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Coffee silverskin fermentation in a single step for sustainable production of carbohydrate-based mixture rich in pectic oligosaccharides

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ABSTRACT

A competitive single-step fermentation using *Bacillus subtilis* 3610 was developed to convert coffee silverskin (CSS) into an oligosaccharide-rich carbohydrate mixture, mainly composed by pectic oligosaccharides (POS). CSS is an abundant by-product of the coffee industry, and POS are widely reported as emerging prebiotics, motivating sustainable production processes from agro-industrial by-products. In this study, OS were produced using CSS and CSS-derived pellets (CP) via a single-step fermentation leveraging the native pectinolytic activity of *B. subtilis* 3610. CP consistently outperformed CSS, achieving higher yields of reducing sugars at the optimal time ($Y_{RS_{max}}$). After optimisation, a $Y_{RS_{max}}$ of 162 ± 7 mg·g^{-1} was obtained at 8 h, 45°C and pH 7.0, with 10 g·L^{-1} CP. The combined hydrolysis of CP with commercial xylanase and pectinase led to a synergetic activity potentially occurring during fermentation, where xylanase may disrupt cell walls to release pectin, while pectinase hydrolyses it into POS. Under optimal conditions, single-step fermentation achieved a significantly higher Y_{RSmax} than the combined enzymatic hydrolysis (141 ± 6 mg·g^{-1} at 12 h, 1 U·mL^{-1} per enzyme). In aerated 3.2 L bioreactors, CP fermentation showed comparable performance to shaken-flask assays (Y_{RSmax} 162 ± 2 *vs.* 162 ± 7 mg·g^{-1}; P_{max} 9.3 ± 0.1 *vs.* 9 ± 1 mg·g^{-1}·h^{-1}, respectively). The carbohydrate-based mixture obtained at optimal time contained a higher content of POS (uronic acids 78.8%mol) with a neutral xyloglucan-derived fraction. This study reinforces single-step fermentation as a simplified and competitive process for carbohydrate-based mixtures that avoid enzyme purchase.

1. Introduction

Prebiotics are of considerable scientific and industrial interest due to their ability to modulate the gut microbiota, which is a key driver of beneficial effects on host health (Gibson et al., 2017). In addition to the most widely known prebiotics as inulin, galacto- or fructo-oligosaccharides (FOS), pectic oligosaccharides (POS) are increasingly recognised as emerging prebiotics (Gullón et al., 2013; Míguez et al., 2016; Tang et al., 2025) and are attractive for food applications due to their good solubility, sweetness and processing stability (Alencar et al., 2025). POS are non-digestible oligosaccharides (OS) that can be obtained by depolymerization of the different structures of pectin (Alencar et al., 2025). The microbial production of OS from agro-industrial by-products is especially promising for the food ingredient sector, as these by-products are inexpensive, and abundant. The global prebiotic ingredients market was valued at USD 7.79 billion in 2025 and is projected to reach USD 12.38 billion by 2030 (Mordor Intelligence, 2025). These trends highlight the need for sustainable and cost-effective bioprocesses for OS production (Martins et al., 2023).

Traditional OS production from lignocellulosic agro-industrial by-products typically involves multiple steps, including chemical or enzymatic pretreatment of biomass followed by separate hydrolysis processes. Chemical methods often rely on harsh reaction conditions that generate toxic by-products (e.g. furfural, 5-hydroxymethylfurfural), whereas enzymatic approaches rely on expensive enzymes (Kumar et al., 2024; Kumari et al., 2024). Amorim et al., (2018), reported, for the first time, the single-step microbial production of arabino-xylooligosaccharides (AXOS) from brewers' spent grain (BSG).

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Cordeiro et al., (2025) further explored this approach to produce a mixture of different OS directly from olive oil by-products, by leveraging the *in situ* microbial production of multiple carbo-hydrolytic enzymes that act on different polysaccharides. These mixtures can offer a broader prebiotic potential than single-family OS by stimulating distinct microbial groups from gut microbiota, promoting cross-feeding and more balanced short chain fatty acids profiles, and yielding synergistic effects that enhance the functional robustness of the microbiota response (Zhang et al., 2024). These advantages position single-step production of carbohydrate-based mixtures as a promising alternative to traditional multi-step, multi-substrate processes.

Coffee silverskin (CSS), the thin tegument that covers coffee beans and removed during the roasting process, is an abundant yet underutilised agro-industrial by-product (Borrelli et al., 2004; Pourfarzad et al., 2013). Recent advancements in biomass processing have led to the development of CSS pellets (CP), which offer several advantages over raw CSS for bioprocessing, including improved handling, enhanced storage stability, and greater transport efficiency, while retaining valuable hemicelluloses content (Barbero-López et al., 2020; Pua et al., 2020). The outer layers of CSS are rich in fibrous tissues composed primarily of cellulose and hemicelluloses, making this residue a promising feedstock for OS production (Arya et al., 2022). Mussatto and Teixeira, (2010) reported CSS as an efficient support and nutrient source to produce FOS and fructofuranosidase by solid-state fermentation with *Aspergillus japonicus*. Additionally, recent studies on lignocellulosic seed coats with compositions similar to CSS have demonstrated the feasibility of extracting and fractionating distinct polysaccharides and OS, including xyloglucan-enriched and pectic fractions (Silva et al., 2024). Although CP is not a classical pectin-rich feedstock, it was selected as a non-conventional substrate to test the robustness of the single-step fermentation strategy for producing complex carbohydrate-based oligosaccharide mixtures enriched in POS from low-cost agro--industrial residues.

Xyloglucans (XG) are neutral hemicelluloses abundant in primary plant cell walls, consisting of a β-(1→4)-glucan backbone substituted with xylosyl residues and additional side-chain decorations depending on origin (Pauly and Keegstra, 2016; Park and Cosgrove, 2015). Beyond their role as structural matrix polysaccharides, XG-derived materials are also attractive from an application standpoint due to their hydration and rheology-modifying properties, which can be relevant for formulation and stability (Shao et al., 2019). In addition, xyloglucan-based mucoprotectant formulations have been evaluated clinically for gastrointestinal symptom management via barrier-related mechanisms, which should be distinguished from direct prebiotic substantiation (Trifan et al., 2019; Perez-Garcia et al., 2023).

In this study, we explored the valorisation of CSS and CP by-products as substrates for oligosaccharide (OS) production via single-step submerged fermentation using *Bacillus subtilis* 3610 in a basal salts' medium. The process performance was assessed in shaken flasks and in 3.7 L bioreactors. The main challenge of this work was to demonstrate that single-step fermentation is a competitive approach to produce complex carbohydrate-based mixture with potential prebiotic effect from alternative by-products, opening its range of application in industry. This strategy provides a promising alternative to conventional production, valorising a major coffee by-product.

2. Methods

2.1. Materials

All chemicals, media, media components and enzymes were obtained from Sigma-Aldrich Chemical Ltd. unless otherwise specified. CP and CSS were supplied by Delta S.A. (Portugal) (Figure S1, Supplementary material). These by-products were grinded with a benchtop mixer, dried overnight at 60°C and stored at room temperature until use (Amorim et al., 2019). The relative humidity content of the residues was < 5% (w/w), being determined with a humidity analyser MAC 50/1/NH device (Amorim et al., 2019). The powders were further stored in a desiccator at room temperature until being used directly as fermentative substrate. The standard XOS was composed of xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6).

2.2. Chemical characterisation of coffee silverskin and coffee silverskin pellets

The CSS and CP were chemically characterised according to AOAC standards. Moisture (AOAC 934.6) and ash contents (AOAC 942.05) were determined. The total protein content was determined using a Micro Kjeldahl block digestion system behr K 24 (ExpotechUSA). A Soxtec 8000 FOSS apparatus was used to determine the quantity of extractives using 80% (v/v) ethanol/water as solvent. The compositions in lignin, cellulose and xylan were determined through acid hydrolysis with 72%(w/w) of sulfuric acid, according to National Renewable Energy Laboratory protocols (NREL/TP-510–42618–42622–4218). The sugars and their degradation products present in the hydrolysates were analysed by High Performance Liquid Chromatography (HPLC) (Section 2.8.3) and used to calculate the cellulose and xylan fraction. The acid soluble lignin was obtained by the analysis of the hydrolysate using a UV–VIS/UV-1280 spectrophotometer (Shimadzu, Kyoto, Japan) at 206 nm. The pectic polysaccharide 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

A pre-inoculum was prepared by cultivating a single colony of *Bacillus subtilis* 3610 wild type, a recognized culture-collection reference strain (NCIB origin), in 4 mL of LB medium (Difco, New Jersey, USA). The cells were incubated at 40°C with agitation at 250 rpm for approximately 2 h until reaching an $OD_{600\ nm}$ of ~1.0. This culture was then diluted to an $OD_{600\ nm}$ of ~0.020 in the fermentation medium.

2.4. Screening the potential of coffee silverskin and coffee silverskin pellets as fermentation substrates

To investigate the potential of CSS and CP as alternative fermentation substrates, 1 g of substrate was added to 50 mL of the minimal medium Vogel's 50 × salts at 2% (v/v) (Vogel, 1956) at pH 7.0. Erlenmeyer flasks (250 mL), containing the CSS or CP–liquid mixture, were sterilised at 121°C during 15 min (Amorim et al., 2018). The fermentation was performed at 45°C, 150 rpm, during 24 h after inoculum preparation and addition as described in Section 2.3.

2.5. Enzymatic hydrolysis of coffee silverskin pellets

To evaluate the potential xylanase-pectinase synergy on carbohydrate-based mixtures production, 20 $g{\cdot}L^{-1}$ of CP were hydrolysed using commercial enzymes: *Aspergillus aculeatus* pectinase P2611 and *Aspergillus oryzae* xylanase X2753 were used individually and combined at a concentration of 1 $U{\cdot}mL^{-1}$ in 0.1 M sodium acetate buffer, pH 4.5, 40°C, and 150 rpm during 24 h. Prior to enzyme addition, the substrate-buffer mixture was sterilised by autoclave as described in Section 2.4. The commercial pectinase was composed by pectin-transeliminase, polygalacturonase and pectinesterase, along with small amounts of hemicellulases and cellulases.

2.6. Single-step fermentation of coffee silverskin pellets for production of carbo-based mixture

2.6.1. Sterilisation effect

The impact of sterilisation method on the single-step fermentation of CP was assessed by comparing autoclaving (Section 2.4) with UV sterilisation. For UV sterilisation, the by-product was irradiated with a 254 nm UV lamp positioned 30 cm away for 1 h and subsequently added to filter-sterilised 2% (v/v) Vogel minimal medium (pH 7.0) with 0.2 μm cellulose membrane filters. The fermentation was then carried at 45°C and 150 rpm for 30 h and the inoculum was prepared as described in Section 2.3. A parallel assay without inoculum was performed as a control.

2.6.2. One-factor-at-a-time screening

The fermentation process conditions for CP were selected using a sequential one-factor-at-a-time (OFAT) screening approach. The individual effects of CP concentration (5, 10, 20, 40 and 60 $g{\cdot}L^{-1}$), initial pH (5.0, 6.0, 7.0 and 8.0) and temperature (30, 37, 45 and 50°C) were evaluated sequentially during 30 h of fermentation. The starter culture was prepared as described in Section 2.3.

2.7. Single-step fermentation of coffee silverskin pellets in bioreactor

The bioreactor experiments were performed in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering) with a working volume of 1 L. The aeration was carried out with a sparger located at the base of the agitator, with a flowrate of 0.5 vvm and a stirring rate of 150 rpm (Braga et al., 2021). Bioreactors were inoculated as follows: a pre-inoculum was prepared as described in Section 2.3, diluted to an OD_{600} of ~0.020 in 50 mL of LB medium, incubated at 40°C and 250 rpm for approximately 8 h until reaching an OD_{600} of ~1.0, and subsequently used to inoculate the fermentation medium.

Prior to inoculation, bioreactors containing a mixture of 10 $g{\cdot}L^{-1}$ CP and 2% (v/v) Vogel medium at pH 7.0 were sterilised at 110°C for 30 min. Process conditions were set according to the optimal parameters identified in shaken flasks (Section 2.6). The fermentations were conducted at 45°C.

2.8. Analytical methods

For all assays, samples from the supernatant were collected centrifuged at 7000 rpm for 7 min and further analysed.

2.8.1. Quantification of reducing sugars

The DNS method was used as a qualitative screening technique to assess total reducing sugars (RS) using galacturonic acid as standard (Miller, 1959). The reducing sugar production yields, Y_{RS} ($mg{\cdot}g^{-1}$), were calculated as the ratio between RS (mg) and the mass of substrate (g) used in the assay. The optimal time corresponded to the time at which the highest value of Y_{RS} was achieved, Y_{RSmax} ($mg{\cdot}g^{-1}$), while the maximum fermentation productivity, P_{max} ($mg{\cdot}g^{-1}{\cdot}h^{-1}$), was calculated by dividing the difference between the Y_{RSmax} and the Y_{RS} at 0 h, by optimal time. The maximum oligosaccharide-associated RS yield, $Y_{OS\text{-}max}$, was calculated as the difference between Y_{RSmax} and Y_{FM}. In the OFAT screening, the selected condition among those tested was identified using a multi-criteria assessment that combined quantitative metrics (Y_{RSmax}, toptimal, and Y_{FM}) with TLC profile interpretation (Section 2.8.2) and practical process operability (e.g., handling/mixing suitability for submerged fermentation).

2.8.2. Thin-Layer Chromatography

Thin-Layer Chromatography (TLC) was used for the qualitative analysis of the OS degree of polymerisation (DP) obtained from CSS and CP (Amorim et al., 2018). Supernatant samples (17 μL) were applied into silica matrix plates (DC-Alufolien Kieselgel 60, Merck), and 2 μL of XOS (2 $g{\cdot}L^{-1}$) was used as standard. A 2:1:1 (v/v/v) mixture of butanol, acetic acid, and water was used as mobile phase (Amorim et al., 2019). The staining solution consisting in 1% (w/v) diphenylamine and 1% (v/v) aniline in acetone was applied, followed by heating the plates at 110°C for 10 min (Dutta and Wu, 2014).

2.8.3. Analysis by high performance liquid chromatography

The HPLC-RI method was used exclusively to quantify free monosaccharides in the supernatants. A volume of 20 μL of sample was eluted using 5 mM H_2SO_4 at a flow rate of 0.6 $mL{\cdot}min^{-1}$ and a temperature of 60°C in an HPLC (Agilent Technologies, USA) with a RI detector (Agilent Technologies, USA) and an Aminex HPX 87 H column (300 × 7.8 mm; Biorad, USA). To quantify the degradation products of xylan and cellulose, the UV detector was used to determine the concentrations of furfural and HMF, respectively.

2.8.4. Partial purification and chemical characterisation of the produced carbohydrate-based mixture

The carbohydrate-based mixture obtained from single-step fermentation of CP under optimal conditions was partially purified using activated charcoal, as described by Amorim et al., (2019), to reduce salt interference. As detailed in Cordeiro et al. (2025), the loss in total sugars (TS) during purification was quantified by phenol-sulfuric acid method and further analysed using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Thermoscientific Dionex – Waltham) and gas chromatography–quadrupole mass spectrometry (GC-qMS, Shimadzu GC–MS QP2010) techniques. POS and XG-related carbohydrates mixture was defined quantitatively by total carbohydrates (phenol–sulfuric acid) complemented by neutral monosaccharide composition (as alditol acetates by GC-FID) and uronic acids (phenylphenol colorimetry). Diagnostic glycosidic linkage signatures were determined by the correspondent partially methylated alditol acetates (GC–MS) and HPAEC-PAD profiles allowed to assess the oligosaccharide structures and categorize them according to charge and degree of polymerization.

2.9. Statistical analysis

Data are reported as mean ± standard deviation from two independent assays. Statistical analyses were performed in GraphPad Prism v10.0.0 (GraphPad Software, San Diego, CA, USA). Comparisons between two independent groups used an unpaired Student's *t*-test. Comparisons among multiple groups defined by a single factor used one-way ANOVA followed by Tukey's multiple-comparisons test. Statistical significance was set at $p < 0.05$. Within each row, values sharing the same superscript letter do not differ significantly.

3. Results and discussion

3.1. Chemical characterisation of coffee silver skin and coffee silver skin pellets

Lignocellulosic agro-industrial by-products, such as coffee silverskin (CSS) and coffee silverskin-derived pellets (CP), are primarily composed of cellulose, hemicelluloses, and lignin (Dutta and Wu, 2014). Table 1 presents the chemical characterisation of both by-products expressed as a percentage of dry weight (% (w/w)).

As expected, CSS and CP exhibit relatively similar chemical compositions (Table 1), since CP is derived from CSS. However, CP exhibited slightly higher levels of xylan (15.5 ± 0.3% (w/w) *vs.* 17.6 ± 0.6% (w/w)) and protein (15.8 ± 0.4% (w/w) *vs.* 18.6 ± 0.3% (w/w)), both of which may contribute to enhanced microbial growth and enzyme activity during fermentation. CP also presents a higher insoluble lignin content (18.1 ± 0.3% (w/w) *vs.* 20.1 ± 0.3% (w/w)). These differences may result from the heating and compression applied during pelletisation, which can also promote the degradation of phenolic compounds

Table 1
Chemical characterisation of coffee silver skin (CSS) and coffee silver skin pellets (CP) in dry weight (% (w/w)).

% (w/w)	CSS	CP
Ashes	2.35 ± 0.04[a]*	2 ± 1[a]
Pectic polysaccharides	2.2 ± 0.1[a]	2.1 ± 0.4[a]
Extractives	19.73 ± 0.04[a]	17.6 ± 0.1[b]
Protein	15.8 ± 0.4[a]	18.6 ± 0.3[b]
Xylan	15.5 ± 0.3[a]	17.6 ± 0.6[a]
Cellulose	18.9 ± 0.1[a]	18.9 ± 0.4[a]
Acid soluble lignin	4.2 ± 0.6[a]	4.9 ± 0.1[a]
Insoluble lignin	18.1 ± 0.3[a]	20.1 ± 0.3[b]

* Results are presented as mean ± standard deviation. Different letters within the same row indicate statistically significant differences between CSS and CP ($p < 0.05$), determined by unpaired Student's *t*-test.

(Borrelli et al., 2004; Pua et al., 2020; Setter et al., 2020) and may therefore explain the lower extractives content observed in CP, 19.73 ± 0.04% (w/w) *vs.* 17.6 ± 0.1% (w/w).

The values herein obtained are consistent with those stated in the literature. Mussatto et al., (2011) and Ballesteros et al., (2014a) reported cellulose contents ranging from 15.0% to 22.6% (w/w), xylan from 10.9% to 20.3% (w/w), and lignin from 19.3% to 27.0% (w/w), for different samples of CSS. Variations may be attributed to differences in the extraction process and the variety or origin of coffee beans used (Ballesteros et al., 2014a). To evaluate the substrates potential for OS production, it is important to consider their polysaccharide composition (Corim and Gabardo, 2021), including their content in pectic polysaccharides. Interestingly, the pectic polysaccharide content in CSS and CP (2.2 ± 0.1% (w/w) and 2.1 ± 0.4% (w/w), respectively) is slightly higher than that reported for olive stones, 1.7 ± 0.1% (w/w), which have already been identified as a promising fermentation substrate for POS and glucurono-xylooligosaccharides (GXOs) production (Cordeiro et al., 2025). CSS is not commonly used as a pectic polysaccharide source, however, the results herein presented agree with the literature, it is reported a pectic polysaccharide content of 1–2% (Ballesteros et al., 2014b; Massaya et al., 2019) who reported a pectic polysaccharide content of 2.3 ± 0.1% (w/w) in CSS. Nonetheless, it is important to note that the pectin quantification method used in this study (Section 2.2) has limitations, since it quantifies only de-esterified carboxyl groups, which may lead to an underestimation of the total pectin content in CSS. Although CSS is not generally regarded as a pectin-rich feedstock, it can still contain pectin-associated domains that can become relevant upon fractionation and subsequent processing (Voragen et al., 2009; Xiao and Anderson, 2013). Overall, the chemical characterisation results suggest that CSS and CP are promising substrates for OS production via single-step fermentation.

3.2. Single-step fermentation for production of carbohydrate-based mixture

3.2.1. Substrate screening

The potential of CSS and CP as substrates for the production of carbohydrate-based mixtures was assessed using single-step fermentation. The yield of reducing sugars (Y_{RS}) was used as a qualitative indicator of fermentation performance, as it reflects the total concentration of mono- and oligosaccharides (OS) with reducing ends. Since YRS does not discriminate between mono- and oligosaccharides, it was used only as a screening metric and interpreted together with Y_{FM} and post-process compositional/structural characterisation to confirm an oligosaccharide-rich profile under the selected conditions. The Y_{RS} profiles obtained for both substrates are presented in Fig. 1.

The Y_{RS} values at 0 h correspond to the reducing sugars released from CSS or CP during the sterilisation process by autoclave (Section 2.6.1), with CP showing the highest value (59 ± 5 mg·g^{-1} *vs.* 42 ± 1 mg·g^{-1}, $p < 0.05$), which may promote more efficiently the biomass

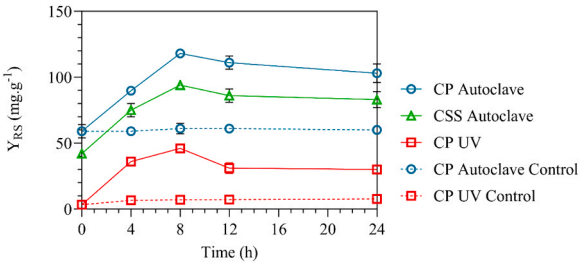

Fig. 1. Reducing sugar production yield (Y_{RS}) for coffee silverskin (CSS) (Δ) and CSS pellets (CP) sterilised by autoclave (○) or UV (□) obtained from the single-step fermentation of 20 g·L^{-1} of substrate by Bacillus subtilis in 2% (v/v) Vogel medium at pH 7.0, 45°C, and 150 rpm. A single-step fermentation of CP sterilised by autoclave and UV without inoculum was performed with autoclave-sterilised medium (Δ). Results represent the average of two independent assays ± standard deviation.

growth at an initial stage. For both CSS and CP under autoclave sterilisation, Y_{RS} increased rapidly from 0 to 8 h, followed by a slight decrease up to 24 h. This profile suggests an initial phase dominated by hydrolysis that leads to OS accumulation, followed by a later phase in which the OS appear to be further hydrolysed into monosaccharides and subsequently consumed, thereby limiting any additional net increase in reducing sugars. In agreement with this interpretation, HPLC indicated low free glucose levels and a slight decrease from 0 h to 8 h (0.64 ± 0.03 mg·g^{-1} at 0 h and 0.42 ± 0.05 mg·g^{-1} at 8 h), suggesting limited monomer accumulation and potential uptake during fermentation. The same behaviour was previously observed by Amorim et al., (2018) during BSG fermentation for AXOS production with *B. subtilis*. In contrast, Liu et al., (2022) did not observe this behaviour during RS production by recombinant *Escherichia coli* in a one-step wheat bran fermentation for XOS production, which is consistent with the fact that metabolic profiles are strongly influenced by the microorganism used, the genetic engineering strategy, the substrate and the specific process conditions. The results shown in Fig. 1, suggest that CP presents the highest potential for OS production by achieving a higher Y_{RSmax} (118 ± 1 mg·g^{-1}) at 8 h when compared to CSS (94 ± 3 mg·g^{-1} at 8 h). Furthermore, CP showed a significantly higher maximum fermentation productivity (Pmax) than CSS (7.4 ± 0.2 vs 6.5 ± 0.2 mg·g^{-1}·h^{-1}, respectively; $p < 0.05$; Table S1, Supplementary material).Despite its slightly higher insoluble lignin content (Table 1), CP likely provides improved substrate accessibility due to the structural alterations induced by pelletisation, along with its higher protein content (Pua et al., 2020). Based on the screening results discussed above, CP was selected for further studies.

3.2.2. Sterilisation effect

Sterilisation is essential for fermentation consistency, but when performed by autoclaving, it may also act as a mild hydrothermal treatment, altering the substrate and releasing compounds that can affect microbial activity (Güleç et al., 2021; Wells et al., 2020). To assess its impact on fermentation, two sterilisation methods were compared: (i) a CP–medium mixture sterilised by autoclaving, and (ii) UV-sterilised CP combined with filter-sterile medium. Two controls using both sterilisation methods in absence of microorganism were also included.

Fig. 1 illustrates the changes in Y_{RS} over time during the single-step fermentation of CP under two sterilisation methods. As expected, both methods produced similar Y_{RS} trends, with values increasing from 0 to 8 h (optimal time) and decreasing thereafter (Fig. 1). Despite that, sterilising by autoclave resulted in a higher Y_{RS} (59 ± 5 mg·g^{-1}) at 0 h, likely due to the solubilisation of CP carbohydrates into the medium. In contrast, UV sterilisation led to a basal value of Y_{RS} at 0 h (3.3 ± 0.2 mg·g^{-1}), indicating that this treatment does not significantly solubilise carbohydrate fractions. The controls in absence of microorganism present Y_{RS} values at 0 h comparable to their corresponding

fermentation condition, e.g. 59 ± 5 mg·g^{-1} autoclave *vs.* 59 ± 3 mg·g^{-1} autoclave control, that the initial release of sugars stems from autoclaving, not microbial activity. Expectably, the Y_{RS} profiles from both controls remained constant over time (Fig. 1), excluding the hypothesis of sugar released caused by the incubation process itself. This evidence demonstrates that the variations in Y_{RS} values in fermented CP arises from microbial activity. Although the Y_{RS} profile trends for fermented CP suggest that the microorganism's metabolism behaves similarly regardless of the sterilisation method used, autoclave sterilisation resulted in superior process performance, resulting in significantly higher values of Y_{RSmax} and P_{max} when compared to UV-treated CP ($p < 0.05$). In particular, the Y_{RSmax} obtained with autoclave (118 ± 1 mg·g^{-1}) was 2.6 times higher than that observed under UV condition (Table S1, Supplementary material). These results suggest that sterilisation by autoclave provides improved substrate accessibility and microbial conversion efficiency, therefore it was selected for further studies.

3.2.3. *Enzymatic hydrolysis of coffee silverskin pellets*

Considering (a) the chemical composition of CP, particularly its contents in pectic polysaccharides and xylans (Table 1); (b) its higher xylan content (17.6 ± 0.6% w/w, Table 1) compared with the BSG (10.3 ± 0.2% w/w) used by Amorim et al., (2018) to produce AXOS; (c) the predominant production of POS, together with GXOS, reported by Cordeiro et al., (2025) through direct fermentation of olive stones with a pectic polysaccharides content (1.7 ± 0.1% (w/w)) similar to that of CP (2.1 ± 0.4% (w/w), Table 1); and (d) the hypothesis proposed by the same authors that microbial xylanase and pectinase may act synergistically even at low pectin levels, although this hypothesis was not experimentally tested — building on these considerations, the potential xylanase-pectinase synergy acting on CP for carbohydrate-based mixtures production was therefore evaluated in this study using commercial pectinase and xylanase, either individually or in combination, in a single-step enzymatic hydrolysis of CP (Figs. 2 and 3).

Fig. 2 shows distinct Y_{RS} profiles reflecting the different enzymatic activity roles during CP hydrolysis: xylanase alone presented a similar Y_{RS} profile to the control (absence of enzymes), whereas pectinase led to a significant increase of the Y_{RS} values (0–12 h) when compared to the control, suggesting that CP pectic polysaccharides are being hydrolysed into POS. Nevertheless, the combined use of pectinase and xylanase resulted in overall higher Y_{RS} values than pectinase alone, particularly at optimal time (12 h), reaching a Y_{RSmax} of 141 ± 6 mg·g^{-1} , while pectinase alone achieved 122 ± 2 mg·g^{-1} (Fig. 2, Table S2, Supplementary material). These results suggest a synergy between xylanase and pectinase activities, xylanase may increase accessibility of pectic polysaccharides to pectinase by breaking xylan–lignin and xylan–pectin

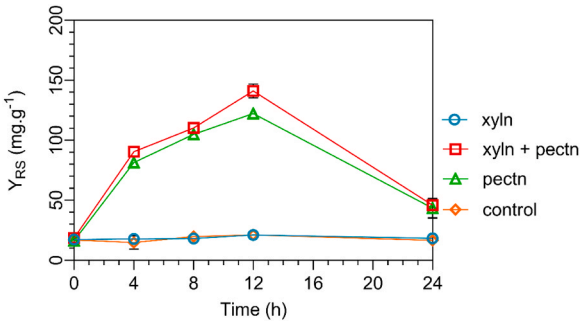

Fig. 2. Reducing sugar production yield (Y_{RS}) obtained from the enzymatic hydrolysis of 20 g·L^{-1} of coffee silverskin pellets (CP) in 0.1 M sodium acetate buffer (pH 4.5), at 40°C, and 150 rpm, using 1 U·mL^{-1} of commercial pectinase from Aspergillus aculeatus (Δ); of commercial xylanase from Aspergillus oryzae (○); and of both enzymes in combination (□). A control without enzymes (◊) was included. Results represent the average of two independent assays ± standard deviation.

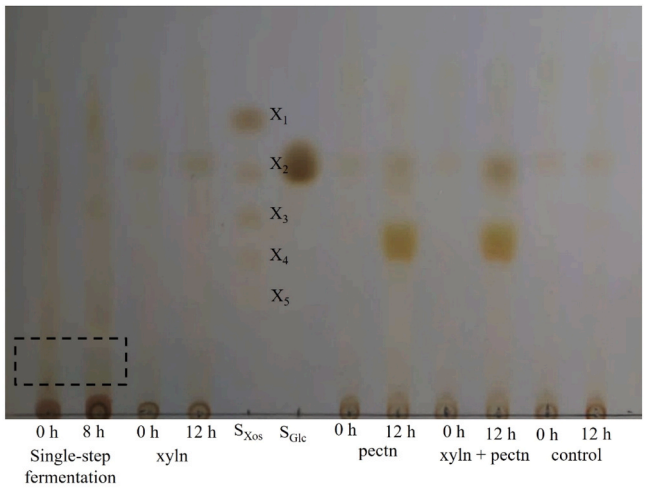

Fig. 3. Qualitative analysis of the supernatants obtained at 0 h and 12 h (optimal time) during enzymatic hydrolysis of 20 g·L^{-1} CP by 1 U·mL^{-1} commercial pectinase from Aspergillus aculeatus (pectn); commercial xylanase from Aspergillus oryzae (xyln); and of both enzymes in combination (xyln+pectn). A control without enzymes was included (control), as well as Glucose (Glc, 2 g·L^{-1}) and a standard mixture (S) containing 2 g·L^{-1} of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5). Mobile phase consisted in butanol:acetic acid:water (2:1:1, v/v/v).

associations in CP cell walls and by reducing steric hindrance around pectin-rich regions, where pectinases de-esterify and depolymerise pectic domains (Li et al., 2014; Kaul et al., 2024).

The TLC qualitative analysis of the supernatants collected at 0 and 12 h during the enzymatic hydrolysis of CP (Fig. 3) corroborates the RS profiles obtained by DNS (Fig. 2). At 12 h, an intense band in the DP 3–4 region was observed only for the assays with pectinase alone or combined with xylanase (Fig. 3), confirming OS formation. Furthermore, at this time a clear band in the glucose region appeared, particularly in the xylanase+pectinase assay (Fig. 3), in agreement with HPLC data showing the presence of monosaccharides, namely 5.4 ± 0.4 mg·g^{-1} of glucose. This highlights a drawback of enzymatic hydrolysis, which generates a higher content of undesired monosaccharides than fermentation, where these sugars are consumed by the microorganism for biomass growth. By contrast, TLC analysis of the supernatants collected during CP fermentation showed only a diffuse band at 8 h in the DP > 5 region (Fig. 3). These results highlight the advantage of using microbial systems that express multiple native enzymes acting synergistically, enabling direct substrate utilisation without the need for prior pretreatment. Single-step fermentation may contribute to in situ enzymatic synergy, where pectinolytic activities act together with xylanases/cellulases to loosen the cell-wall network and improve substrate accessibility, which may contribute to the mobilisation of uronic-acid–rich oligosaccharide structures.

3.2.4. *One-factor-at-a-time screening*

A one-factor-at-a-time screening was performed to evaluate the individual impact of key process parameters (CP concentration, pH, and temperature) on production of carbohydrate-based mixtures via single-step fermentation. The Y_{RS} profiles obtained during the sequential optimisation of each tested variable are presented in Figs. 4–6. The effect of CP concentration on the fermentation performance over time was evaluated within a 5–60 g·L^{-1} range at pH 7.0 and 45°C, and the results are shown in Fig. 4.

For all the tested CP concentrations, comparable profiles were observed, suggesting a consistent two-step metabolism by *B. subtilis*, with Y_{RS} increasing up to the optimal time (8 h), followed by a slight decrease thereafter (Fig. 4). Despite this similar evolution overtime, significant differences in the Y_{RS} values were observed across the tested

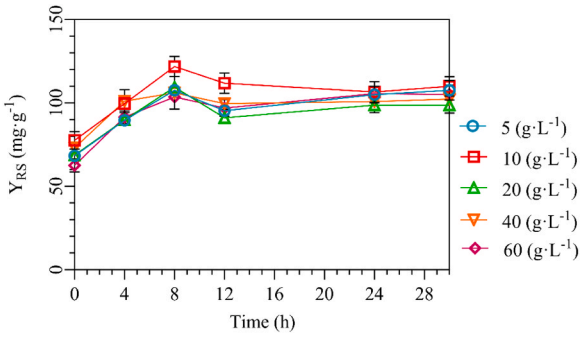

Fig. 4. Reducing sugar production yield (Y_{RS}) for the concentration optimisation of substrate obtained from the single-step fermentation of 5 (○), 10 (□), 20 (Δ), 40 (▽) and 60 (◇) g·L^{-1} of coffee silverskin pellets by Bacillus subtilis in 2% (v/v) Vogel medium at pH 7.0, 45°C, and 150 rpm. Results represent the average of two independent assays ± standard deviation.

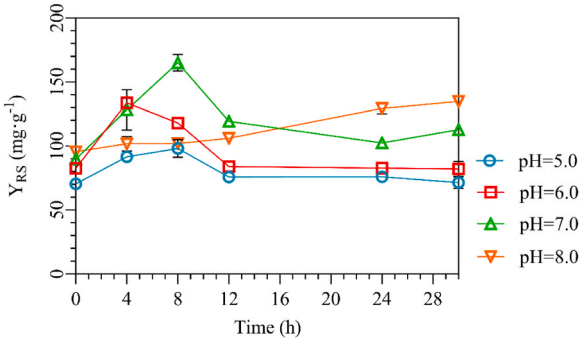

Fig. 5. Reducing sugar production yield (Y_{RS}) for the pH optimisation obtained from the single-step fermentation of 10 g·L^{-1} of coffee silverskin pellets by Bacillus subtilis in 2% (v/v) Vogel medium at 45°C, and 150 rpm. Results represent the average of two independent assays ± standard deviation.

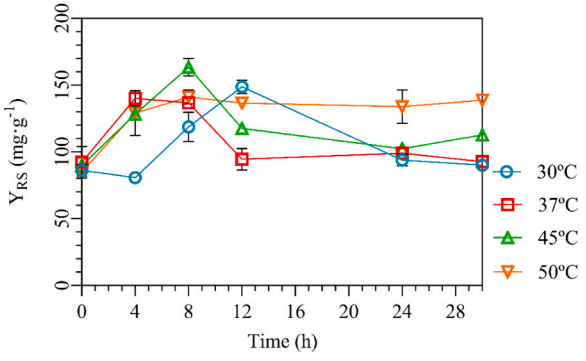

Fig. 6. Reducing sugar production yield (Y_{RS}) for the temperature optimisation obtained from the single-step fermentation of 10 g·L^{-1} of coffee silverskin pellets by Bacillus subtilis in 2% (v/v) Vogel medium at 30 (○), 37 (□), 45 (Δ), and 50°C (▽), pH 7.0, and 150 rpm. Results represent the average of two independent assays ± standard deviation.

substrate concentrations, of particular relevance at the start of fermentation (0 h) and at the optimal time, 8 h, (Table S3, Supplementary material). As expected, increasing the CP concentration led to an increase in RS released during the autoclave sterilization (Section 3.2.2), with values at 0 h ranging from 0.34 ± 0.01 g·L^{-1} at 5 g_{CP}·L^{-1} to 6 ± 1 g·L^{-1} at 60 g_{CP}·L^{-1} (Table S3, Supplementary material). Nevertheless, increasing the CP concentration did not result in a proportional increase in RS at the optimal time (8 h). The highest Y_{RSmax} (61 ± 3 mg·g^{-1}) and P_{max} (5.5 ± 0.3 mg·g^{-1}·h^{-1}) values were obtained with 10 g·L^{-1} CP (Table S4, Supplementary material). On one hand, higher CP concentrations may have caused mass-transfer and aeration limitations, reflected in lower Y_{RSmax} values, likely due to the increased viscosity and density of the mixture at higher substrate loads (Figueiredo et al., 2017; Helianti et al., 2016; Moteshafi et al., 2016). On the other hand, the initial higher RS content (0 h) resulting from increased CP concentrations may have contributed to inhibition by the substrate for the microbial metabolism. Regarding the yield of free monosaccharides at optimal time, Y_{FM}, the minimum quantifiable value obtained was observed for 10 g·L^{-1} of CP (0.9 ± 0.2 mg·g^{-1}), while superior CP concentrations resulted in higher Y_{FM} values, with 60 g·L^{-1} of CP reaching 1.63 ± 0.05 mg·g^{-1} (Table S3, Supplementary material). Collectively, these results indicate that 10 g·L^{-1} is the optimal substrate concentration, balancing efficiency in RS production and process productivity, while avoiding generation of undesired free monosaccharides and inhibition by the substrate and process limitations caused by higher solids loading.

Environmental factors such as pH and incubation temperature can significantly influence bacterial growth, enzyme production and activity (Kallel et al., 2015; Ho and Heng, 2014). To investigate the impact of the initial pH on fermentation performance, a pH range of 5.0–8.0 was tested at 45°C, using 10 g·L^{-1} CP (previously determined as optimal concentration, Fig. 4 and Table S3, Supplementary material). Fig. 5 presents the Y_{RS} values obtained overtime and Table S3 (Supplementary material) summarises the key process performance indicators (optimal time, Y_{RSmax}, P_{max} and Y_{FM}).

In contrast to the optimisation of CP concentration (Fig. 4), varying the initial pH produced distinct Y_{RS} profiles (Fig. 5), indicating that pH exerts a stronger influence on process modulation. Although alkaline conditions were more favourable for sugar extraction during autoclave processing, as reflected by the highest Y_{RS} at 0 h (1.1 ± 0.2 mg·g^{-1}) for pH 8.0, which could initially promote microbial growth, the overall process performance appeared to decline under this condition, Fig. 5 and Table S3 (Supplementary material). At pH 8.0 the optimal time shifted significantly to 24 h, resulting in the lowest $P_{\Delta Y}$ value among all tested conditions (1.4 ± 0.3 mg·g^{-1}·h^{-1}, Table S3, Supplementary material). However, the highest Y_{RSmax} (165 ± 1 mg·g^{-1} at 8 h) was observed for pH 7.0, Table S3 (Supplementary material). These results reinforce the high impact of pH variation on process performance.

The optimal pH implies a compromise between the pH conditions more favourable for sugars extraction during sterilization (alkaline conditions), *B. subtilis* growth (in minimal medium is usually optimal at pH 7.0 (Jaacks et al., 1989), enzyme production and activity (for pectinases and xylanases the optimal range is comprised between pH 4.0 and 6.0 (Grange et al., 1996; Shrestha et al., 2022; Yardimci and Cekmecelioglu, 2018). Thus, it is consistent that the best-performing conditions in this study were pH 6.0 and pH 7.0 (Fig. 5; Table S3, Supplementary material). When comparing the two conditions, pH 6.0 yielded the highest P_{max} value (13 ± 1 mg·g^{-1}·h^{-1}, Table S3 Supplementary material), owing to its shorter optimal time (4 h). However, the corresponding Y_{RSmax} was significantly lower ($p < 0.05$) than that obtained at pH 7.0, with values of 134 ± 2 mg·g^{-1} and 165 ± 1 mg·g^{-1} at 8 h, respectively. Regarding Y_{FM} (0.67 ± 0.04 mg·g^{-1} at pH 6.0 and 1.1 ± 0.1 mg·g^{-1} at pH 7.0), both conditions resulted in low levels of undesired free sugars (Table S3, Supplementary material). Considering the crucial importance of achieving a competitive product titre for future industrial application, pH 7.0 was selected as the optimal condition for further studies.

Several studies have also identified pH 7.0 as optimal for pectinase and xylanase production by *Bacillus* spp. Shrestha et al., (2022), reported the highest xylanase and pectinase activities at pH 7.0 using 1% (w/v) orange peel. Swain and Ray., (2010), likewise found pH 7.0 to be optimal for the production and activity of a thermostable exo-polygalacturonase (a pectinase) by *B. subtilis* CM5. The same optimal pH was also reported by Pandey and Gupta (2023), for xylanase production by *B. subtilis* in submerged fermentation of different

substrates, and by Yardimci et al. (2018), who used co-cultures of *B. subtilis* and *Kluyveromyces marxianus* to ferment hazelnut shells for xylanase production. On the other hand, Liu et al., (2022) reported an optimal pH of 7.98 for recombinant *E. coli* harbouring an alkaline xylanase for XOS production from wheat bran, leveraging the favourable effect on sugar extraction of alkaline conditions.

Temperature is also a critical parameter influencing both microbial metabolism and the catalytic efficiency of enzymes, thereby directly affecting overall process performance. Fig. 6 shows the Y_{RS} values obtained under different temperatures (30, 37, 45, and 50°C) during single-step fermentation performed at pH 7.0 with 10 g·L^{-1} of CP. As expected, for all the tested temperatures, the Y_{RS} at 0 h did not differ significantly ($p > 0.05$) Table S3 (Supplementary material), however variations in the Y_{RS} profiles appear overtime Fig. 6. At 30°C, the production and accumulation of RS occurred 4 h later than in the other conditions, resulting in the highest optimal time (12 h) and the lowest corresponding P_{max} (5.2 ± 0.2 mg·g^{-1}·h^{-1}). Although *B. subtilis* grows optimally between 30 and 37°C (Korsten and Cook, 1996), *Bacillus* sp., pectinases, and xylanases exhibit maximal activity between 37 and 60°C (Haile and Ayele, 2022; Phukon et al., 2024; Shrestha et al., 2022), which may possibly explain the limited fermentation performance observed at 30 °C. At this temperature, during the first 0–4 h, the microbial metabolism is likely directed toward cell division, resulting in a decrease in Y_{RS} due to the consumption of sugars released during sterilisation (Y_{RS} at 0 h), Fig. 6. In contrast, at temperatures above 30°C, the microbial metabolism may possibly be partitioned between cellular growth and the production and activity of hydrolytic enzymes, resulting in an increase of the Y_{RS} at an initial stage of fermentation (0–4 h), Fig. 6.

However, at 50°C, a significant decrease in Y_{RSmax} (141 ± 13 mg·g^{-1} at 8 h) was observed compared with 45°C (163 ± 13 mg·g^{-1} at 8 h; $p < 0.05$), which corresponded to the highest yield obtained in this assay, Table S3 (Supplementary material). This reduction suggests that 50°C may negatively affect the balance between the optimal temperatures required for biomass growth and for enzyme production and activity. When comparing the 37°C and 45°C conditions, the highest P_{max} was obtained at 37°C (15 ± 3 mg·g^{-1}·h^{-1} at 4 h *vs.* 9 ± 1 mg·g^{-1}·h^{-1} at 8 h). However, the Y_{RSmax} achieved at 45°C was approximately 1.2-fold higher ($p < 0.05$), Table S3. (Supplementary material) the importance of attaining a competitive product titre, together with the improved mixing, enhanced substrate dissolution, and reduced risk of contamination by fast-growing mesophilic microorganisms, 45°C was selected for subsequent studies.

Remarkably, after variable-by-variable optimisation the single-step fermentation of CP yielded a significantly higher Y_{RSmax} (163 ± 13 mg.g^{-1} at 8 h; $p < 0.05$) than the one obtained by direct enzymatic hydrolysis with the simultaneous use of commercial pectinase and xylanase at 1 U·mL^{-1} (141 ± 6 mg·g^{-1} at 12 h, section 3.2.3, Table S2, Supplementary material). Plus, resulted in a residual production of monosaccharides (0.87 ± 0.01 mg·g^{-1}, Table S3 Supplementary material) compared with the enzymatic hydrolysis (8.2 ± 0.1 mg·g^{-1}, Table S2, Supplementary material), suggesting that this production approach may be competitive from an industrial standpoint. Furthermore, CP used in this study exhibited greater potential than olive stones to produce carbohydrate-based mixtures via single-step fermentation, achieving a Y_{RS} of 60 ± 3 mg·g^{-1} at 12 h under optimal conditions (Cordeiro et al., 2025). These findings suggest the potential robustness and adaptability of single-step fermentation for OS production, enabling the valorisation of different agro-industrial by-products.

3.3. Quantification and chemical characterisation of the carbohydrate-based mixture produced by single-step fermentation

The supernatant collected at the optimal time of CP fermentation under the conditions selected in Section 3.2.4 was partially purified with activated charcoal (Section 2.8.4) to eliminate salt interference in the characterisation of the resulting carbohydrate-based mixture, leading to a total sugars' (TS) loss of approximately 33%. This mixture was composed by 355.2 μg·mg^{-1} of carbohydrates, being uronic acids the predominant sugar residue (78.8%mol), followed by arabinose (6.1% mol), galactose (4.4%mol), rhamnose (3.7%mol), glucose (2.9%mol), xylose (2.3%mol), and mannose (1.6%mol). The nature of glycosidic linkages of the carbohydrates was determined performing a methylation analysis using three different methods: a methylation analysis with a dialysis with a cut off membrane of 0.5 kDa and 1 kDa, and without performing the dialysis step, a liquid-liquid extraction step was done instead of the dialysis (Table 2).

The different approaches provide information about the molecular weight of the carbohydrates (0.5 kDa and 1 kDa) and allows to infer if the carbohydrates have uronic acids on their structure, since with the methodology without dialysis, the acidic structures are separated from the neutral structures during the liquid-liquid extraction step and only the carbohydrates which structures are not linked to uronic acids will be analysed. The glycosidic linkage composition of the OS mixture produced has not been yet reported. The glycosidic linkage analysis shows that the major differences between the three methodologies is mostly related with the total amount of galactose, higher using the methylation methodologies with dialysis (37.3–57.5%), and with the total amount of arabinose, mannose, and glucose, higher using the methylation methodologies with no dialysis (22.2%, 16.6%, and 12.2%, respectively).

The significant decrease of 4-Gal*p* in the methylation analysis with no dialysis, as well as the decrease of the majority of Gal residues, indicates that these residues may be part of acidic carbohydrates that were dissolved in the aqueous phase during the liquid-liquid extraction step of the methylation procedure. Indeed, the higher amount of 4-Gal*p*, mainly

Table 2
Glycosidic linkage analysis of carbohydrate-based mixture produced by single-step fermentation of coffee silverskin pellets from methylation procedure using dialysis (0.5 kDa and 1 kDa cut off membranes) and without dialysis.

Glycosyl linkage	0.5 kDa	RSD	1 kDa	RSD	No Dialysis	RSD
2-Rha*p*	2.8	2	11.1	18	6.2	33
3-Rha*p*	0.4	6	1.0	28	0.6	15
2,4-Rha*p*	5.8	9	7.0	13	n.d.	n.d.
Total	**9.0**		**19.1**		**6.8**	
t-Fuc*p*	1.7	6	3.3	14	3.2	14
Total	**1.7**		**3.3**		**3.2**	
t-Ara*f*	6.8	4	11.7	11	10.1	17
2-Ara*f*	0.7	7	1.4	24	3.2	6
3-Ara*f*	0.8	3	1.4	28	2.8	2
5-Ara*f*	2.1	8	2.8	6	4.7	13
3,5-Araf	0.4	11	0.6	15	1.4	3
Total	**10.8**		**17.9**		**22.2**	
t-Xyl*p*	3.9	2	8.3	10	9.5	15
4-Xyl*p*	9.7	8	3.5	20	4.4	6
2,3-Xyl*p*	0.6	9	2.2	9	1.0	13
3,4-Xyl*p*	0.5	13	1.6	38	4.5	4
Total	**14.7**		**15.6**		**19.4**	
t-Man*p*	3.1	1	2.0	12	10.8	15
2-Man*p*	n.d.	n.d.	n.d.	n.d.	2.4	5
6-Man*p*	n.d.	n.d.	n.d.	n.d.	3.4	5
Total	**3.1**		**2.0**		**16.6**	
t-Gal*p*	3.1	0	6.0	12	5.3	10
2-Gal*p*	1.1	14	0.9	9	n.d.	n.d.
3-Gal*p*	1.7	4	2.7	2	1.0	16
4-Gal*p*	45.1	5	16.1	42	6.3	28
6-Gal*p*	2.2	4	4.2	13	3.0	15
2,4-Gal*p*	0.7	6	2.1	1	n.d.	n.d.
3,4-Gal*p*	1.7	6	2.8	8	1.9	34
3,6-Gal*p*	1.9	0	2.5	12	2.1	8
Total	**57.5**		**37.3**		**19.6**	
t-Glc*p*	1.8	2	3.2	3	6.1	0
2-Glc*p*	n.d.	n.d.	n.d.	n.d.	2.4	14
4-Glc*p*	n.d.	n.d.	n.d.	n.d.	1.8	7
4,6-Glc*p*	1.3	5	1.5	22	1.9	6
Total	**3**		**4.7**		**12.2**	

n.d.: not detected

in the method using dialysis with 0.5 kDa (45.1%) but also in 1 kDa (16.1%) cut off, as well as the presence of 2-Rha*p* (2.8–11.1%) and 2,4-Rha*p* (5.8–7%), are in agreement with the presence of pectic polysaccharides, namely type I rhamnogalacturonan (RG-I), containing repeating units of 1,2-α-L-Rha*p*-(1,4)-α-D-Gal*p*A as backbone (Cardoso et al., 2002). These polysaccharides have side chains such as galactan, arabinogalactan, and arabinan. The higher relative amount of 4-Gal*p* in methylation analysis with dialysis agrees with the presence of galactan side chain that consists of (β1→4)-Gal*p* residues. 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 4.7% of 5-Ara*f* and 1.4% 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 relative amount of these acidic polysaccharides decreased. Thus, the OS mixture produced is mainly composed by pectic related carbohydrates (78.8% mol of uronic acids). Those uronic acids could also be part of homogalacturonan pectic polysaccharides, which presence was not possible to infer from methylation analysis, since the reduction of the carboxylic group was not performed. Xylose residues are in relative higher abundance using the methylation method without dialysis, with indicates that are part of neutral oligo/polysaccharides. Indeed, the occurrence of 4,6-Glc*p*, together with 4-Glc*p*, t-Fuc*p* and t-Xyl*p* could indicate the presence of xyloglucans (XG) (Silva et al., 2024).

The OS mixture produced was analysed via HPAEC-PAD Fig. 7, to evaluate the carbohydrate profile. Moreover, 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.

Fig. 7 shows a region of neutral mono- and OS in the beginning of the chromatogram, possible corresponding to the XG, and a region of charged OS, possible corresponding to POS, since they have the same retention time as OS produced from the partial hydrolysis of polygalacturonic acid. The xylanase possibly was able to destructure the cell tissues and the polysaccharides, such as pectic polysaccharides, are more available to be extracted to culture broth. An analysis of neutral mono- and OS (DP1-DP5) of fermented CP was performed by GC-MS (Fig. 8), and it is composed by 6.5 μg·mg^{-1} of these carbohydrates (DP1-DP3).

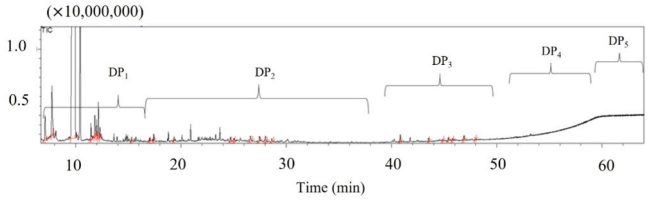

Fig. 8. Chromatogram of the oligosaccharides profile of the carbohydrate-based mixture produced from coffee silverskin pellets sample (DP1-DP5) analysed by GC-MS.

It was observed the presence of, arabinose, mannose, glucose, and galactose. Moreover, possible apiose other was also detected, characteristic of rhamnogalacturonan type II, a highly branched polysaccharide with a backbone of (1α→4)-D-Gal*p*A units substituted by four heteropolymeric side chains containing eleven different sugar residues (Coimbra et al., 1994). Despite of the low amount, it was possible to detect 9 different pentoses and hexoses disaccharides, and 7 different trisaccharides.

The concentration of carbohydrates in the purified mixture obtained herein (355.2 μg·mg^{-1}) corresponds to a yield of 145 mg of carbohydrate-based OS per g of CP. When compared with other sustainable methods for production of OS with possible prebiotic effects (Table S4, Supplementary material), this yield is higher than the range reported for single-step fermentation of agro-residues (38–60 mg·g^{-1}) (Amorim et al., 2018, 2019; Liu et al., 2022; Cordeiro et al., 2025) highlighting its promising potential to compete with traditional enzymatic hydrolysis processes (98–226 mg·g^{-1}) (Embaby et al., 2016; Babbar et al., 2017; Orrego et al., 2024). Furthermore, the supernatant purification protocol used in this study (2.8.4.) still offers room for optimisation, which could further improve both the quantification and structural characterisation of the resulting mixture.

Overall, the recovered product corresponds to an oligosaccharide-rich carbohydrate mixture containing 355.2 μg·mg^{-1} total carbohydrates, dominated by an acidic/uronic-acid fraction (78.8 mol% uronic acids), followed by arabinose (6.1 mol%), galactose (4.4 mol%), rhamnose (3.7 mol%), glucose (2.9 mol%), xylose (2.3 mol%) and mannose (1.6 mol%). Glycosidic linkage analysis (Table 2) supports the presence of pectic polysaccharide-associated structures, with RG-I signatures (e.g., 2-Rha/2,4-Rha and arabinan/galactan-type side-chain structural

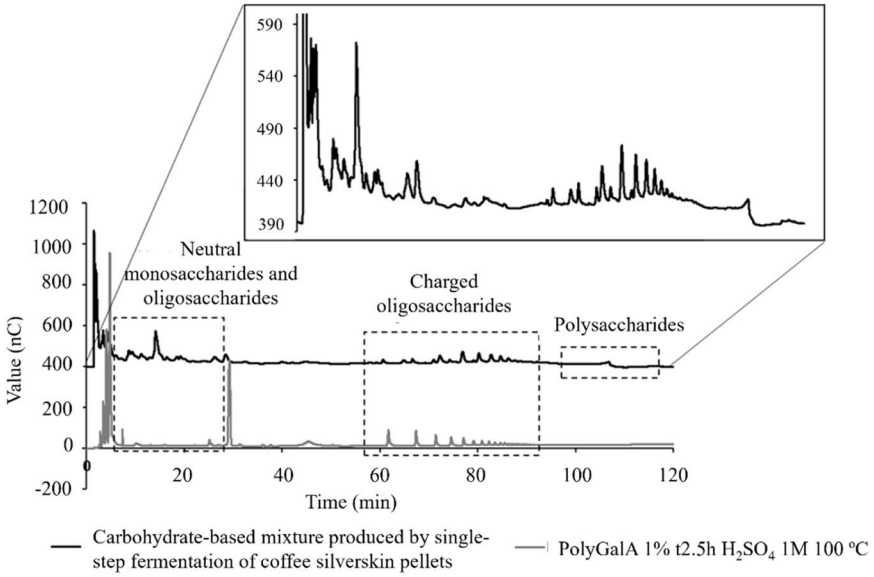

Fig. 7. Carbohydrate-based mixture produced from coffee silverskin pellets (black line) and hydrolysed polygalacturonic acid (grey line) analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

signatures), together with a neutral fraction consistent with xyloglucan-related carbohydrates. Consistently, the HPAEC-PAD profile (Fig. 7) shows an early region associated with neutral carbohydrates and a later region enriched in charged oligosaccharides aligned with a polygalacturonic-acid hydrolysate reference, supporting the coexistence of neutral and acidic (POS-rich) components within the final mixture. Consistently, the HPAEC-PAD profile (Fig. 7) shows an early region associated with neutral carbohydrates and a later region enriched in charged oligosaccharides aligned with a polygalacturonic acid hydrolysate reference, supporting the coexistence of neutral and acidic (POS-rich) components within the final mixture.

3.4. Single-step fermentation of coffee silverskin pellets in bioreactor

B. subtilis is a fast-growing aerobic microorganism, and its metabolism imposes high oxygen demands during active fermentation (Helianti et al., 2016). In industrial settings, oxygen transfer often becomes a limiting factor, constraining process performance and requiring controlled aeration and appropriate bioreactor design. To address these considerations and evaluate the scalability of the process, single-step fermentation of CP was conducted under aerated bioreactor conditions, using the optimal conditions determined for shake-flask (Section 3.2.4): 10 $g{\cdot}L^{-1}$ of CP, 45°C and pH 7.0, Fig. 9.

The Y_{RS} profile obtained in the bioreactor experiments was comparable to that observed at flask scale, exhibiting the same two-step metabolic behaviour of *B. subtilis*: during the first 0–4 h, the rate of RS production exceeded its consumption, whereas after the optimal time (8 h) RS consumption became predominant (Fig. 9). Additionally, *in situ* autoclave sterilisation of the fermentation medium in the bioreactor did not result in significant differences in sugar release, as indicated by comparable Y_{RS} at 0 h ($p > 0.05$), Fig. 9 and Table S5 (Supplementary material).

Although the optimized bioreactor process reached 1.62 $g{\cdot}L^{-1}$ of RS at 8 h, this value should be interpreted cautiously, since RS is a screening metric that reflects total soluble carbohydrates with reducing ends and does not directly quantify specific OS. In this work, the process was operated at 10 $g{\cdot}L^{-1}$ CP, selected as the best compromise between fermentation performance, low free monosaccharide formation and process operability, since higher solids loading did not improve yields proportionally and likely imposed mass-transfer/aeration limitations. Importantly, after partial purification, the process yielded 145 mg of carbohydrate mixture per g of CP, which compares favourably with other single-step fermentation studies summarized in Table S5. Therefore, while the current RS concentration remains modest and requires further improvement for industrial implementation, the results demonstrate the feasibility of producing a structurally complex carbohydrate-based mixture from CP by single-step fermentation. From an industrial perspective, this may represent a critical limitation, as

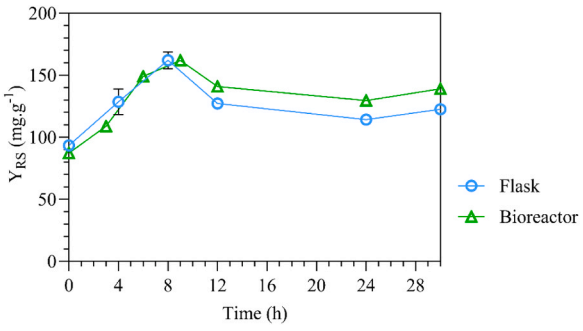

Fig. 9. Reducing sugar production yield (Y_{RS}) for the single-step fermentation of coffee silverskin pellets (CP) by Bacillus subtilis in 2% (v/v) Vogel using 10 g. L^{-1} of CP at 45°C and pH 7.0 at flask scale (○), and with aeration (Δ). Results represent the average of two independent assays ± standard deviation.

sterilisation performance changes markedly with scale due to heat transfer constraints, and our results (Section 3.2.2) show that the sugars released during sterilisation are important to support initial microbial growth. However, this drawback could, if necessary, be mitigated by supplementing the medium with an additional rapidly consumed carbon source. Furthermore, air supply at industrial scale is likely to be a critical process parameter, as sufficient oxygen must be delivered to meet, and not limit, the microorganism's demand. In fact, in a preliminary assay performed in a 2 L DASGIP Parallel Bioreactor System (Eppendorf, Hamburg, Germany) at 200 rpm and without air supply, the optimal time increased to 12 h and Y_{RSmax} and P_{max} decreased by approximately 1.3- and 4-fold, respectively compared with the aerated condition described in Table S5. Industrially, the oxygen demand challenge can be addressed through strategies such as optimising impeller type and agitation speed to increase the volumetric mass transfer coefficient for oxygen, using more efficient sparger designs, operating at moderate overpressure, or applying oxygen-enriched air or pure oxygen at peak demand. In addition, dissolved oxygen cascade control, staged aeration along the reactor height, or the use of multiple smaller, well-aerated reactors in parallel can help maintain adequate oxygen transfer. The aerated bioreactor achieved similar performance to the flask assay in terms of Y_{RSmax} (162 ± 2 $mg{\cdot}g^{-1}$) and P_{max} (9.3 ± 0.1 $mg{\cdot}g^{-1}{\cdot}h^{-1}$), $p > 0.05$ (Table S5). This confirms the reproducibility and potential scalability of the process under aerated conditions. Remarkably, Y_{FM} was approximately 20-fold higher in the enzymatic assay (Y_{FM} = 8.2 $mg{\cdot}g^{-1}$ at 8 h) than in autoclaved CP fermentation (≤0.42–0.80 $mg{\cdot}g^{-1}$ at 8 h), which is consistent with microbial consumption of the released monosaccharides. From a product perspective, Y_{OSmax} achieved by single-step fermentation under optimal flask and reactor conditions was approximately 1.2-fold higher than the enzymatic assay (161.5 and 161.2 $mg{\cdot}g^{-1}$ at 8 h for flask and reactor, respectively, versus 132.8 $mg{\cdot}g^{-1}$ at 12 h), indicating a higher OS-associated RS yield.

4. Conclusion

This work demonstrates that CP are a promising substrate for producing complex carbohydrate-based mixture by single-step submerged fermentation with *B. subtilis* 3610. Under optimised conditions (10 $g{\cdot}L^{-1}$ CP, pH 7.0, 45°C), the process achieved a Y_{RSmax} of 162 ± 7 $mg{\cdot}g^{-1}$, outperforming both coffee silverskin fermentation and combined commercial xylanase–pectinase hydrolysis of CP. The autoclave sterilisation of low-cost liquid basal medium containing CP simultaneously supports microbial growth and promote mild hydrothermal pretreatment, enhancing substrate accessibility. The resulting mixture is enriched in pectic oligosaccharides (78.8% mol uronic acids) with a neutral xyloglucan-derived fraction, offering broad prebiotic potential. Comparable performance in aerated bioreactors confirms process robustness and scalability. Overall, this study positions single-step fermentation of CP as a cost-effective and sustainable strategy for valorising coffee by-products into potential next-generation prebiotic ingredients and provides a solid basis for future structure–function, microbiome, and techno-economic assessments. The production of carbohydrate-based mixture paves the way to future prebiotic assays using these compounds to develop valuable food ingredients.

CRediT authorship contribution statement

Manuel A. Coimbra: Writing – review & editing, Validation, Resources, Methodology. **António M. Peres:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition. **Andreia S. Ferreira:** Writing – original draft, Methodology, Investigation. **Elisabete Coelho:** Writing – review & editing, Validation, Resources, Methodology. **Ana Cordeiro:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Andreia Fernandes:** Methodology, Investigation. **Cláudia Amorim:**

Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Lígia R. Rodrigues:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2026.04.001.

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