}.h^{-1}) resulted in a lower $P_{\Delta Ymax}$ (1.4 ± 0.2 mg.g^{-1}.h^{-1}) and statistically similar to that obtained for pH 6 (1.3 ± 0.2 mg.g^{-1}.h^{-1}). Regarding the free monosaccharides production, low Y_{FM} values were obtained for pH 6.0 to 8.0, representing approximately 5.1 % of the concentration of the TS_{max}. The highest $P_{\Delta Ymax}$ (2 ± 1 mg.g^{-1}.h^{-1}) was achieved at 12 h for pH 7.0, being selected as the most suitable pH value for OS production by one-step fermentation of OLSp.

The optimum pH for this fermentation process would imply a compromise between the pH conditions more favourable for sugars extraction, the optimal pH for the growth of *B. subtilis* (pH 5.5) and the optimal pH for enzymes activity, reported to be between 4 and 6 for xylanase (Grange et al., 1996) and between 6 and 8 for pectinases (Doan et al., 2021). However, other studies also reported an optimum pH around 7.0 for *Bacillus* xylanase production using different substrates and approaches. Bakry et al. (2024) achieved the maximum production of xylanase using *Bacillus haynesii* in a mineral salt broth medium supplemented with xylan (2 g.L^{-1}, *w/v*) at pH 7.0. Alokika and Singh (2020) studied the optimisation of xylanase production by *B. substilis* JJBS250, reporting a maximum production of xylanase also at pH 7.0 using sugarcane bagasse as substrate. The production of POS is typically conducted in two stages: pectin extraction and enzymatic hydrolysis. However, the pH employed in each process is dependent on the organism used in the production of the enzymes and their respective optimal pH range (Cano et al., 2020). In a previous study, Doan et al. (2021) demonstrated the POS production by the enzymatic hydrolysis of different substrates (banana peel, rice bran, orange peel, spent coffee grounds or wheat bran) by pectinases from *Bacillus amyloquefaciens*. In that study, the optimum pH of the enzyme, and consequently the optimal pH for POS production, was found to be approximately 6.0.

Temperature, like pH, is another crucial factor that significantly influences microbial growth, as well as enzymes production and activity (Tarafdar et al., 2021), thus its optimisation is of utmost importance. Fig. 2D presents the Y_{TS} values obtained from the one-step fermentation by recombinant *B. subtilis* at pH 7.0, 20 g.L^{-1} of OLSPp and at different

temperatures (30, 37, 45 and 50 °C). Table 4 summarizes the main process performance indicators obtained for each tested temperature.

The lowest temperatures tested (30 and 37 °C) increased significantly the optimal time (30 and 24 h, as shown in Fig. 2D), leading to similar $P_{\Delta Ymax}$ values (1.3 ± 0.2 and 1.3 ± 0.1 mg.g^{-1}.h^{-1}, respectively, Table 4). The optimal temperature growth for *B. subtilis* ranges between 30 and 37 °C (Korsten & Cook, 1996) and the optimal temperature for the xylanase production from by-products using *Bacillus* sp. is reported to be around 35–37 °C (Irfan et al., 2016; Kallel et al., 2015). However, for the pectinase production it is around 50 °C (Oumer & Abate, 2017). Within the scope of a one-step fermentation process for a mixture of OS production, the optimal temperature for the xylanase activity (40–60 °C according to Karunya et al. (2021)) and pectinase activity (45–50 °C according to Doan et al. (2021)), as well as the optimal temperature for sugar extraction and proper medium mixing come along as important variables, for instance to provide the conditions for a favourable biomass boost at the begin of the fermentation. This may possibly explain the highest $P_{\Delta Ymax}$ (1.9 ± 0.1 mg.g^{-1}.h^{-1}) being achieved at 45 °C, after 12 h of fermentation (Table 4). Although the Y_{TS} profiles obtained for 45 and 50 °C were similar (Fig. 2D), and their Y_{TSmax} were statistically equivalent (60 ± 3 and 61 ± 4 mg.g^{-1}, respectively, Table 4), at 45 °C a significant higher $P_{\Delta Ymax}$ was achieved ($p < 0.05$, Table 4), besides allowing a more cost-effective bioprocess. For these reasons, 45 °C was selected as the optimal temperature.

The production of XOS by one-step fermentation has been reported using different microorganisms and substrates, such as de-starched wheat bran by a recombinant *Escherichia coli* or wheat middling's by *B. subtilis,* reaching a production of 53–80 $mg_{XOS}.g^{-1}_{substrate}$ and 64 $mg_{RS}.g^{-1}_{substrate}$, respectively (Liu et al., 2022; Reque et al., 2019).

POS production by fermentation was also reported by Embaby et al. (2016) that described the fermentation of citrus peels by *Aspergillus* sp. achieving a 57 % conversion yield. Babbar, Dejonghe, et al. (2016) reported an enzymatic hydrolysis of the pectin extracted from onion skins, producing 13.2–14.4 % (*w*/w).

The production of a mixture of OS using OPLp by hydrothermal treatment allowed obtaining approximately 13 % of neutral OS (Lama-Muñoz et al., 2012b). Although the composition of the OS is similar to that obtained in the present study, this is the first instance in which a mixture of OS has been produced by one-step fermentation using OLSp. Amorim et al. (2018) optimised a one-step production of AXOS from BSG by the same recombinant *B. subtillis*, producing 27.5 ± 0.8 $mg_{reducing\ sugars}\ g^{-1}_{substrate}$ at 12 h using the same conditions as found in this study: 20 g L^{-1} of OLSp at pH 7.0 and 45 °C. These observations suggest a potential robustness of this process technology to produce OS valorising different by-products. Following the optimisation process, it is of interest to establish the precise nature of the sugars produced. To this end, the sugars present in the fermentation medium, after 12 h, were quantified and characterised.

Table 4
Optimal time (h), maximum total sugar concentration, TS_{max} (g.L^{-1}), maximum total sugar production yield, Y_{TSmax} (mg.g^{-1}), maximum fermentation productivity, $P_{\Delta Ymax}$ (mg.g^{-1}.h^{-1}), and yield of free monosaccharides, Y_{FM} (mg.g^{-1}), obtained from the one-step fermentation of 20 g.L^{-1} of olive stones powder by *Bacillus subtilis* with the xylanase gene *xyn2* from *Trichoderma reesei* in 2 %(*v*/v) Vogel medium at 150 rpm and pH 7.0 and 30, 37, 45 and 50 °C. Results represent the average of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p < 0.05$) according to one-way ANOVA followed by the Tukey's test.

Temperature (°C)	Optimal time (h)	TS_{max} (g.L^{-1})	Y_{TSmax} (mg.g^{-1})	$P_{\Delta Ymax}$ (mg.g^{-1}.h^{-1})	Y_{FM} (mg.g^{-1})
30	30	1.5 ± 0.1^{a}	73 ± 6^{a}	1.3 ± 0.2^{a}	n.d.
37	24	1.4 ± 0.1^{b}	69 ± 3^{b}	1.3 ± 0.1^{a}	n.d.
45	12	1.2 ± 0.1^{c}	60 ± 3^{c}	1.9 ± 0.1^{b}	n.d.
50	12	1.2 ± 0.1^{c}	61 ± 4^{c}	1.6 ± 0.4^{c}	n.d.

n.d.: not detected.

3.4. Quantification and chemical characterisation of OS produced by *B. subtilis* with the xylanase gene *xyn2* from *T. Reesei*

The OLSp fermented sample contained 175.4 μg.mg^{-1} of polysaccharides, being uronic acids the predominant sugar residue (72.1 %mol), followed by xylose (11.6 %mol), arabinose (5.5 %mol), glucose (3.9 %mol), rhamnose (3.5 %mol), and mannose (1.3 %mol) (Table S3).

To disclose the carbohydrate structure of the low molecular weight OS produced during fermentation, ranging from DP2 to DP4 (Fig. S2), methylation analyses using three different approaches were performed to recover the compounds with molecular weight > 0.5 kDa and > 1 kDa, and neutral OS (Table 5). The methylation analysis with liquid-liquid extraction isolates the neutral permethylated carbohydrates, which structures are not linked to uronic acids.

The glycosidic linkage analysis shows that the major differences between the three methodologies relies mainly on the total amount of xylose, higher in neutral oligosaccharide analysis (51 %) in comparison with the method using dialysis (25.5–27.6 %), where neutral OS were recovered from both neutral and acidic oligosaccharide structures. Concerning galactose, the opposite was observed, higher in >0.5 kDa (57.9 %), followed by >1 kDa (37.5 %), and in neutral OS (7.3 %), in accordance with the fact that galactose residues are side chains of pectic polysaccharides composed of galacturonic acid residues.

The xylose is a component of xylans present in the OLSp, mainly from the slightly branched glucuronoxylan, where the backbone is composed

Table 5
Glycosidic linkage analyses (%mol) of olive stone one-step fermentation from methylation procedure using dialysis (>0.5 kDa, >1 kDa and neutral oligosaccharides.

Glycosyl linkage	>0.5 kDa	>1 kDa	Neutral OS
2-Rha*p*	1.0	7.8	7.5
3-Rha*p*	0.4	1.0	0.5
2,4-Rha*p*	tr	tr	tr
Total	**1.4**	**8.7**	**8.0**
t-Fuc*p*	1.0	1.8	2.3
Total	**1.0**	**1.8**	**2.3**
t-Ara*f*	4.6	8.5	7.7
2-Ara*f*	0.2	1.0	0.9
3-Ara*f*	0.7	1.6	2.7
5-Ara*f*	0.9	2.9	3.0
3,5-Ara*f*	0.5	1.2	2.1
Total	**7.0**	**15.1**	**16.4**
t-Xyl*p*	4.9	8.8	18.9
4-Xyl*p*	15.0	11.8	28.9
2,3-Xyl*p*	0.4	1.6	
2,4-Xyl*p*	4.7	3.1	3.2
3,4-Xyl*p*	0.5	2.2	
Total	**25.5**	**27.6**	**51.0**
t-Man*p*	4.8	5.1	6.8
2-Man*p*			1.5
Total	**4.8**	**5.1**	**8.3**
t-Gal*p*	3.0	3.2	2.1
2-Gal*p*	1.0	1.0	
3-Gal*p*	1.2	0.9	
4-Gal*p*	49.6	24.8	2.9
6-Gal*p*	0.7	1.7	1.1
2,4-Gal*p*	0.5	2.3	
3,4-Gal*p*	1.0	2.0	
3,6-Gal*p*	0.8	1.5	1.2
Total	**57.9**	**37.5**	**7.3**
t-Glc*p*	1.0	2.7	4.9
2-Glc*p*			1.7
4,6-Glc*p*	1.4	1.5	
Total	**2.4**	**4.1**	**6.6**

tr - trace.

by -(β1 → 4)-xylose branched with a glucuronic acid substituted at *O*-2, with a molar ratio of 9:1 (Xyl:GlcA) (Coimbra et al., 1995). It was possible to observe the relative increase of 4-Xyl*p* and the decrease of substituted 2,4-Xyl*p* in neutral oligosaccharide fraction in relation to the methylation with 0.5 kDa dialysis, as the glucuronic acid is linked to the *O*-2 of the xylose residues, indicative of the presence of neutral XOS. The amount of about 18.9 % of terminally-linked xylose could indicate the presence of short chain XOS.

The significant decrease of 4-Gal*p* in the methylation of neutral OS, as well as the decrease of most Gal residues, indicates that these residues are part of acidic carbohydrates. The higher amount of 4-Gal*p,* mainly in 0.5 kDa (49.6 %) but also in 1 kDa (24.8 %), as well as the presence of 2-Rha*p* (1–7.8 %), agree with the presence of pectic polysaccharides containing the repeating units of 2-L-Rha*p*-(α1 → 4)-α-D-Gal*p*A as backbone (Cardoso et al., 2002). These polysaccharides have side chains such as galactan, arabinogalactan, and arabinan. The galactan side chain consists of (β1 → 4)-Gal*p* residues, in agreement with the higher relative amount of 4-Gal*p* in methylation analysis with dialysis. The presence of 3,4-Gal*p* and 5-Ara*f* in the sample may be due to arabinogalactan-I, which consists of a -(β1 → 4)-Gal*p* backbone substituted with -(α1 → 5)-L-Ara*f* residues attached to the *O*-3 position of galactosyl units (Vierhuis, 2002). The arabinan chain could also be present due to the occurrence 3.0 % of 5-Ara*f* and 2.1 % of 3,5-Ara*f*. The arabinans consist of -(α1 → 5)–Ara*f* backbone, that could be substituted at *O*-2 or *O*-3 or at both positions (Cardoso et al., 2002). The pectic carbohydrates mainly corresponds to OS with lower molecular weight, since when the cut off increased, the amount of these acidic polysaccharides decreased.

Despite the presence of GXOS produced by the action of the xylanases on the glucuronoxylans, the OLSp after fermentation is mainly composed by pectic related carbohydrate side chains. In addition, HPAEC-PAD showed a chromatographic profile consistent with the presence of pectic oligosaccharides, comparable with the oligosaccharides released by the acid hydrolysis of a polygalacturonic acid standard (Fig. 3). The presence of pectic polysaccharides is corroborated by 72.1 %mol of uronic acids measured in the sample (Table S3), which can be mainly attributed to galacturonic acid, as the contribution of glucuronic acid from glucuronoxylans should be very low, as inferred by the 11.6 % mol of xylose in the sample (Table S3). The low amount of XOS in OLSp sample can possibly be due to the low yield of the extraction from the highly lignified OLS tissues. Other hypothesis is related with the extensive enzymatic hydrolytic action that led to the production of monomers or disaccharides of xylose that could be consumed by the bacteria.

To confirm the presence of GXOS and POS, the sample was analysed via HPAEC-PAD (Fig. 3). As a reference, a sample of polygalacturonic acid resulted from a hydrolysis with 1 M H_2SO_4 at 100 °C for 1 h was also analysed in the same conditions.

From Fig. 3 it is possible to observe a region of neutral mono and OS at lower retention time, possibly corresponding to the GXOS, and a region of charged OS, possibly corresponding to POS, since they have the same retention time as OS produced from the partial hydrolysis of polygalacturonic acid.

To corroborate the presence of both POS and GXOS, an analysis of neutral mono and OS (DP1-DP5) of fermented OLSp sample was performed by reduction, acetylation, and analysis by GC–MS (Fig. S3). According to the GC–MS analysis, the sample OLSp fermented is composed of 5.61 $\mu g.mg^{-1}$ of the mono and OS (DP1-DP4). It was observed the presence of rhamnose, arabinose, xylose, mannose, glucose, and galactose. Moreover, possible apiose was also detected, characteristic of rhamnogalacturonan type II (Vierhuis, 2002). Despite the low amount, it was possible to detect 18 different pentose and hexose disaccharides, 9 trisaccharides, and 1 tetrasaccharide (Table S4). The OS profile of OLSp corroborates the presence of GXOS, although in small amounts and in a mixture with other neutral and acidic OS.

This study successfully demonstrates the feasibility of a one-step fermentation process for converting OLS into a high-value mixture of prebiotic OS, marking an important advancement toward more versatile and sustainable bioprocesses. OLS, abundantly available in Mediterranean regions as low-cost fermentative substrate, offer a favourable combination of lignocellulosic and pectic components compared to

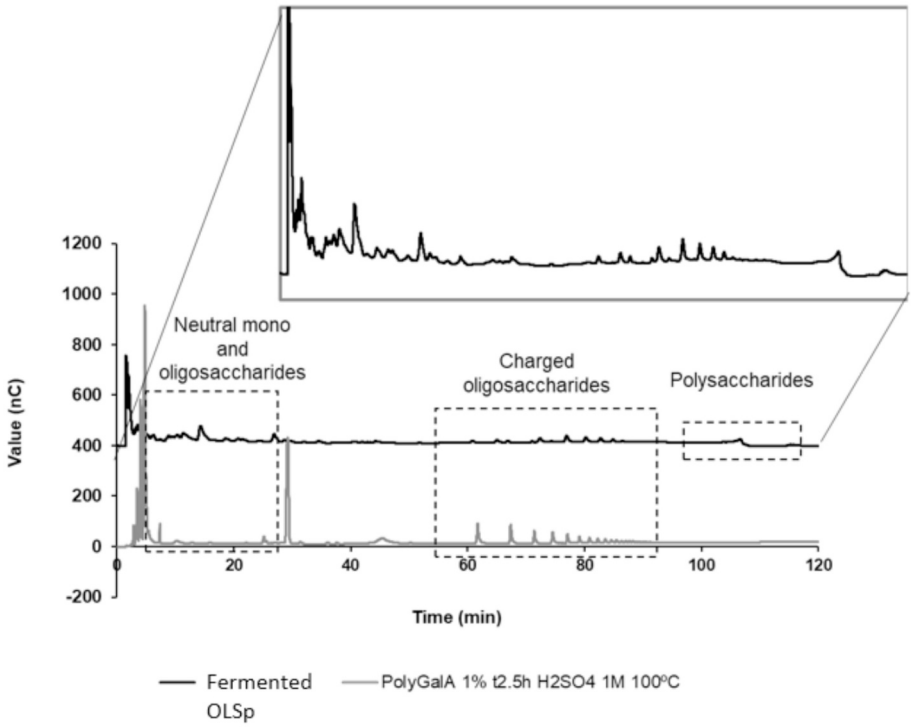

Fig. 3. Fermented OLSp (black line) and hydrolysed polygalacturonic acid (grey line) analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

other substrates—such as the xylan in wheat bran or the pectin in citrus peels —which predominantly yield only XOS or POS, respectively (Embaby et al., 2016; Liu et al., 2022). This enables the co-production of GXOS and POS, which aligns with growing evidence that OS mixtures provide enhanced or synergistic prebiotic functionalities compared to their individual use (Dong et al., 2023). Furthermore, the predominance of low-degree polymerization (DP2–4) OS presents advantageous physicochemical properties, including high solubility and mild sweetness, which are desirable for food applications (Ali et al., 2025).

This work provides important groundwork and proof of concept for the direct microbial bioconversion of OLS. The inherent complexity of OLS lignocellulosic matrix posed specific challenges regarding substrate accessibility and enzymatic synergy. The process overcomes these challenges by leveraging a recombinant *B. subtilis* strain expressing heterologous xylanase alongside its native pectinolytic enzyme system. The results open new directions for exploring the molecular mechanisms underpinning microbial bioconversion, including the roles of xylanases and pectinases during fermentation. Investigating gene expression dynamics and enzyme activity profiles could further refine strain engineering strategies, such as targeted enzyme overexpression and synthetic pathway optimization. Additionally, the process developed here lays the basis for scale-up and techno-economic assessment, which is essential for validating this approach's competitiveness against conventional processes and produce economic and easily accessible prebiotic oligosaccharides able to be used as food ingredients.

4. Conclusions

The chemical analysis highlighted the potential of producing OS, particularly GXOS and POS, from olive oil industry by-products, specifically OLP and OLS, which are rich in lignocellulose and pectin. Using a novel one-step fermentation process with genetically engineered *Bacillus subtilis* incorporating a xylanase gene, OLSp emerged as the most effective substrate for OS production. Optimal conditions (20 $g{\cdot}L^{-1}$ OLSp, pH 7.0, 45 °C) yielded 60 ± 3 $mg{\cdot}g^{-1}$ of sugars after 12 h of fermentation. This one-step fermentation offers a sustainable, efficient alternative for prebiotic compound production, without the need for commercial enzymes. The findings underscore the viability of using OLS as a cost-effective substrate for generating a mixture of prebiotics (including GXOS, in small amounts, and various neutral and acidic OS), promoting a circular economy within the olive oil sector. Future work will prioritize process scale-up to generate the necessary data for a robust techno-economic assessment. This will enable evaluation of cost-effectiveness, time efficiency, and sustainability relative to existing OS production processes from other agricultural residues.

CRediT authorship contribution statement

Ana Cordeiro: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Andreia Fernandes:** Methodology, Investigation. **Andreia S. Ferreira:** Writing – review & editing, Methodology, Investigation. **Elisabete Coelho:** Writing – review & editing, Validation. **Manuel A. Coimbra:** Writing – review & editing, Validation, Resources. **Sara C. Silvério:** Writing – review & editing, Investigation. **Vasco Cadavez:** Resources, Methodology. **António M. Peres:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis. **Lígia R. Rodrigues:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Cláudia Amorim:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.146278.

Data availability

Data will be made available on request.

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