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An integrated SSF strategy enables superior valorization of apple pomace into 2,3-butanediol using *Paenibacillus polymyxa*

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A B S T R A C T

The valorization of agro-industrial residues is fundamental to developing a sustainable circular bioeconomy. This study establishes a robust framework for converting apple pomace, an abundant lignocellulosic waste, into the high-value platform chemical 2,3-butanediol (2,3-BDO) using the non-pathogenic bacterium *Paenibacillus polymyxa*. A comprehensive optimization was conducted through the systematic comparison of Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) strategies. Key process parameters, including hydrothermal pretreatment conditions (time and solids loading) and synergistic enzyme loading, were optimized for each configuration. Although the optimized SHF process yielded a higher product concentration (24.2 g/L at 16 % solids), the integrated SSF configuration (21.0 g/L at 11 % solids) demonstrated superior overall process efficiency, yielding 238 kg of 2,3-BDO per ton of dry apple pomace versus 189 kg for SHF. Further optimization of enzyme loading in the SSF process enhanced this yield to 268 kg/ton. To maximize productivity, a fed-batch strategy was implemented for the optimal SSF configuration, which culminated in a final concentration of 32.7 g/L. This corresponded to an overall mass yield of 371 kg of 2,3-BDO per ton of pomace. This work, to our knowledge, represents the first comprehensive optimization of 2,3-BDO production from apple pomace using *P. polymyxa*. It is concluded that the integrated SSF process, despite achieving lower batch titers, represents a more promising route for industrial application due to its operational simplicity and higher material conversion efficiency. These findings provide a critical foundation for advancing circular biorefinery models based on fruit waste.

1. Introduction

Effective management of agro-industrial waste represents a critical environmental challenge within the global food supply chain [1]. These residues, typically high in moisture and chemically unstable, are prone to uncontrolled degradation, which contributes to greenhouse gas emissions and the contamination of soil and water [2]. Fruit and vegetable processing accounts for approximately 14.8 % of agro-food waste, with apple pomace emerging as a notably abundant and underutilized by-product [2–5]. The apple processing industry is growing due to the high worldwide demand for apple juice and cider [4]. Global apple production exceeded 113 million tons, and in the European Union, annual apple production currently stands at approximately 12.2 million tons [6]. Spain is the sixth largest producer in the European Union, harvesting more than 600,000 tons [7]. Depending on its use, 15 % of total apple production is used for cider production [8], so, Spain produces more than 90,000 tons of cider apples. Apple pomace, the primary solid residue from apple juice, cider, and purée production, constitutes 25–30 % of the original fruit mass [9]. Worldwide, apple pomace production exceeds 4 million tons annually [9], with Spain contributing approximately 160,000 tons [4]. In Asturias, over 24,000 tons of apples are fermented each year for cider, underscoring the regional significance of this residue [4].

Owing to its rich composition—comprising fermentable sugars, dietary fiber, pectin, and phenolic compounds—apple pomace offers substantial potential for biotechnological valorization [3,4,10,11]. Current applications include pectin recovery, ethanol production, and organic fertilizer, or animal feed, among others [1]. Among the potential valorization pathways, microbial synthesis of 2,3-butanediol (2,3-BDO) stands out as a versatile option with applications across the polymer,

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pharmaceutical, cosmetic, and fuel sectors [12,13]. It functions as a precursor for compounds including 1,3-butadiene, methyl ethyl ketone, and diacetyl, and is employed in the production of inks, perfumes, plasticizers, and antifreeze agents [3,12]. Its high calorific value (27.2 kJ/g), low vapor pressure (0.23 hPa), and freezing point of −60 °C further reinforce its suitability as a biofuel or fuel additive [13]. With global market demand estimated at 32 million tons per year [14] and a current price of approximately $3.23/kg [13], the development of cost-effective and sustainable bioprocesses for 2,3-BDO production is of growing industrial interest.

Fermentative synthesis of 2,3-BDO from renewable substrates has emerged as a viable alternative to petrochemical routes [13]. In recent years, the number of studies on the microbial conversion of renewable sugars from waste into 2,3-BDO has increased [13,15–18]. Although bacterial genera such as *Klebsiella*, *Enterobacter*, and *Serratia* exhibit high conversion efficiencies, their classification as biosafety level 2 organisms restricts industrial use due to potential pathogenicity [12,19]. Conversely, *Paenibacillus polymyxa*, a GRAS (Generally Recognized as Safe) microorganism, can produce 2,3-BDO at competitive yields [12]. Previous studies using lignocellulosic hydrolysates—such as poplar wood, wheat straw, corn stover, and switchgrass—have reported 2,3-BDO concentrations ranging from 18 to 32 g/L, although often under restrictive conditions or with limited substrate conversion [20–22]. Investigations using fruit and vegetable waste, including banana and red beet residues, have achieved yields between 0.4 and 0.5 g/g [16,17]. Nonetheless, challenges persist in optimizing fermentation conditions, mitigating inhibitory effects from complex substrates, and enhancing process scalability [12,13,19].

The bioconversion of apple pomace into 2,3-BDO entails several key stages: pretreatment, enzymatic hydrolysis, and fermentation. Pretreatment is essential to increase the accessibility of cellulose and hemicellulose to enzymatic attack, thereby enhancing sugar release [18]. To ensure environmental and economic sustainability, pretreatment strategies must avoid the use of toxic or expensive chemicals [18]. Additionally, sugar concentration must be carefully controlled, as excessively high levels can lead to substrate inhibition and increased medium viscosity, negatively affecting microbial performance [23]. These factors highlight the importance of optimizing each stage of the process to enable efficient and scalable 2,3-BDO production from apple pomace.

This study examines the bioconversion of apple pomace into 2,3-BDO using *Paenibacillus polymyxa*, comparing two process configurations: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). We hypothesize that an integrated SSF approach can offer superior process efficiency compared to SHF, despite potential limitations in substrate loading. To evaluate this, we optimized hydrothermal pretreatment conditions for both configurations, refined the enzymatic hydrolysis step for SSF, and implemented fed-batch feeding to explore productivity limits. This work constitutes the first comprehensive investigation into 2,3-BDO production from apple pomace, offering insights into process optimization, microbial performance, and the valorization of apple pomace from fruit-processing residues within a circular bioeconomy framework.

2. Material and methods

2.1. Raw material

Apple pomace was kindly supplied by the Regional AgriFood Research and Development Service (SERIDA) (Asturias, Spain). The apple pomace was received in vacuum-sealed packages with a moisture content of humidity of 4.5 % for storage. Prior to use, the particle size was reduced to 1–4 mm with a domestic grinder (Taurus, Lérida, Spain).

2.2. Experimental design to pretreatment optimization in autoclave

Based on preliminary fermentation tests (data shown in Supplementary Material Table S1), a hydrothermal pretreatment of the apple pomace in an autoclave was identified as necessary to enhance the production of 2,3-BDO. A key aspect of this study was the use of the entire slurry post-pretreatment, eliminating a solid-liquid separation step. This approach is advantageous for lignocellulosic biomasses that require pretreatment since it allows the use of a single hydrolysate, containing both cellulosic and hemicellulosic sugars, to obtain 2,3-BDO by fermentation, thereby simplifying the process by eliminating a separation stage [24].

The pretreatment of the apple pomace was performed in an autoclave (Model MED-12, Selecta, Spain). Briefly, the raw material was placed into 250 mL Erlenmeyer flasks at the designated solid load (5–15 % w/v, dry matter) for each experiment. Subsequently, water was added to a total volume of 60 mL and the flasks were closed. Thereafter, a thermal treatment in a steam autoclave (at 121 °C) was performed. The heating time was modified according to each experiment (5–60 min). Finally, the resulting pretreated slurry was stored at room temperature. Once it has cooled down to room temperature, the next stage of the process is immediately prepared to produce 2,3-BDO by fermentation.

A central composite experimental design was employed ($\alpha = 1.41$) to determine the optimal experimental conditions to maximize total monosaccharide concentration and 2,3-BDO concentration (in SHF configuration) and only 2,3-BDO concentration (in SSF configuration). Accordingly, a design of 13 experimental runs (Table 1) was performed for each fermentation configuration, SHF and SSF, with 5 replicates at the central point. Pretreatment time in autoclave (10–30 min) and solid load (5–15 % s/L) were chosen as independent variables. Table 1 reports the coded and uncoded values of factors in the experimental design. The design and statistical analysis of the experiments were performed using the commercial software Statgraphics Centurion XIX. The optimal experiments were performed in triplicate, and the average results are shown.

2.3. Saccharification and fermentation

2.3.1. Microorganism and culture conditions

Paenibacillus polymyxa DSM 635, from the German collection of microorganisms (DSMZ, Leibniz, Germany), was used in the 2,3-BDO fermentation. It was maintained and grown according to the protocol described by Ref. [16]. The inoculum was prepared by growing the strain in Häßler medium [25] in a 250 mL Erlenmeyer flask containing 100 mL of the medium, until it reached an optical density of 0.6 using a Uvmini-1240

Table 1
Experimental conditions for optimizing pretreatment variables in autoclave (121 °C) for apple pomace in SHF fermentation (enzymatic hydrolysis of 24 h, Viscozyme 15 FPU/g dry matter, 150 rpm, 50 °C) and SSF fermentation. The coded and uncoded values of the factors in the experimental designs are shown in parentheses.

Run	Pretreatment time (min)	Solid load (% s/L)
1	30 (+1)	5 (−1)
2	10 (−1)	5 (−1)
3	20 (0)	17 (+1.41)
4	10 (−1)	15 (+1)
5	20 (0)	10 (0)
6	6 (−1.41)	10 (0)
7	20 (0)	10 (0)
8	20 (0)	10 (0)
9	20 (0)	3 (−1.41)
10	20 (0)	10 (0)
11	34 (+1.41)	10 (0)
12	20 (0)	10 (0)
13	30 (+1)	15 (+1)

spectrophotometer (Shimadzu, Kyoto, Japan). The inoculum was grown at 37 °C and 200 rpm for 24 h in an orbital shaker (Comecta Optic Ivymen system).

2.3.2. Saccharification and fermentation of pretreated solid through SHF

After the apple pomace pretreatment, the resulting slurry was utilized for enzymatic hydrolysis and subsequent fermentation to 2,3-BDO. The enzymatic hydrolysis was carried out in an orbital shaker (Comecta Optic Ivymen system) at 50 °C, 150 rpm and for 24 h. The enzyme loading used was 15 Filter Paper Units (FPU)/g of pretreated solid, using the pretreated slurry obtained in each experimental run as the substrate. The choice of enzyme loading is based on the data included in the article by Ref. [17]. In this study, several enzyme loads (between 10 and 15 FPU/g of substrate) and two different enzymes (Viscozyme and Cellic CTec 2), individually or mixed, are compared for the enzymatic hydrolysis of a waste product that may be like ours, in this case, banana waste or whole bananas. When using a single enzyme, these authors select an enzyme load of 15 FPU/g of substrate. The aim is to achieve greater saccharification efficiency when using a single enzyme, and, on the other hand, a higher enzyme load may not be economically viable. The Viscozyme enzyme (Novozymes A/S, Denmark) was added directly to the 250 mL Erlenmeyer flasks containing the pretreated slurry, and the initial pH was adjusted to 6 with 1 M NaOH or 1 M HCl. After 24 h, the collected samples (1 mL per sample) were centrifuged and analyzed for monosaccharide content. In addition, enzyme blanks were performed under the same conditions to correct for the monosaccharide content of the commercial enzyme preparation.

In the same Erlenmeyer flasks, fermentation assays were carried out containing a working volume of 75 mL at 37 °C and 200 rpm in an orbital shaker (Comecta Optic Ivymen system). At the beginning of fermentation, the initial pH was adjusted to a value of 6 and was not controlled during fermentation. The inoculum volume was 10 % v/v. Samples were withdrawn at different time points (0, 14, 18, 24, 38, 42, 48, 62 and 134 h), centrifuged (13,400 rpm, 5 min, Mini Spin, Eppendorf, Germany) and their supernatants were analyzed for their content of sugars, 2,3-BDO and by-products.

2.3.3. Saccharification and fermentation of pretreated solids through SSF

The slurry of pretreated apple pomace was subjected to simultaneous saccharification and fermentation (SSF) to produce 2,3-BDO. The fermentation process was conducted at 37 °C and 200 rpm in 250 mL Erlenmeyer flasks. For each substrate loading employed, the Viscozyme enzyme was used at a dose of 15 FPU/g of pretreated substrate. The enzyme and inoculum (10 % v/v) were added at the same time, and the initial pH was adjusted to 6. The samples were taken and processed in the same way as in SHF fermentation. The 2,3-BDO fermentation yield (g/g) was calculated as the ratio of the 2,3-BDO concentration (g/L) to the concentration of sugars (g/L) consumed during fermentation at the time of maximum concentration of 2,3-BDO. In the SSF configuration, the slurry was used directly. The initial sugar concentration was therefore determined from a separate enzymatic hydrolysis performed under identical conditions. The 2,3-BDO productivity (g/L·h) was calculated as the ratio between the 2,3-BDO concentration (g/L) and the fermentation time (h) at which this 2,3-BDO concentration was reached.

2.3.4. Experimental design to enzymatic load optimization in SSF configuration

A central composite design was proposed ($\alpha = 1.41$) to determine the optimal enzyme loading to maximize the final 2,3-BDO concentration obtained. Viscozyme enzyme loading (3–15 FPU/g pretreated solid) and Cellic CTec2 enzyme loading (3-15-FPU/g pretreated solid) were the selected factors (Table 2). The design comprises 13 experimental runs, including one central point with five replicates. Table 2 reports the coded and uncoded values of factors in the experimental design. The experimental design was generated and the data were analyzed using Statgraphics Centurion XIX. The optimal experiment was replicated in triplicate, and the mean results are shown.

2.3.5. Fed-batch fermentation under optimal conditions for SHF and SSF configurations

In fed-batch fermentation, experiments were conducted for both the SHF and SSF configurations, under the respective optimal pretreatment conditions, and with the enzyme load optimized for SSF. The fermentations were carried out as described in the previous sections, but with pulses of concentrated glucose (500 g/L) added into the medium at 24, 48 and 72 h.

2.4. Optimization by Response Surface Methodology

Design of Experiments combined with Response Surface Methodology is a recognized methodology for optimization [26]. In this work, the study employed three Design of Experiments approach employing a Central Composite Design to systematically investigate the effects of process parameters on key response variables. The factorial design generated a total of thirteen experimental runs, each conducted in triplicate to ensure reliability and reproducibility. Additionally, five central points were included as replicates, enabling accurate estimation of pure experimental errors, a critical parameter used to assess model lack of fit. Response Surface Methodology is a mixture of mathematical and statistical techniques that is useful for evaluate the effects of several

Table 2
Experimental conditions for the optimization of enzyme loading (15 FPU/g Viscozyme and 17 FPU/g Cellic CTec2) in SSF fermentation of apple pomace. The coded and uncoded values of the factors in the experimental designs are shown in parentheses.

Run	Viscozyme (FPU/g pretreated solid)	Cellic CTec2 (FPU/g pretreated solid)	2,3-BDO[a] (g/L)	Time[b] (h)	2,3-BDO yield (g/g)	2,3-BDO productivity (g/L·h)	Acetoin[c] (g/L)	Ethanol[c] (g/L)
1	15 (+1)	3 (−1)	15.7 ± 0.3	38	0.38	0.41	4.8 ± 0.3	1.4 ± 0.1
2	9 (0)	9 (0)	16.3 ± 0.2	38	0.40	0.43	4.2 ± 0.1	1.4 ± 0.0
3	9 (0)	9 (0)	16.3 ± 0.2	48	0.35	0.34	9.6 ± 0.3	0.4 ± 0.0
4	9 (0)	9 (0)	19.0 ± 0.4	48	0.39	0.40	7.8 ± 0.1	0.5 ± 0.0
5	9 (0)	0.51 (−1.41)	13.3 ± 0.1	14	0.32	0.95	0.8 ± 0.0	2.3 ± 0.2
6	9 (0)	17.49 (+1.41)	19.4 ± 0.5	48	0.38	0.41	8.0 ± 0.3	0.6 ± 0.0
7	0.51 (−1.41)	9 (0)	14.2 ± 0.3	24	0.35	0.59	2.5 ± 0.1	1.4 ± 0.1
8	3 (−1)	3 (−1)	15.2 ± 0.2	18	0.38	0.84	1.4 ± 0.2	1.7 ± 0.3
9	9 (0)	9 (0)	16.8 ± 0.4	24	0.40	0.70	1.6 ± 0.2	2.1 ± 0.2
10	3 (−1)	15 (+1)	16.7 ± 0.4	24	0.42	0.70	1.0 ± 0.0	1.8 ± 0.1
11	17.49 (+1.41)	9 (0)	17.4 ± 0.2	24	0.39	0.72	1.1 ± 0.1	2.1 ± 0.1
12	15 (+1)	15 (+1)	24.1 ± 0.4	42	0.45	0.57	4.1 ± 0.3	1.9 ± 0.0
13	9 (0)	9 (0)	22.3 ± 0.3	38	0.46	0.59	4.5 ± 0.2	1.3 ± 0.2

[a] 2,3-BDO maximum concentration.
[b] Time for maximum 2,3-BDO concentration.
[c] Acetoin and ethanol concentration at time for maximum 2,3-BDO concentration.

independent variables on the system response without the need of a predetermined relationship between the objective function and the variables [27]. The experimental results for the randomized design points were loaded and quadratic equations were obtained relating the responses with independent variables.

To evaluate the impact of the factors and their interactions, an Analysis of Variance (ANOVA) was performed. Based on these analyses, insignificant variables were identified. The quality of the resulting model was assessed using *p*-values to evaluate factor relevance.

2.5. Analytical methods

To determine the chemical composition of the apple pomace before pretreatment, the standard analytical methods of the National Renewable Energy Laboratory (NREL) [28,29] were used. Subsequently, the same methods were used to characterize the solids obtained after pre-treatment under optimal conditions for both SHF and SSF configurations. The determinations were carried out in triplicate and the mean values are reported.

The concentration of monosaccharides (glucose, galactose, arabinose and rhamnose) and fermentation products (2,3-BDO, acetoin and ethanol) were measured by High Performance Liquid Chromatography (HPLC). A refractive index detector (Waters 2414, Milford, MA, USA) and an Aminex HPX-87H column were employed. The operating conditions of the column were 60 °C and 0.01N H_2SO_4 as the mobile phase, with a flow rate of 0.6 mL/min. As the Aminex H column (Bio-Rad, Alcobendas, Madrid) does not separate the monosaccharides xylose, fructose, mannose and galactose, their combined concentration was quantified as a single peak. All samples were centrifuged at 13,400 rpm for 5 min and filtered through 0.2 μm nylon filters, prior to injection.

2.6. Statistical analysis

Data from the design of the experiments were analyzed with the Statgraphics Centurion XIX software. The statistical significance was evaluated by analysis of variance (ANOVA) with significance at $p < 0.05$. The Tukey multiple range test was used to find significantly different means and were performed using Microsoft Excel.

3. Results and discussion

3.1. Characterization of apple pomace

The chemical characterization of the apple pomace (Table 3) revealed a substantial content of carbohydrates, at approximately 53 % (w/w), comprising 39.0 % structural carbohydrates, mainly glucan (19.6 %). This value is consistent with the findings of [30], who reported that the structural carbohydrate content (glucan, xylan and arabinan) in apple pomace is approximately 36 %. Additionally, 9.8 % of non-structural carbohydrates were found in the extractives, mainly fructose and glucose. This composition suggests that apple pomace is a promising feedstock for the enzymatic conversion of polysaccharides into fermentable sugars [17]. The acid-soluble lignin content was relatively low (1.7 %), whereas the acid-insoluble lignin content was substantially higher (22.7 %). Consequently, a pretreatment step is likely necessary to improve the accessibility of these structural carbohydrates for enzymatic hydrolysis.

In terms of extractives content, the apple pomace contained 23.7 %, a relatively lower amount compared to other fruit and vegetable wastes such as carrot discard with 43.1 % extractives [31] or discarded red beetroot with 66.1 % [16]. The monosaccharides found were, in descending order of abundance, fructose, glucose, rhamnose and arabinose. This finding is consistent with [4], who reported that low molecular weight carbohydrates, mainly fructose and glucose, are found in this apple by-product. Rhamnose and arabinose monomers can also be found in small amounts, as apple pomace contains these monosaccharides in the insoluble dietary fiber part [4]. Finally, the ash content was low (2.0 %).

Table 3
Chemical composition of raw apple pomace and solids recovered after optimal pretreatment for both SHF and SSF configurations (% dry weight).

Component		Raw apple pomace	Pretreated solid in SHF optimal conditions	Pretreated solid in SSF optimal conditions
Glucan		19.6 ± 0.1	31.6 ± 0.8	29.7 ± 0.5
Galacturonan		15.5 ± 0.0	8.2 ± 0.2	10.7 ± 0.2
Galactan		10.6 ± 0.1	11.8 ± 0.3	12.6 ± 0.1
Arabinan		5.0 ± 0.4	1.4 ± 0.2	3.0 ± 0.0
Ramnan		3.8 ± 0.2	2.9 ± 0.1	3.0 ± 0.1
Extractives in ethanol		14.2 ± 1.6	–	–
Extractives in water[a]		9.5 ± 1.0	–	–
Lignin	Acid-soluble lignin	1.7 ± 0.0	1.9 ± 0.0	2.0 ± 0.1
	Acid-insoluble lignin	22.7 ± 0.3	33.0 ± 0.7	29.8 ± 0.3
Total ash		2.0 ± 0.0	0.7 ± 0.1	0.8 ± 0.0

Tukey's pairwise comparisons were performed between the different chemical compositions (raw apple pomace, pretreated solid in SHF, and SSF optimal conditions). All values are significant at $p < 0.05$.

[a] Composition of extractives in water (% dry weight): glucose (3.1 ± 0.4). fructose (5.3 ± 1.4). arabinose (0.6 ± 0.0) and rhamnose (0.8 ± 0.1).

Paniagua-García et al. [32] reported a similar composition of carbohydrates (59.8 %), lignin (19.80 %) and extractives (16.6 %) in apple pomace. In Ref. [11] a lower content of structural carbohydrates (approximately 44 %) was observed, as well as a similar lignin (18.3 %) and ash (2.6 %) content in the characterization of apple pomace.

3.2. Optimization of autoclave pretreatment for apple pomace fermentation to 2,3-BDO

The following sections describe the pretreatment optimization results and evaluate the efficiency of two subsequent fermentation strategies: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF).

3.2.1. SHF fermentation from pretreated apple pomace

To maximize 2,3-BDO production and sugar recovery after the enzymatic hydrolysis step within the SHF fermentation strategy, two variables were optimized: pretreatment time and solid-liquid ratio. The results, in terms of sugar concentration after 24 h of enzymatic hydrolysis, maximum 2,3-BDO concentrations reached in the SHF process, as well as the concentrations of the by-products acetoin and ethanol, are summarized in Table 4.

Monosaccharide concentrations ranged from 17.0 g/L (run 9) to 70.9 g/L (run 13), which resulted in final 2,3-BDO concentrations between 5.7 g/L (run 9) and 29.8 g/L (run 3). A strong positive correlation was observed between solids loading and sugar concentration, with values increasing from 17.0 g/L at 3 % solids (run 9) to 67.2–70.9 g/L at 15–17 % solids (runs 3, 4, 13). In contrast, the pretreatment time (from 6 to 34 min) showed a negligible effect (Standardized Pareto chart in Table S5 a)), with sugar concentrations remaining within a narrow range of 42.4–50.1 g/L at a constant 10 % solids loading. These results clearly indicate that solids loading was the dominant factor influencing sugar release, whereas pretreatment time had only a minor impact within the studied range.

The overall sugar recovery yield—the mass of sugars recovered per 100 g of dry raw material—ranged from 23.2 % (run 9) to a maximum of

Table 4
Experimental results of design of experiments to optimize pretreatment conditions in autoclave (121 °C) for apple pomace in SHF fermentation and SSF fermentation.

Run	SHF					SSF			
	Monosaccharides[a] (g/L)	Time[b] (h)	2,3-BDO[c] (g/L)	Acetoin[d] (g/L)	Ethanol[d] (g/L)	Time[b] (h)	2,3-BDO[c] (g/L)	Acetoin[d] (g/L)	Ethanol[d] (g/L)
1	20.7 ± 1.6	10	7.7 ± 0.4	2.4 ± 0.1	1.3 ± 0.0	14	9.0 ± 0.3	2.6 ± 0.0	1.0 ± 0.1
2	24.8 ± 1.2	10	8.4 ± 0.2	2.2 ± 0.0	1.7 ± 0.0	14	9.3 ± 0.1	2.7 ± 0.0	1.0 ± 0.0
3	67.2 ± 2.1	62	29.8 ± 0.2	6.5 ± 0.3	3.3 ± 0.1	14	<1.0	<1.0	<1.0
4	68.2 ± 2.3	38	4.0 ± 0.3	5.4 ± 0.2	0.4 ± 0.0	14	<1.0	<1.0	<1.0
5	50.1 ± 1.8	38	20.9 ± 0.5	5.4 ± 0.1	1.2 ± 0.1	38	18.9 ± 0.9	5.6 ± 0.5	1.0 ± 0.1
6	42.4 ± 1.5	24	8.5 ± 0.1	13.3 ± 0.7	1.6 ± 0.3	38	19.5 ± 0.2	5.0 ± 0.4	1.1 ± 0.0
7	50.0 ± 1.7	38	17.6 ± 0.4	4.8 ± 0.3	0.9 ± 0.1	48	15.9 ± 0.8	6.1 ± 0.6	0.9 ± 0.0
8	45.5 ± 2.0	24	17.0 ± 0.3	2.8 ± 0.0	2.4 ± 0.2	62	6.0 ± 0.6	4.5 ± 0.2	0.1 ± 0.0
9	17.0 ± 1.6	8	5.7 ± 0.2	1.8 ± 0.0	0.2 ± 0.0	14	4.7 ± 0.2	3.2 ± 0.3	0.6 ± 0.1
10	49.3 ± 2.2	38	18.8 ± 0.4	5.0 ± 0.1	1.0 ± 0.0	42	16.4 ± 0.4	7.5 ± 0.2	0.8 ± 0.1
11	46.2 ± 1.5	28	19.2 ± 0.3	3.2 ± 0.1	1.9 ± 0.1	48	15.8 ± 0.5	2.3 ± 0.4	0.2 ± 0.0
12	43.2 ± 2.1	24	18.7 ± 0.4	2.8 ± 0.2	2.6 ± 0.2	38	17.9 ± 0.3	6.1 ± 0.0	0.9 ± 0.2
13	70.9 ± 1.3	48	27.3 ± 0.4	5.4 ± 0.1	2.7 ± 0.1	134	2.1 ± 0.2	2.3 ± 0.3	0.1 ± 0.1

[a] Glucose + galactose + arabinose + rhamnose (total monosaccharides after 24 h of enzymatic hydrolysis).
[b] Fermentation time for maximum 2,3-BDO concentration.
[c] 2,3-BDO maximum concentration.
[d] Acetoin and ethanol concentration at fermentation time for maximum 2,3-BDO concentration.

83.7 % (run 5). These values are considerably higher than the 26–37 % yields reported by Ref. [33] for apple pomace under different pretreatment conditions. The highest yields in this study (82.1–83.7 %) were consistently achieved near the central point conditions (runs 2, 5, 7, 10), indicating the existence of an optimal process window. However, complex interactions were observed: at low solids loading (5 %), increasing pretreatment time from 10 to 30 min decreased the yield (from 82.4 % to 66.7 %), suggesting sugar degradation. Conversely, at a fixed 10 min pretreatment, increasing the solids from 5 % to 15 % also decreased the recovery yield (from 82.4 % to 75.0 %), likely due to mass transfer limitations at higher slurry densities.

These results are consistent with, and in some cases improve upon, findings reported in the literature. For instance Ref. [34], investigated the hydrothermal pretreatment of depectinized apple pomace, optimizing temperature (180–220 °C), time (10–30 min), and solids loading (2.5–5 % w/v). Their optimal conditions of 180 °C and 10 min, but at a low 2.5 % solids loading, yielded 21.3 g/L of glucose. This concentration is considerably lower than those achieved in the present study, likely because our higher solids loadings outweighed the effect of their more severe pretreatment temperature.

In a different approach [33], utilized an enzymatic pretreatment with laccase followed by hydrolysis. At a solids loading of 15 % (w/v), their process yielded a sugar concentration of 51.8 g/L and a sugar yield of 0.34 g/g, values that are comparable to the optimal ranges found in our work. Furthermore, the strategy of using the whole pretreated slurry is supported by the work of [35]. They reported that using the entire slurry from a dilute acid pretreatment (1 % H_2SO_4, 121 °C) nearly doubled the concentration of reducing sugars compared to using only the washed solid fraction. Consequently, a higher availability of fermentable sugars logically translates into a greater potential for product formation.

In the SHF configuration, the final 2,3-BDO concentration showed a strong positive correlation with both solids loading and pretreatment time (Standardized Pareto chart in Table S5 b)). At low solids loading (3 %, run 9), the concentration was only 5.7 g/L, whereas at the highest loading (17 %, run 3), it reached a maximum of 29.8 g/L. The impact of pretreatment time was also significant; at a constant 10 % solids loading, increasing the time from 6 min (run 6) to 34 min (run 11) more than doubled the 2,3-BDO concentration from 8.5 g/L to 19.2 g/L. This effect was even more pronounced at 15 % solids, where a longer pretreatment (30 min) boosted the concentration to 27.3 g/L, compared to just 8.4 g/L after 10 min.

2,3-butanediol yields and productivities are detailed in Table 4. The 2,3-BDO yield ranged from a low of 0.10 g/g in runs 4 and 6 to a maximum of 0.48 g/g in run 3. Notably, several experimental conditions resulted in yields of approximately 0.4 g/g. A clear trade-off between the final titer and productivity was observed. Fermentations with high solids loadings required extended times (up to 62 h); while the highest productivity (0.84 g/L·h) was achieved in a rapid, low-solids fermentation (run 2, 8 h). This was linked to sugar consumption, which was generally high (>85 %) and peaked at the central point conditions (up to 95.7 %). The poor performance of run 4, which had the lowest consumption, yield, and productivity, is likely due to high viscosity hindering mass transfer at a high solids loading with insufficient pretreatment.

By-product formation remained low under most conditions. Acetoin concentrations ranged from 1.8 to 6.5 g/L, except for a spike to 13.3 g/L in run 6. This outlier is attributed to a metabolic shift toward acetoin triggered by rapid glucose depletion, a phenomenon previously described by Ref. [22]. Ethanol was consistently a minor by-product, with all concentrations remaining between 1.0 and 3.3 g/L.

3.2.1.1. Optimization of pretreatment conditions to fermentation of pretreated solid through SHF. To model the process and understand the influence of the operational variables, second-order polynomial equations were developed for both monosaccharide release (Eq. (1)) and final 2,3-BDO concentration (Eq. (2)).

$$Monosaccharide\ concentration\left(\frac{g}{L}\right) = -9.24421 - 0.20726\cdot t$$

$$+7.17495\cdot S - 0.166721\cdot S^2;$$

$$R^2 = 97.3\%;\ R^2 adjust = 95.3\%;\quad Eq.\ 1.$$

$$2,3-BDO\ concentration\left(\frac{g}{L}\right) = -0.418437 + 0.272368\cdot t$$

$$+1.58633\cdot S - 0.0250372\cdot t^2 + 0.12015\cdot t\cdot S - 0.162893\cdot S^2;$$

$$R^2 = 97.2\%;\ R^2 adjust = 94.9\%;\quad Eq.2.$$

Statistical analysis ($p < 0.05$) revealed contrasting effects for each response. The ANOVA results are displayed in Table S2 of supplementary material. The values observed and predicted by the model are listed in the supplementary material (Table S4 a)). For monosaccharide concentration, solids loading (S) was the sole significant positive factor. The dominance of this factor is visually confirmed by the response surface plot (Fig. 1a), where monosaccharide concentrations are shown to increase from below 20 g/L to over 70 g/L as solids loadings rise from 3 % to 17 %, largely independent of the pretreatment time. Conversely, for 2,3-BDO concentration, all factors—pretreatment time (t), solids

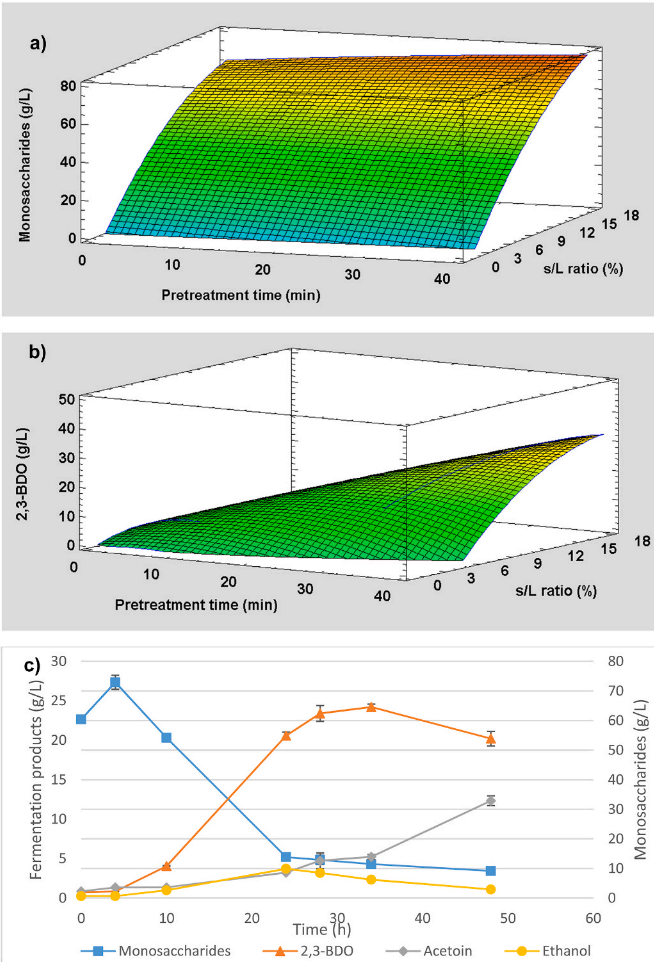


Fig. 1. Response surface for the SHF fermentation. Effect of pretreatment time and solids loading (autoclave, 121 °C) on a) monosaccharides concentration and b) 2,3-BDO concentration. c) Time-course profile of substrates and products during SHF fermentation under optimized pretreatment conditions (31 min at 121 °C, 16 % w/v solids loading).

loading (S), and their interaction (t·S)—were statistically significant. The synergistic nature of this interaction is evident in the corresponding response surface (Fig. 1b), which illustrates that the highest 2,3-BDO concentrations (>25 g/L) are confined to the region where both solids loading (>15 %) and pretreatment time (>25 min) are maximized.

Given that the primary objective of this stage is to maximize sugar availability, a multi-response optimization was conducted to simultaneously maximize both sugar release and 2,3-BDO production. This analysis identified optimal conditions of 31 min and a 16 % (w/v) solids loading. Under these parameters, the model predicted a monosaccharide concentration of 71.4 g/L and a 2,3-BDO titer of 27.4 g/L. To validate these predictions, triplicate experiments were performed under the optimal conditions, yielding an average monosaccharide concentration of 75.5 g/L and a final 2,3-BDO concentration of 24.2 g/L (Fig. 1c). The close agreement between the predicted and observed values confirms the model's accuracy and robustness. The selection of a high optimal solids loading (16 %) is consistent with the work of [18], who also concluded that high solids loading is a critical parameter for improving sugar yields from biomass for 2,3-BDO production.

Characterization of the solid residue recovered after the optimal SHF pretreatment (45.2 % total gravimetric recovery) revealed significant compositional changes (Table 2). The glucan content increased substantially from 19.6 % in the raw material to 31.6 % in the pretreated solid, while the relative lignin content also rose to 33.0 %. This enrichment of more recalcitrant fractions is consistent with the partial solubilization of other components like hemicellulose and extractives during pretreatment, as reported by Ref. [10]. Furthermore, the final lignin content is remarkably similar to the 35.8 % reported by Ref. [34] for apple pomace pretreated under different, but also thermally intensive, conditions (140 °C, 5 min). The solid recovery of 45.2 % in this work also falls squarely within the range of 40–50 % reported by Ref. [18] for macroalgae pretreated at moderate temperatures (30–90 min), supporting the effectiveness of our chosen conditions. This confirms that combining high solids loadings with moderate pretreatment temperatures is an effective strategy for maximizing sugar concentration while maintaining reasonable solid recovery.

3.2.2. *SSF fermentation from pretreated apple pomace*

For the SSF process, the autoclave pretreatment was optimized using the maximum 2,3-BDO concentration as the sole response variable, with the full experimental results for each run detailed in Table 3. Final 2,3-BDO concentrations varied significantly, ranging from less than 1 g/L in failed runs (runs 3, 4, and 13) to a maximum of 19.5 g/L, which was achieved in run 6. A critical divergence from the SHF configuration was observed regarding the influence of solids loading (Standardized Pareto chart in Table S5 c)). While a low loading of 3 % yielded a modest 4.7 g/L of 2,3-BDO (run 9), increasing the solids to 17 % (run 3) resulted in complete fermentation failure, in stark contrast to the positive trend seen in the SHF process. For successful fermentations (i.e., at solids loadings below 15 %), the time required to reach the maximum 2,3-BDO concentration followed a similar trend to SHF, with higher solids loadings requiring longer fermentation times. The effect of the pretreatment time was also distinct; at a constant 10 % solids loading, a shorter pretreatment of 6 min (run 6) produced the highest 2,3-BDO concentration of 19.5 g/L, outperforming the 15.8 g/L obtained after a much longer 34-min pretreatment (run 11). This non-linear relationship, which suggests the existence of an optimal pretreatment time, was confirmed across all experiments at 10 % solids loading (runs 5–8 and 10–12), where longer times did not necessarily lead to higher product concentrations. The consistent failure at high solids loadings (>15 %) is attributed to the high initial slurry viscosity, a known challenge in SSF that severely hinders mass transfer and microbial activity before saccharification can proceed.

The success or failure of these fermentations correlated directly with substrate utilization. Sugar consumption plummeted to as low as 6.2 % and 6.8 % in the high-solids runs (runs 3 and 13, respectively), confirming that fermentation was severely inhibited. In stark contrast, all successful experiments exhibited high sugar consumption, consistently above 82 %. The highest consumption values of 94.8 % (run 5) and 93.7 % (run 6) were achieved near the optimal window of 10 % solids loading and a pretreatment time between 6 and 20 min. This highlights that while higher solids provide more potential substrate, the initial high viscosity in the SSF process creates a critical barrier to microbial activity, a known challenge for this configuration.

By-product formation remained minimal in all successful experiments, with acetoin concentrations between 1.0 and 7.5 g/L and ethanol concentrations between 1.0 and 1.1 g/L (Table 3).

Yield and Productivity were calculated at the time of maximum 2,3-BDO concentration and are detailed in Table 4. Productivities were near zero in the failed high-solids runs, while successful experiments showed values between 0.3 and 0.5 g/(L·h), peaking at 0.64–0.66 g/(L·h) in low-solids, rapid fermentations (runs 1 and 2). The 2,3-BDO yields in the successful runs were comparable to the SHF process, ranging from 0.3 to 0.5 g/g, with an average value of 0.4 g/g.

3.2.2.1. *Optimization of pretreatment conditions to fermentation of pretreated solid through SSF.*

To optimize the SSF process, a quadratic model was developed to predict the final 2,3-BDO concentration based on pretreatment time (t) and solids loading (S). The resulting model (Eq.

(3)) demonstrated a strong predictive capacity. The values observed and predicted by the model are listed in the supplementary material (Table S4 b)).

$$2,3-BDO\ concentration\left(\frac{g}{L}\right) = -18.873 + 0.435685\cdot t$$

$$+6.969823\cdot S - 0.306929\cdot S^2;$$

$$R^2 = 96.2\%;\ R^2 adjust = 92.5\%;\quad Eq.\ 3.$$

Here, t is the pretreatment time (min), and S is the solid-liquid ratio (% w/v). As shown by the R^2 and adjusted R^2 values (Eq. (3)), as well as the confidence level (95 %, $p < 0.05$), a good agreement was reached between the experimental and predicted values for the response.

Statistical analysis ($p < 0.05$) of the model (ANOVA results are displayed in Table S2) revealed that, while the linear terms for time (t) and solids loading (S) were not statistically significant, the quadratic term for solids loading (S^2) exerted a significant negative influence. This indicates that while increasing solids loading is initially beneficial, excessively high loads become inhibitory. This trend is clearly visualized in the response surface plot (Fig. 2a), which shows that peak 2,3-BDO concentrations are achieved at intermediate, rather than maximum, solids loading values. Multi-response optimization identified optimal conditions of 7 min and an 11 % (w/v) solids loading, predicting a 2,3-BDO concentration of 19.5 g/L. Experimental validation under these conditions yielded a final concentration of 21.0 g/L (Fig. 2b), confirming the model's reliability with less than 5 % deviation. The lower optimal solids loading for SSF compared to SHF (11 % vs. 16 %) aligns with observations by Ref. [18], who noted that loadings above 10 % can increase slurry viscosity to levels that impede proper mixing.

The chemical changes underlying these process outcomes were investigated by characterizing the solid residue after the optimal SSF pretreatment, which had a gravimetric recovery of 45.1 %. The pretreated solid was significantly enriched in glucan (29.7 % vs. 19.6 % initially) and lignin, while retaining a considerable galactan content (12.6 %) (Table 3). This compositional shift is a direct result of the hydrothermal pretreatment preferentially degrading and solubilizing the more labile hemicellulose fraction. This phenomenon is well-documented in the literature; studies by Refs. [11,34,36] all report that the amorphous, branched structure of hemicellulose makes it highly susceptible to degradation by hot liquid water, leading to a relative increase in cellulose and lignin in the remaining solid.

The chemical changes underlying these process outcomes were investigated by characterizing the solid residue after the optimal SSF pretreatment, which had a gravimetric recovery of 45.1 %. The pretreated solid was significantly enriched in glucan (29.7 % vs. 19.6 % initially) and lignin, while retaining a considerable galactan content (12.6 %) (Table 3). This compositional shift is a direct result of the hydrothermal pretreatment preferentially degrading and solubilizing the more labile hemicellulose fraction. This observation aligns with the general principle reported by Ref. [11], who stated that increasing pretreatment temperature and time leads to higher glucan and lignin content as hemicellulose is removed. More specifically [36], identified xylan as the primary component removed from apple pomace during hot liquid water treatment. The mechanistic basis for this selective degradation was explained by Ref. [34], who noted that the unique structural properties of hemicellulose—its amorphous nature, low degree of

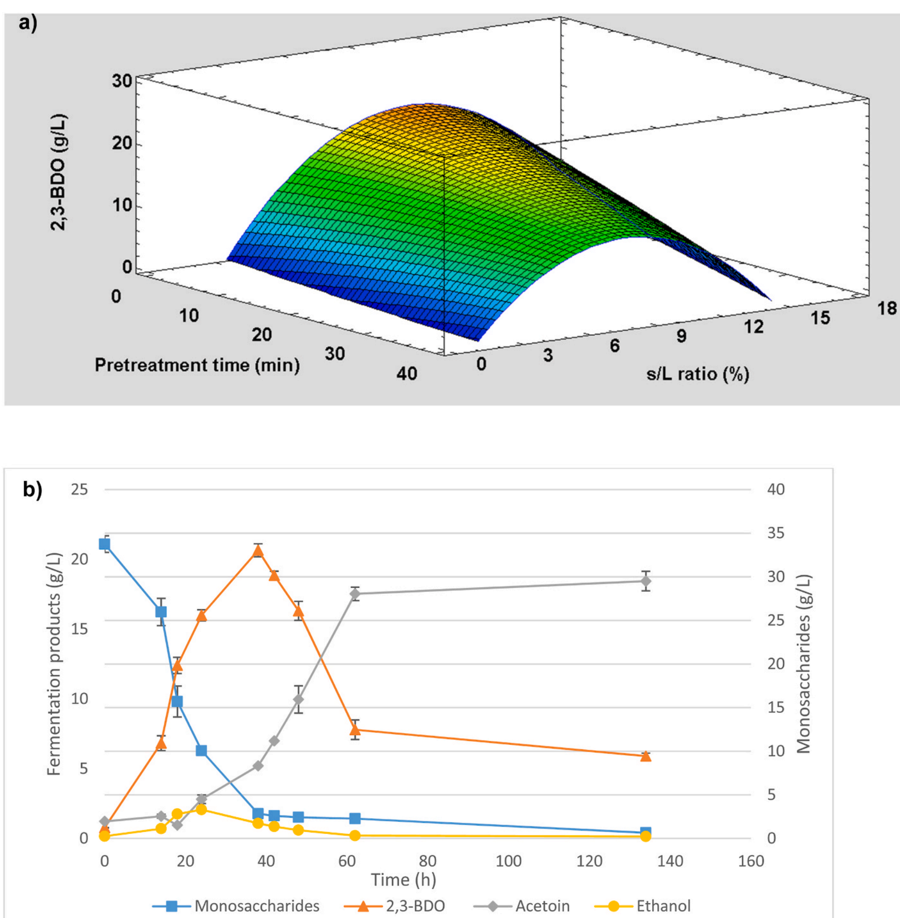


Fig. 2. Response surface for the SSF fermentation. Effect of pretreatment time and solids loading (autoclave, 121 °C) on a) 2,3-BDO concentration. b) Time-course profile of substrates and products during SSF fermentation under optimized pretreatment conditions (7 min at 121 °C, 11 % w/v solids loading).

polymerization, and highly branched chains—make it more susceptible to thermal degradation than crystalline cellulose.

3.2.3. Comparative of SSF and SHF processes in optimal pretreatment conditions

A direct comparison of the two process configurations, each operating under its respective optimal pretreatment conditions, revealed a critical trade-off between final product titer and overall process efficiency. The more intensive pretreatment required for the SHF process (31 min at 16 % solids) was necessary to overcome the lignocellulosic recalcitrance at high solids loading, consistent with the principle that longer times facilitate biomass decomposition [18]. In contrast, the SSF process was limited to a lower solids loading (11 % w/v) and a shorter optimal pretreatment time (29 min), as higher solids led to viscosity issues. Consequently, the SHF configuration achieved a higher final 2,3-BDO concentration of 24.2 g/L, compared to 21.0 g/L for the SSF process. However, when evaluated on an overall mass balance, the SSF strategy was superior, yielding 23.8 g of 2,3-BDO per 100 g of raw apple pomace, a significant improvement over the 18.9 g/100 g obtained with SHF.

Analysis of the fermentation kinetics (Figs. 1c and 2b) provided further insight. The SHF process reached its maximum concentration in 34 h, slightly faster than the SSF process at 38 h. This faster fermentation time explains its higher volumetric productivity (0.71 vs. 0.55 g/L·h), as the sugars were fully available from the start. Conversely, the SSF configuration demonstrated a higher conversion yield (0.43 g/g vs. 0.38 g/g) and more complete sugar consumption (92.5 % vs. 83.6 %), probably because the simultaneous removal of sugars by the microorganism alleviated enzymatic inhibition. By-product formation was similar in both optimized strategies, with acetoin concentrations of approximately 5.2 g/L and ethanol concentrations between 1 and 2 g/L.

The performance of the processes developed in this study can be benchmarked against relevant reports in the literature. The 24.2 g/L obtained via SHF is comparable to the 26.1 g/L reported by Ref. [23] from food waste hydrolysates with *Bacillus licheniformis*, validating the high titer potential. This result is also comparable to the 23.4 g/L achieved by Ref. [21] with *P. polymyxa*; however, that study required a more complex approach, involving an acid pretreatment (1 % H_2SO_4, 121 °C, 60 min) and a cocktail of four different enzymes. The milder, chemical-free pretreatment employed in this work therefore represents a more streamlined and potentially cost-effective alternative. Similarly, the 21.0 g/L obtained from the SSF process developed here significantly surpasses the results from other studies using *P. polymyxa* on pretreated brewer's spent grain. For instance Ref. [37], obtained 11.4 g/L of 2,3-BDO using a harsh acid pretreatment (1.25 % H_2SO_4, 155 °C, 17 min). Under similar acid pretreatment conditions [38], reported an even lower 2,3-BDO titer of only 3.7 g/L, which was accompanied by a high concentration of acetoin (7.4 g/L). Considering the superior overall mass yield, milder pretreatment conditions, and operational simplicity of a one-pot process, the SSF strategy is deemed more advantageous for industrial scale-up. Therefore, further optimization of the SSF process, specifically focusing on enzyme loading, was pursued.

3.3. Optimization of enzyme loading to obtain 2,3-BDO from the pretreated solid under optimal conditions through SSF fermentation

A critical factor for the economic viability of lignocellulosic biorefineries is the enzyme loading, which presents a trade-off between hydrolysis efficiency and process cost [30,35]. To address this, the loading of a synergistic enzyme cocktail comprising Viscozyme and Cellic CTec2, a combination previously shown to be effective for hydrolyzing fruit and vegetable waste [31], was optimized. This study examines the saccharification of carrot waste using three enzymes separately and/or in combination. The results show that, when a single enzyme is used, Viscozyme leads to higher recoveries of glucose, galactose, and arabinose than Cellic CTec2. In addition, this enzyme can hydrolyze both cellulosic and hemicellulosic sugars. This study also considered the use of enzyme mixtures, observing that the highest sugar content after enzymatic hydrolysis was achieved using a mixture of Cellic CTec 2 and Viscozyme enzymes. The optimization was performed on the SSF process operating under its previously determined optimal pretreatment conditions, with the final 2,3-BDO concentration as the response variable. Enzyme loadings for both preparations were varied between 3 and 15 FPU/g of pretreated substrate, as detailed in the experimental design (Table 5).

The experimental results, summarized in Table 5, show that 2,3-BDO concentrations ranged from 13.3 g/L (run 5) to a maximum of 24.1 g/L (run 12). The highest concentration was achieved with the maximum combined loading of both enzymes (15 FPU/g each). The individual contribution of each enzyme was also significant (Standardized Pareto chart in Table S5 d)). Increasing the Viscozyme loading from its axial point minimum (0.5 FPU/g, run 7) to its maximum (17 FPU/g, run 11) raised the 2,3-BDO concentration from 14.2 g/L to 17.4 g/L. A more pronounced effect was observed for Cellic CTec2, where increasing the loading across the same range (run 5 vs. run 6) enhanced the final titer from 13.3 g/L to 19.4 g/L.

On the other hand, productivity ranged from 0.34 g/L·h (run 3) to a peak of 0.95 g/L·h (run 5). Notably, the highest productivity occurred at the lowest loading of Cellic CTec2, a result directly linked to its short fermentation time (14 h). In contrast, the highest 2,3-BDO yields (0.45–0.46 g/g) were obtained in runs 12 and 13, which corresponded to high loadings of both enzymes. Across most experiments, the yield values were consistent, ranging between 0.32 and 0.46 g/g. By-product formation remained low and consistent with previous experiments, with acetoin concentrations between 1.0 and 9.6 g/L and ethanol concentrations around 1–2 g/L (Table 5).

The experimental results from the enzyme loading optimization were fitted to a quadratic model (Eq. (4)) to predict the final 2,3-BDO concentration based on the loadings of Viscozyme (V) and Cellic CTec2 (C). The model demonstrated a strong predictive capacity. The values observed and predicted by the model are listed in the supplementary material (Table S4 b)).

$$2,3-BDO\ concentration\left(\frac{g}{L}\right)=15.7115-0.475001\cdot V-0.116703\cdot C$$

$$+0.0474306\cdot V\cdot C;$$

$$R^2=97.2\%;\ R^2 adjust=93.6\%;\quad Eq.4.$$

Statistical analysis ($p < 0.05$) of the model revealed that all factors, including the interaction term (V·C), had a significant positive influence on the 2,3-BDO concentration (ANOVA results are shown in Table S2). The response surface plot (Fig. 3a) visually confirms this synergistic effect, showing that the highest titers are achieved with high loads of both enzymes. While the Cellic CTec2 (C) loading exerted the most significant positive impact, likely due to its efficient glucose release, the contribution of Viscozyme (V) was also critical. Its role in reducing slurry viscosity and improving mass transfer is a crucial factor in SSF processes, as highlighted by Ref. [34]. This is further supported by previous work from Ref. [31], who demonstrated that Viscozyme can rapidly fluidize vegetable slurries. This synergistic requirement was confirmed in our preliminary tests, where using only Cellic CTec2 led to inferior results (data shown in Supplementary Material Table S3).

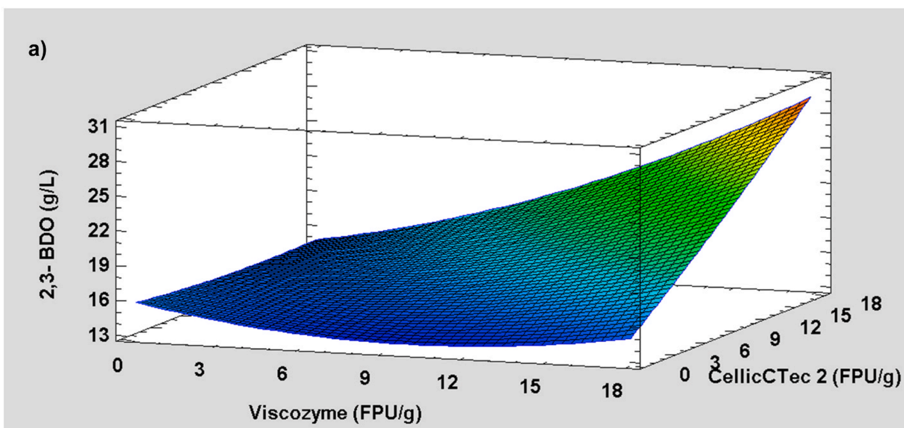
The observation that increasing enzyme concentrations provides diminishing returns aligns with previous studies on apple pomace hydrolysis. For instance Ref. [33], described a hyperbolic relationship between Cellic CTec2 concentration and glucose release, with saccharification rates plateauing around 20 FPU/g. Similarly [36], concluded that the economic benefits of using Cellic CTec2 at concentrations above 20 FPU/g for pretreated apple pomace were negligible, emphasizing the importance of a cost-benefit analysis in enzyme selection.

Multi-response optimization identified optimal conditions of 15

Table 5
Results of design of experiments to optimize pretreatment conditions in autoclave (121 °C) for apple pomace in SHF fermentation and SSF fermentation. All data are referred to fermentation time for maximum 2,3-BDO concentration.

Run	SHF			SSF		
	Monosaccharides[a] (g/L)	2,3-BDO yield[b] (g/g)	2,3-BDO productivity[c] (g/L·h)	Monosaccharides[a] (g/L)	2,3-BDO yield[b] (g/g)	2,3-BDO productivity[c] (g/L·h)
1	4.4 ± 0.2	0.47	0.77	3.7 ± 0.1	0.53	0.64
2	3.5 ± 0.1	0.39	0.84	3.8 ± 0.1	0.44	0.66
3	5.5 ± 0.1	0.48	0.48	63.0 ± 0.0	–	–
4	30.2 ± 0.4	0.10	0.10	55.1 ± 0.0	–	–
5	2.9 ± 0.2	0.44	0.55	2.6 ± 0.2	0.40	0.50
6	7.2 ± 0.4	0.24	0.35	2.7 ± 0.1	0.49	0.51
7	2.2 ± 0.3	0.37	0.46	8.9 ± 0.3	0.39	0.33
8	6.5 ± 0.1	0.44	0.71	27.6 ± 0.2	0.33	0.10
9	2.2 ± 0.1	0.39	0.72	1.7 ± 0.0	0.31	0.34
10	2.3 ± 0.0	0.40	0.50	4.1 ± 0.1	0.36	0.39
11	4.9 ± 0.1	0.47	0.69	11.8 ± 0.2	0.46	0.33
12	2.6 ± 0.0	0.46	0.78	4.9 ± 0.1	0.47	0.47
13	4.9 ± 0.1	0.41	0.57	66.0 ± 0.2	0.44	0.02

[a] Total monosaccharides (g/L) at fermentation time for maximum 2,3-BDO concentration.
[b] Yield calculated as 2,3-BDO maximum concentration/(total monosaccharides after 24 h of HE-monosaccharides at time of maximum 2,3-BDO concentration).
[c] Productivity calculated as 2,3-BDO maximum concentration/time for maximum 2,3-BDO concentration.



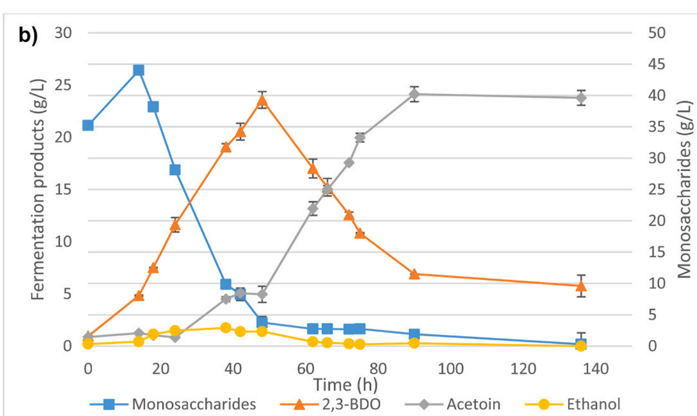


Fig. 3. Effect of enzyme loading on the SSF process. (a) Response surface plot showing the effect of Viscozyme and Cellic CTec2 loading on the final 2,3-BDO concentration. (b) Time-course profile of substrates and products during SSF fermentation under optimized conditions (pretreatment: 7 min, 11 % w/v solids; enzyme loading: 15 FPU/g Viscozyme and 17 FPU/g Cellic CTec2).".

FPU/g of Viscozyme and 17 FPU/g of Cellic CTec2, predicting a 2,3-BDO concentration of 25.6 g/L. Experimental validation under these conditions yielded a final concentration of 23.6 g/L (Fig. 3b), confirming the model's reliability with less than 5 % deviation.The optimized SSF process developed here yields results that compare favorably with other reports on 2,3-BDO production from apple pomace and similar fruit wastes. For example [39], reported concentrations of 12.8–16.5 g/L and yields of 0.13–0.17 g/g using depectinized apple pomace hydrolysate of 40 g/L of glucose (pretreated in autoclave (121 °C, 20 min, enzymatic hydrolysis (multienzyme preparation from *Aspergillus niger* IBT 90, enzyme/substrate ratio 1 mL/10 g solid substance, 50 °C, 48 h)) with *Bacillus* species in 46 h of fermentation. The significantly higher concentrations and yields achieved in the present study underscore the effectiveness of the integrated pretreatment and fermentation strategy.

The results are also comparable to those obtained from other fruit wastes using the same microorganism; for instance Ref. [17], reported 2,3-BDO titers of 15.0 g/L from banana peel and 26.6 g/L from whole banana, both in an SSF process (37 °C, 48h, solid loading 10 %, 10 FPU/g substrate Cellic CTec 2 and 10 FPU/g substrate Viscozyme).

3.4. Fed-batch production of 2,3-BDO

To enhance final product concentrations and mitigate potential substrate inhibition, a fed-batch strategy was implemented for both optimized configurations, as this approach is known to facilitate downstream processing and improve productivity [40].

In the SHF fed-batch process, the 2,3-BDO concentration reached a maximum of 40.1 g/L in 58 h, with a productivity of 0.69 g/(L·h) and a yield of 0.42 g/g (Fig. 4a). In contrast, the SSF fed-batch configuration achieved a final concentration of 32.7 g/L in 58 h, with a lower productivity of 0.56 g/(L·h) but a superior conversion yield of 0.49 g/g (Fig. 4b). Acetoin was the main by-product, with concentrations of 5.4 g/L (SHF) and 2.7 g/L (SSF) at the point of maximum 2,3-BDO concentration. Notably, at later stages of fermentation (192 h), acetoin levels increased significantly. This indicates the conversion of 2,3-BDO back to acetoin, a metabolic shift previously reported by Ref. [40]. The trigger for this conversion is glucose depletion in the medium, as explained by Ref. [22], an effect which is counteracted by the glucose pulses in a fed-batch strategy. Ethanol remained a minor by-product in both systems, with final concentrations of 3.9 g/L for the SHF configuration and 2.7 g/L for the SSF configuration.

The performance of the developed fed-batch processes can be contextualized by comparison with the relevant literature. For instance Ref. [41], achieved a very high titer of 87.8 g/L using *Bacillus licheniformis* from depectinized apple pomace in a fed-batch system. However, their process was considerably more complex, involving an autoclave pretreatment (121 °C, 20 min), a prolonged 48 h enzymatic hydrolysis, separation of the hydrolysate, and multiple glucose pulses during fermentation, which increases both operational complexity and cost. In another study using *P. polymyxa* [34], reported a concentration of 71.7 g/L in 54 h, achieving a high productivity of 1.33 g/(L·h) and a yield of 0.39 g/g. However, this benchmark performance was achieved using a pure glucose medium, which is not economically feasible for bulk chemical production. Furthermore, while some studies using pathogenic microorganisms report higher titers, the use of the non-pathogenic *P. polymyxa* in this work is a significant advantage for industrial safety and application [40].

In summary, the implementation of a glucose-based fed-batch strategy successfully enhanced 2,3-BDO production in both SHF and SSF configurations, significantly improving final concentrations while maintaining high conversion yields. This approach effectively mitigates the substrate inhibition often observed in high-concentration batch fermentations with *P. polymyxa*, as demonstrated by Ref. [31], proving its potential for developing a more intensive and industrially relevant bioprocess.

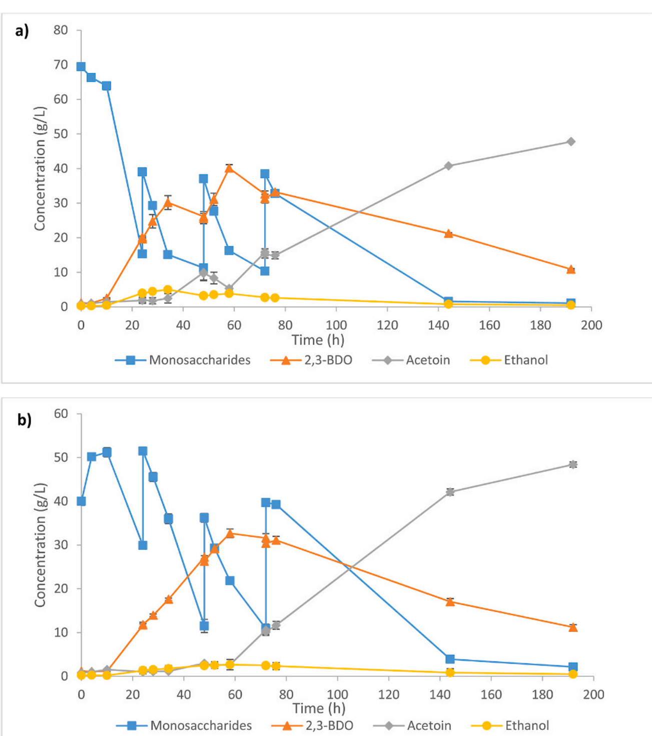


Fig. 4. Fed-batch fermentation performance. Time-course profiles of substrates and products are shown for (a) the optimized SHF configuration and (b) the fully optimized SSF configuration.

3.5. Mass balance in different configurations for 2,3-BDO production

To assess the overall process efficiency and compare the different strategies, a comprehensive material balance was conducted for each configuration. All calculations were based on a starting input of 100 kg of dry apple pomace, and the results are summarized in Fig. 5.

The analysis of the batch configurations revealed significant differences in overall yield. From the initial 100 kg of dry apple pomace, the optimized SHF process yielded 18.9 kg of 2,3-BDO. In contrast, the standard SSF process was more efficient, producing 23.8 kg, and this further improved to 26.8 kg after the optimization of enzyme loading. The fed-batch strategy significantly enhanced these mass yields. Under fed-batch conditions, the SHF process yielded 31.3 kg of 2,3-BDO, while the enzyme-optimized SSF process achieved the highest overall yield of 37.1 kg.

These overall process yields are comparable to those reported for other waste-to-2,3-BDO processes. For example [23], reported a yield of 0.28 kg of 2,3-BDO per kg of dry bakery waste using *Bacillus licheniformis*. This aligns well with the 0.27 kg/kg achieved in the enzyme-optimized batch SSF process and is surpassed by the 0.37 kg/kg obtained in the fed-batch SSF configuration. This comprehensive mass balance analysis, which provides critical data on process inputs and outputs, is essential for future techno-economic assessments. The high overall yields demonstrate that apple pomace can be effectively valorized into 2,3-BDO, confirming the industrial potential of the developed bioprocess.

4. Conclusion

This investigation provides the first systematic comparison and multi-stage optimization of these process configurations for this specific feedstock and microorganism. The findings offer a critical roadmap for designing scalable biorefinery processes based on apple pomace, directly contributing to circular economy objectives.This study has demonstrated a viable and robust pathway for valorizing apple pomace, a low-value agro-industrial residue, into the platform chemical 2,3-butanediol utilizing the non-pathogenic *Paenibacillus polymyxa*. A systematic comparison of bioprocessing strategies revealed that, while a Separate Hydrolysis and Fermentation (SHF) configuration achieved higher product titers in batch mode, enabling by its tolerance for greater solids loading (16 %), the integrated Simultaneous Saccharification and Fermentation (SSF) process emerged as the more advantageous strategy from an industrial perspective. Despite operating at a lower optimal solids loading (11 %), the SSF strategy resulted in a higher overall mass yield (26.8 kg of 2,3-BDO per 100 kg of dry apple pomace) and streamlined the workflow into a single-vessel process. This outcome is significant, as it underscores that final product concentration alone is not the sole metric for process viability; rather, overall mass yield and

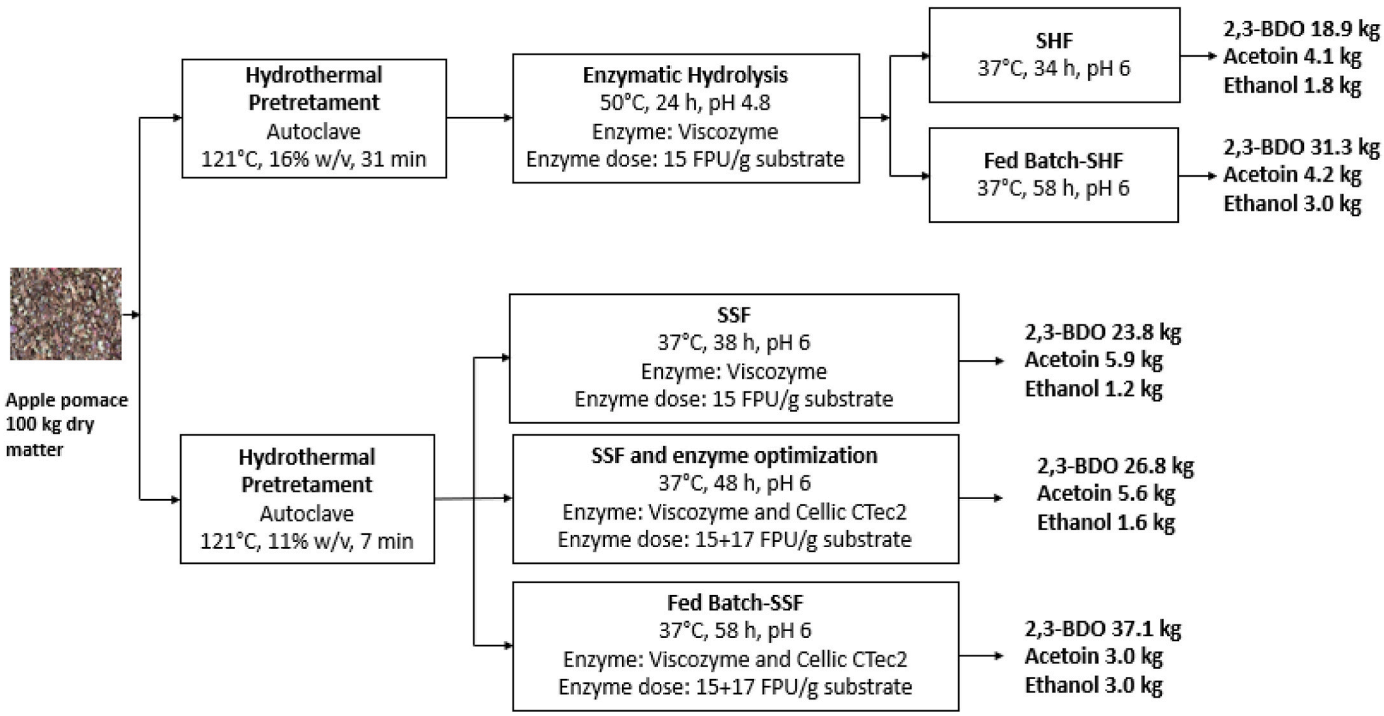


Fig. 5. Overall process yield comparison based on mass balance from a basis of 100 kg of dry apple pomace for the five process configurations developed: optimized batch SHF, fed-batch SHF, optimized batch SSF, enzyme-optimized SSF, and fed-batch SSF.

operational simplicity are critical drivers for economic feasibility. This work presents the first comprehensive optimization of an SSF process for 2,3-BDO production from apple pomace utilizing *P. polymyxa*. The high overall yield of 37.1 kg of 2,3-BDO per 100 kg of apple pomace, achieved via an enzyme-optimized (15 FPU/g of Viscozyme and 17 FPU/g of Cellic CTec2), fed-batch SSF process with 11 % s/L solids loading for SSF, provides a solid foundation for future research. Future work should focus on advanced bioprocess engineering, particularly the development of sophisticated feeding strategies (e.g., pH-stat or model-based) for precise control of substrate levels, which would aim to maximize 2,3-BDO concentrations while suppressing by-product formation. Furthermore, integrating an efficient and cost-effective downstream purification strategy represents the critical next step toward demonstrating a complete bench-scale biorefinery process. Finally, a comprehensive techno-economic analysis (TEA) and life cycle assessment (LCA) will be essential to rigorously quantify the economic viability and environmental benefits of this circular economy model.

CRediT authorship contribution statement

Cristina Barrios: Writing – original draft, Data curation. **Juan Carlos López Linares:** Supervision, Funding acquisition, Conceptualization. **Mónica Coca:** Writing – review & editing, Methodology. **Susana Lucas:** Writing – review & editing, Methodology. **María Teresa García-Cubero:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2026.108965.

Data availability

Data will be made available on request.

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