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Enhancing 2,3-butanediol and acetoin production from brewer's spent grain hemicellulosic hydrolysate through bacterial co-cultivation

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ABSTRACT

This study evaluated bacterial co-cultivation as a strategy to mitigate brewer's spent grain (BSG) hemicellulosic hydrolysate toxicity, aiming to enhance 2,3-butanediol (2,3-BDO) and acetoin production through fermentation. Co-culture of *Paenibacillus polymyxa* with *Pseudomonas alloputida* or *Rhodococcus* sp. was assessed using synthetic medium and BSG hydrolysate. Attention was given to removing inhibitory compounds, including lignin-derived phenolics, hydroxymethylfurfural, furfural, and acetic acid, through microbial detoxification during co-cultivation. Various fermentation temperatures (30, 34, and 37 °C) and initial cell concentrations (OD_{600} of 0.05 and 0.1) were tested. Both *P. polymyxa* and *Rhodococcus* sp. effectively removed inhibitory compounds present in the medium. Co-cultures with *Rhodococcus* sp. exhibited higher sugar consumption rates (1.01 vs 0.88 g/L·h) than *P. polymyxa* monoculture, efficiently utilizing glucose, xylose, and arabinose, producing 2,3-BDO and acetoin. In co-culture with *Rhodococcus* sp., concentration (3.7 g/L), yield (0.14 g/g) and productivity (0.10 g/L·h) of 2,3-BDO at 34 °C considerably surpassed that of the *P. polymyxa* monoculture, with an increase of up to 48 %. These findings highlight the potential of co-cultures, especially with *Rhodococcus* sp., to alleviate inhibitory compound impacts when using complex media for fermentation. This study represents the first exploration of 2,3-BDO and acetoin production from BSG hemicellulosic hydrolysates using co-cultures.

1. Introduction

Brewer's spent grain (BSG) is the most abundant solid residue generated from brewing (approx. 20 kg of wet BSG is generated for every 100 liters of beer produced) representing up to 85 % of the total industry's residues [1]. Despite BSG can be used as animal feed, its high moisture content, typically around 80 %, poses significant challenges in terms of storage and utilization. Consequently, it is prone to rapid spoilage and is often discarded in landfills [2]. However, due to the sugar-rich composition, this abundant and underutilized resource presents a significant opportunity to produce biofuels and bioproducts, including valuable compounds such as 2,3-butanediol (2,3-BDO) and acetoin.

2,3-BDO is a versatile bulk chemical known for its exceptional properties. It is colorless, odorless, transparent, hygroscopic, biodegradable, and highly soluble in water, alcohols, ketones, and ethers [3]. In addition, it is a key platform chemical with diverse applications across various industries such as in the production of solvents, cosmetics, and synthetic rubber [4,5]. It also serves as an antifreeze agent due to its low freezing point. Additionally, it acts as a food additive and plays a dual role in the fuel industry as both a liquid fuel and an "octane booster," enhancing gasoline performance [3,5,6]. Acetoin is a naturally occurring compound present in fruits, vegetable flours, wine, and various food products. It serves as a flavor enhancer in the food industry and a fragrance agent in cosmetics. Additionally, it plays an important role in the synthesis of α-hydroxyketone derivatives, including optically active ones, and is also used in the manufacturing of liquid crystal composites and pharmaceuticals [7].

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Currently, the industrial production of 2,3-BDO primarily relies on petroleum-derived hydrocarbons, such as butane and 2-butene [8]. In contrast, acetoin is synthesized through various chemical processes, including diacetyl partial reduction, 2,3-BDO selective oxidation, butan-2-one oxidation, alkaline hydrolysis, and acetaldehyde hydrogenation [9]. However, recent trends indicate a growing interest in the biological production of both 2,3-BDO and acetoin. Several factors are driving this shift, including the increasing cost of petroleum, environmental concerns, and safety considerations in food and cosmetic applications [3,5,6]. 2,3-BDO and acetoin-producing microorganisms belong to the genera *Paenibacillus, Bacillus*, *Ralstonia*, *Klebsiella*, and *Enterobacter*, among others [10–13]. Among these bacteria, *Paenibacillus polymyxa* stands out due to its non-pathogenic classification (biosafety level 1). In addition, this bacterium has shown high production yields for both 2,3-BDO and acetoin and can metabolize C5 and C6 sugars. These features make it suitable for the industrial production of 2,3-BDO and acetoin from lignocellulose-derived sugars [14].

The exploitation of lignocellulosic materials for microbial fermentation requires the use of pretreatment. To this purpose, dilute acid pretreatment has been widely used due to its effectiveness in solubilizing hemicellulosic sugars [15]. However, the release of inhibitory compounds during pretreatment, along with the monomeric sugars, poses a significant challenge in the fermentation of lignocellulosic hydrolysates as such inhibitors, including formic and acetic acids, furfural, hydroxymethylfurfural (HMF), and phenolic compounds, can be highly toxic to microorganisms [16]. To address this challenge, this work proposes the utilization of co-cultures with microorganisms capable of metabolizing potential inhibitory compounds. This approach could eliminate the need of detoxification steps such as activated charcoal, overliming, or ion-exchange resins, reducing process costs while increasing the yields and productivities of the primary fermentation product [17–19]. *Pseudomonas alloputida* and *Rhodococcus* sp. strains can degrade furans and phenolic compounds [20–22], which make these strains excellent candidates for utilization in co-cultures to detoxify hemicellulosic hydrolysates.

In this context, this study assessed the effectiveness of co-cultures involving *P. polymyxa* and either *P. alloputida* or *Rhodococcus* sp., to remove potential inhibitors from BSG hemicellulosic hydrolysate with the ultimate goal of enhancing the production of 2,3-BDO and acetoin. Different temperatures and initial cell concentrations were used for fermentation and the most suitable co-cultivation conditions were selected.

2. Materials and methods

2.1. Raw material

Brewer's spent grain (BSG) was kindly provided by Carlsberg Breweries (Copenhagen, Denmark). The biomass, derived from an 80 % barley malt and 20 % barley mixture, was stored at −20 °C until use. Prior to dilute acid pretreatment, BSG was oven-dried at 60 °C to a moisture content below 10 %. The biomass was then milled and sieved to 1 mm particle size.

2.2. Hemicellulosic hydrolysate

BSG hemicellulosic hydrolysate was obtained by dilute acid pretreatment using 1.25 % (w/v) sulfuric acid, 1:8 (w/v) solid:liquid ratio, for 17 min at 155 °C [1]. Reactions were carried out in a 600-mL Parr reactor (Parr Instr. Co., IL, USA). After pretreatment, the slurry was subjected to centrifugation (9000 g, 4 °C, 10 min, Sorvall Lynx 6000 Centrifuge) to remove the residual solid. Then, the pH of the supernatant was adjusted to 6.5 and the precipitate formed was removed by vacuum filtration. The liquid fraction obtained (BSG-H) was analyzed by HPLC for its content in sugars (glucose, xylose, and arabinose) and lignocellulose-derived inhibitory compounds (acetic and formic acids, furfural, 5-hydroxymethylfurfural (HMF), and phenolic compounds).

To increase the carbohydrate content, BSG-H was three times concentrated (BSG-CH) using a rotary evaporator (IKA, Staufen, Germany). The BSG-CH then obtained was filter-sterilized using 0.2 µm vacuum PES filter units (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and its sugar and inhibitor contents were also determined by HPLC.

2.3. Microorganism, inoculum, and fermentation media

Three bacterial strains were employed in this work: *Paenibacillus polymyxa* DSM 365 (P), *Pseudomonas alloputida* DSM 6125 (Pput), and *Rhodococcus* sp. DSM 43001 (R). The microorganisms were obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). Strains were stored in glycerol (20 % sterile glycerol) at −80 °C.

The microorganisms were cultivated separately using an M9 minimal pre-culture medium consisting of: salt solution (12.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl), trace elements (0.05 g/L EDTA, 0.083 g/L $FeCl_3.6H_2O$, 0.084 mg/L $ZnCl_2$, 0.013 mg/L $CuCl_2.2H_2O$, 0.01 mg/L $CoCl_2.6H_2O$, 0.01 mg/L HBO_3, and 0.016 mg/L $MnCl_2.4H_2O$), 0.1 mM $MgSO_4$, 0.01 mM $CaCl_2.2H_2O$, and 4 % glucose. The M9 medium was sterilized by filtration using 0.2 µm vacuum PES filter units (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). *P. polymyxa* was cultivated at 37 °C, while *P. alloputida* and *Rhodococcus* sp. were grown at 30 °C. Assays were carried out at 250 rpm for 48 h in 50 mL Erlenmeyer flasks containing 10 mL of M9 medium.

Three semi-defined media (SM) were used for fermentation: 1. a semi-defined medium prepared with 55 g/L of glucose (G-SM), which is the preferred sugar for *P. polymyxa* [4]; 2. a semi-defined medium consisting of C5 and C6 sugars (xylose, 13.5 g/L; arabinose, 7.0 g/L; and glucose, 2.4 g/L), which was named mixed-sugars semi-defined medium (XAG-SM); and 3. a three-fold concentrated medium (xylose, 42.1 g/L; arabinose, 18.8 g/L; and glucose, 10.2 g/L), which was called concentrated mixed-sugars semi-defined medium (XAG-C-SM). The sugar composition of XAG-SM was determined based on the characterization of BSG-H. All semi-defined media were supplemented with 10 g/L yeast extract, 0.2 g/L $MgSO_4$, 3 g/L $(NH_4)_2SO_4$, 100 mM potassium phosphate buffer (pH 6), and 3 mL trace elements [23]. BSG-H and its concentrated version (BSG-CH) were also used as fermentation media directly, without supplementation with additional components, unlike semi-defined media.

To be used as fermentation medium, all media were sterilized by filtration (using 0.2 µm vacuum PES filter units).

2.4. Fermentation assays

Fermentations were carried out both using monocultures of each microorganism and co-cultures (*P. polymyxa* with *P. alloputida* or *P. polymyxa* with *Rhodococcus sp*). Additional assays containing only medium without any microbial inoculation were also carried out as negative controls. For fermentation, an inoculum load of 0.05 OD_{600} (optical density units) was used for each microorganism, both in single cultures and co-cultures (P + Pput or P + R). In addition, 0.1 OD_{600} of *P. alloputida* and *Rhodococcus* sp. were also used in co-cultures with *P. polymyxa* (P + $Pput^+$ or P + R^+) to observe whether there was any effect related to the initial inoculum load. Three different temperatures (30 °C, 34 °C and 37 °C) were tested, as 37 °C is considered the optimal temperature for *P. polymyxa* [23,24], while *P. alloputida* and *Rhodococcus* sp. are reported to work optimally in the range of 28–30 °C [25]. The fermentation assays were carried out in a Growth Profiler 960 (Enzyscreen, Heemstede, The Netherlands) using 96-well microplates with a working volume of 250 µL and headspace of 100 µL for each well. A stirring speed of 250 rpm was used in all cases. During fermentation, data were collected every 20 min for 72 h.

The experimental design and sampling approach used for fermentation were precisely structured to ensure the accuracy and reliability of

data collection. In brief, each plate was prepared to have triplicates (within three wells) in all conditions tested. This setup allowed us to replicate each unique experimental condition a total of 30 times (30 wells in independent plates). This high level of replication facilitated the collection of samples at precise time points throughout the 72-h fermentation process, by withdrawing one plate at each time (0, 12, 16, 20, 24, 28, 32, 36, 42, 48, and 72 h). The collected samples underwent centrifugation (13,500 rpm for 10 min) and the obtained supernatants were used for quantification of sugars, 2,3-BDO, ethanol, acetoin, HMF, furfural, and total phenolic compounds.

The cell content was estimated by converting the data of the growth profiler into cell dry weight (CDW; g/L). In the growth profiler, the data of bacterial growths are expressed as green value (GV) units. At the end of each experiment, the GV units were converted to OD_{600} values using Eq. (1):

$$OD = a(GV_{value} - GV_{blank})^b + c(GV_{value} - GV_{blank})^d + e(GV_{value} - GV_{blank})^f \quad (1)$$

where the constants were the following: for *P. polymyxa:* $a = 0.0199$, $b = 1$, $c = 3.87 \times 10^{-5}$, $d = 2.06$, $e = 1.828 \times 10^{-19}$, $f = 9.13$, and $GV_{blank} = 9.04$; for *P. alloputida:* $a = 0.0118$, $b = 1$, $c = 2.59 \times 10^{-5}$, $d = 2.38$, $e = 3.915 \times 10^{-19}$, $f = 8.95$, and $GV_{blank} = 9.29$; for *Rhodococcus* sp.*:* $a = 0.0218$, $b = 1$, $c = 2.68 \times 10^{-5}$, $d = 1.74$, $e = 2.905 \times 10^{-19}$, $f = 9.19$, and $GV_{blank} = 8{,}66$; for *P. polymyxa* +*Rhodococcus* sp.*:* $a = 0.02085$, $b = 1$, $c = 3.27 \times 10^{-5}$, $d = 1.9$, $e = 2.366 \times 10^{-19}$, $f = 9.16$, and $GV_{blank} = 8.8$; for *P. polymyxa*+*P. alloputida* $a = 0.0158$, $b = 1$, $c = 3.23 \times 10^{-5}$, $d = 2.22$, $e = 2.8715 \times 10^{-19}$, $f = 9.04$, and $GV_{blank} = 9.165$. In the co-cultures, it was assumed that the two bacteria contributed equally to the growth. The equations that correlated GV units to OD were obtained by performing a calibration of each strain in the LB medium.

For the CDW determination, each strain was cultivated in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) until stationary phase and then centrifuged (13,000 rpm for 5 min). The cell pellet was resuspended in fresh LB medium to concentrate the suspension and the OD_{600} value was measured. Calibration curves were constructed using CDW and OD_{600} data. The slope values obtained for each strain were used to calculate the CDW of the growth profiler cultures.

2.5. Analytical methods

The content of extractives, structural carbohydrates, lignin, and ash in BSG were determined according to the National Renewable Energy Laboratory (NREL) analytical methodology [26–28]. The total starch content was analyzed by the "Total Starch Assay Kit" method (Megazyme, Ireland), while the total protein content was measured by the Kjeldahl acid digestion method (described in AOAC (1990) 955.04), applying 6.25 as conversion factor.

The concentration of sugars (glucose, xylose, and arabinose), inhibitory compounds (acetic acid, formic acid, furfural, and HMF), and fermentation products (2,3-BDO, ethanol, and acetoin) were determined by High-Performance Liquid Chromatography (HPLC) using a Dionex Ultimate 3000 high-performance liquid chromatograph HPLC + focused system (Dionex Softron GmbH, Germering, Germany). A refractive index detector (Shodex RI-101) and a Bio-Rad Aminex column HPX-87H (300 mm × 7.8 mm) (at 60 °C) were used, being 5 mM H_2SO_4 the mobile phase employed with a flow of 0.6 mL/min. The total content of phenolic compounds was measured by the Folin-Ciocalteu method.

Analytical determinations were carried out in triplicate and average results are shown.

2.6. Data analysis

The statistical differences were determined by analysis of variance (ANOVA) at a confidence level of 95 % ($p < 0.05$). The Tukey multiple range test was used to find significantly different means.

3. Results and discussion

3.1. Composition of BSG and BSG hydrolysates

BSG used in the present study was composed of (wt%, dry matter): 17.4 ± 1.8 cellulose, 16.1 ± 0.2 hemicellulose, 16.4 ± 0.1 acid-insoluble lignin (AIL), 1.8 ± 0.3 acid-soluble lignin (ASL), 14.9 ± 2.4 extractives, 3.8 ± 0.1 ash, 2.2 ± 0.1 acetyl groups, 7.9 ± 0.9 fat, 1.8 ± 0.1 starch, and 17.4 ± 0.3 protein. After dilute acid pretreatment, xylose was the most abundant sugar present in the hemicellulosic hydrolysate, representing 60 % of the total sugars in BSG-H (Table 1). Phenolic compounds (1.50 g/L) and acetic acid (0.58 g/L) showed the highest concentrations among the inhibitory compounds identified in the hydrolysate. Acetic acid is typically released from the hemicellulosic structure, while phenolic compounds are produced due to the partial breakdown of lignin [16,19].

To increase the content of sugars in the hydrolysate (BSG-H) and potentially enhance the production of 2,3-BDO and acetoin, BSG-H was concentrated approximately three times, resulting in a hydrolysate (BSG-CH) with higher content of sugars than BSG-H (Table 1). However, along with the higher sugar content, higher levels of inhibitory compounds were also obtained since these compounds, except furfural, are not volatile under the conditions applied for hydrolysate concentration (rotary evaporator at 60 °C under vacuum condition).

3.2. Production of 2,3-BDO and acetoin from glucose-rich semi-defined medium

To evaluate the ideal combination of medium, temperature, and bacteria for the production of 2,3-BDO and acetoin, three semi-defined culture (SM) media were used in the fermentation tests at 30 °C, 34 °C, and 37 °C, using bacterial monocultures and co-cultures. Results revealed no production of 2,3-BDO and acetoin in any of the SM media fermented at 30 ºC. However, *P. polymyxa* was able to produce 2,3-BDO and acetoin, both in monoculture and in co-culture with *Rhodococcus* sp., at 34 °C and 37 °C (Fig. 1). The highest concentrations of 2,3-BDO (12.6 g/L) and acetoin (13.3 g/L) were achieved in monoculture at 37 °C, with an increase of 90.9 and 29.1 %, respectively, considering the monoculture at 34 ºC. Similar to other bacteria such as *Bacillus licheniformis* and *Raoultella ornithinolytica* B6, *P. polymyxa* synthesizes 2,3-BDO and acetoin only under specific conditions of pH, temperature, and agitation [29,30], which could explain the results observed at 37 °C, since this is considered the optimal temperature for *P. polymyxa* [23,24].

Co-culture of *P. polymyxa* with *Rhodococcus* sp. did not significantly ($p > 0.05$) affect the production of 2,3-BDO (37 ºC: 12.2–12.6 g/L; 34 ºC: 6.6–6.8 g/L) and acetoin (37 ºC: 12.4–13.3 g/L; 34 ºC: 10.3–10.5 g/L), except when an initial OD concentration of 0.1 of *Rhodococcus* sp. was used ($P + R^+$) (for example, at 37 ºC, 9.6 vs 12.2–12.6 g/L 2,3-BDO, and 9.7 vs 12.4–13.3 g/L acetoin) (Fig. 1). In all cases studied, an initial similar rate of 2,3-BDO production was observed, followed by a decrease in its concentration over the time course of culturing, particularly in the

Table 1
Composition of BSG hemicellulosic hydrolysates in the original (BSG-H) and concentrated (BSG-CH) forms.

Component	BSG-H	BSG-CH
Sugars (g/L)		
Glucose	2.20 ± 0.11	7.93 ± 0.25
Xylose	14.61 ± 0.15	38.68 ± 0.34
Arabinose	7.41 ± 0.08	16.71 ± 0.14
Inhibitors (g/L)		
Formic acid	0.05 ± 0.01	0.18 ± 0.00
Acetic acid	0.58 ± 0.05	2.17 ± 0.09
HMF	0.02 ± 0.00	0.23 ± 0.05
Furfural	0.21 ± 0.02	0.26 ± 0.01
Total phenolics	1.50 ± 0.12	3.24 ± 0.15

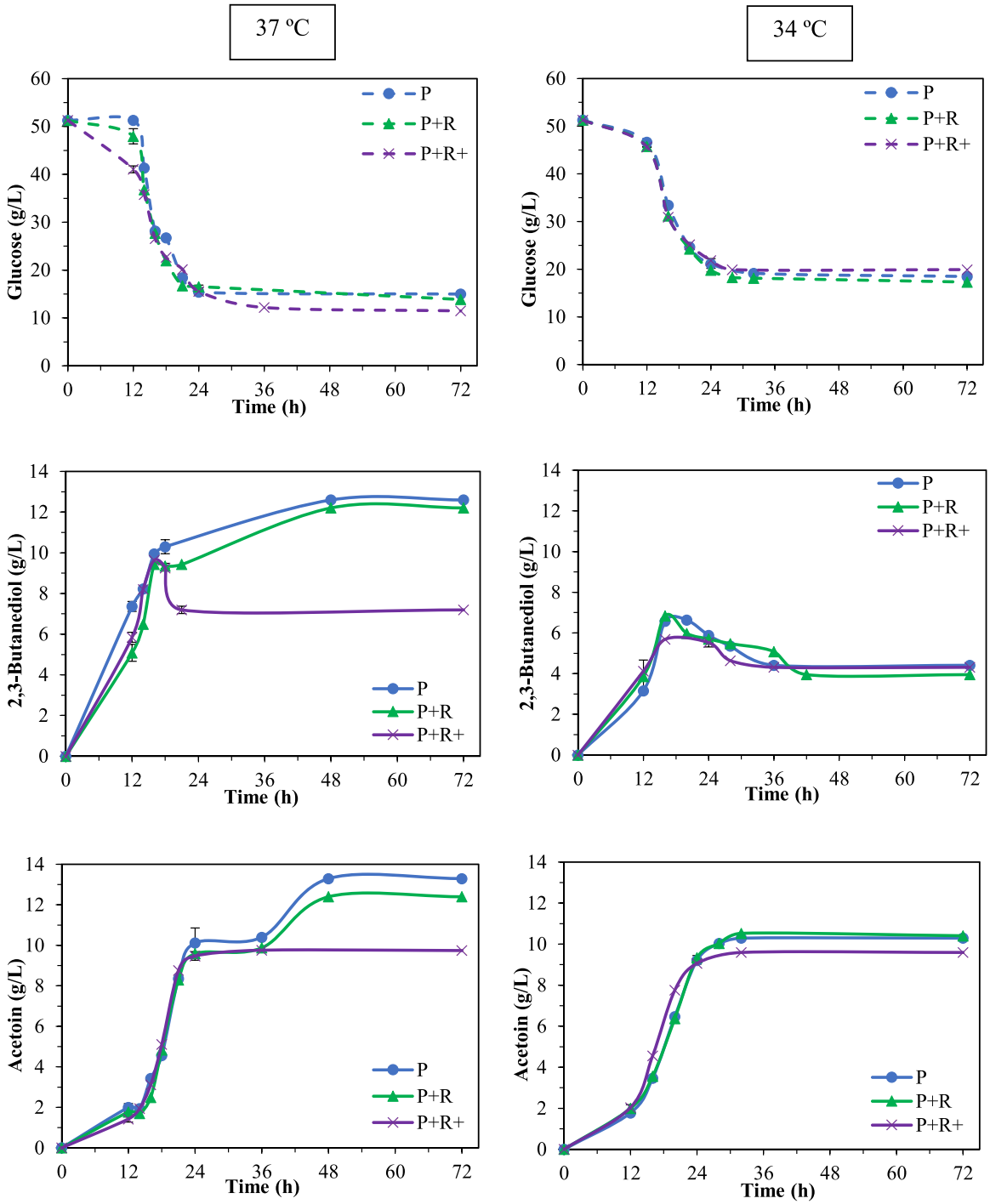

Fig. 1. Fermentation using glucose semi-defined medium (G-SM) at 37 °C and 34 °C, by single cultures of *P. polymyxa* (P) and in co-culture with *Rhodococcus* sp. (R). In co-culture, *Rhodococcus* sp. was inoculated at initial optical densities of 0.05 (P + R) or 0.1 (P + R^{+}). Average values and standard deviation for samples by triplicate are shown.

case of P + R+ at 37 ºC. In this case, the unbalance in the initial inoculum size might have altered the interaction dynamics between the strains, leading to an advantage in the substrate consumption by *Rhodococcus* sp., and reducing the product formation, as a consequence. The decrease in 2,3-BDO concentration over the time course of culturing could also be due to a change in the balance 2,3-BDO↔acetoin, since it coincides with an increase in acetoin production through the fermentation process. These findings highlight the importance of ensuring favorable conditions for *P. polymyxa* during the initial growth phase of co-culture.

3.3. Production of 2,3-BDO and acetoin from mixed-sugars semi-defined medium

In all the fermentations using semi-defined medium containing mixture of C5 and C6 sugars (XAG-SM), most of the sugars were rapidly depleted within 16–20 h, except for the single cultures of *P. alloputida* and *Rhodococcus* sp., where the maximum consumptions were 34 % and 30 %, respectively, at 34 °C (Fig. 2). The substrate consumption rates may be related to the type of monosaccharides present in the medium, as unlike glucose, which undergoes glycolysis, C5-sugars such as xylose

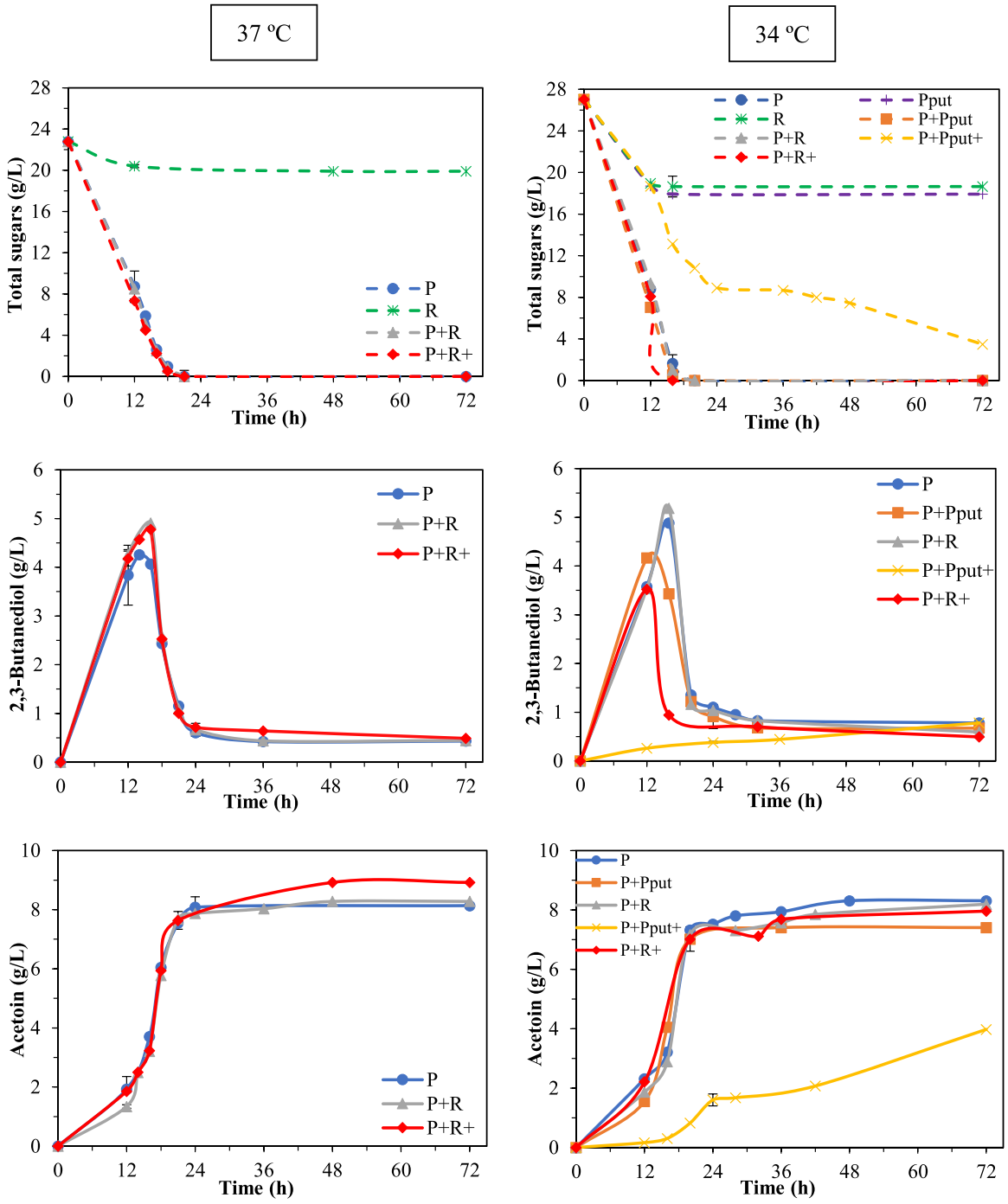

Fig. 2. Fermentation assays using mixed-sugars (C5 and C6) semi-defined medium (XAG-SM) at 37 °C and 34 °C by single cultures of *P. polymyxa* (P), *P. alloputida* (Pput), and *Rhodococcus* sp. (R), as well as by co-cultures of *P. polymyxa* + *P. alloputida* (P + Pput) and *P. polymyxa* + *Rhodococcus* sp. (P + R). Inoculum loads of *P. alloputida* and *Rhodococcus* sp. in co-culture were 0.05 OD_{600} (P + Put and P + R) and 0.1 OD_{600} (P + Put^{+} and P + R^{+}). Average values and standard deviation for samples by triplicate are shown.

and arabinose are challenging for most bacteria as they require additional enzymes and metabolic adaptation [31].

It is worth mentioning that in the present study, *P. polymyxa* co-metabolized the pentose sugars with a similar consumption rate (data not shown). This was also observed by Okonkwo et al. [4], who fermented wheat straw hydrolysates using *P. polymyxa* DSM 365. This ability of *P. polymyxa* is useful when hemicellulosic hydrolysates are used for fermentation, as C5 sugars are particularly abundant in such media. Moreover, in co-culture, this ability of *P. polymyxa* may provide a competitive advantage over other bacteria, such as *P. alloputida* and *Rhodococcus,* which did not use C5 sugars (data not shown).

When the concentration of total sugars was low, *P. polymyxa* started consuming the produced 2,3-BDO, leading to an increase in the production of acetoin (Fig. 2). Such behavior was not observed when glucose semi-defined medium (G-SM) was used (Fig. 1) probably because the initial concentration of glucose was higher than the total concentration of sugars used in XAG-SM (Fig. 2), and glucose was not totally consumed in that case. Overall, there was no significant difference ($p > 0.05$) between monoculture and co-culture on the production of 2,3-BDO (37 ºC: 4.3–4.9 g/L; 34 ºC: 3.5–5.2 g/L) and acetoin (37 ºC:

8.1–8.9 g/L; 34 ºC: 7.4–8.3 g/L), except for the co-culture P + Pput^{+} at 34 °C, which resulted in significantly ($p < 0.05$) lower concentrations of 2,3-BDO (0.8 vs 3.5–5.2 g/L) and acetoin (4.0 vs 7.4–8.3 g/L) (Fig. 2).

This result suggests that the dominance of *P. alloputida* in the co-culture overcame the metabolic advantage of *P. polymyxa* to use xylose and arabinose, causing a negative impact on the production of 2,3-BDO.

Since the use of *P. putida* and *Rhodococcus* sp. in co-culture with *P. polymyxa* did not negatively influence the production of 2,3-BDO and acetoin, this fermentation process can be considered robust to produce such compounds. In addition, it can be of interest for industrial applications, because it led to a very fast maximum production of 2,3-BDO, within 12–16 h in practically all cases (Table 1s - Supplementary material).

In concentrated C5-C6 semi-defined medium (XAG-C-SM), xylose, glucose, and arabinose were present in 42 g/L, 7.9 g/L, and 18.8 g/L, respectively. With this composition, glucose was completely metabolized, while arabinose consumption varied between 60 % and 97 %, and xylose consumption between 43 % and 67 %. This sugar utilization pattern can be attributed to an excess of xylose in the fermentation medium compared to glucose and arabinose, as well as to a preference of *P. polymyxa* for glucose and arabinose, regulated by carbon catabolite

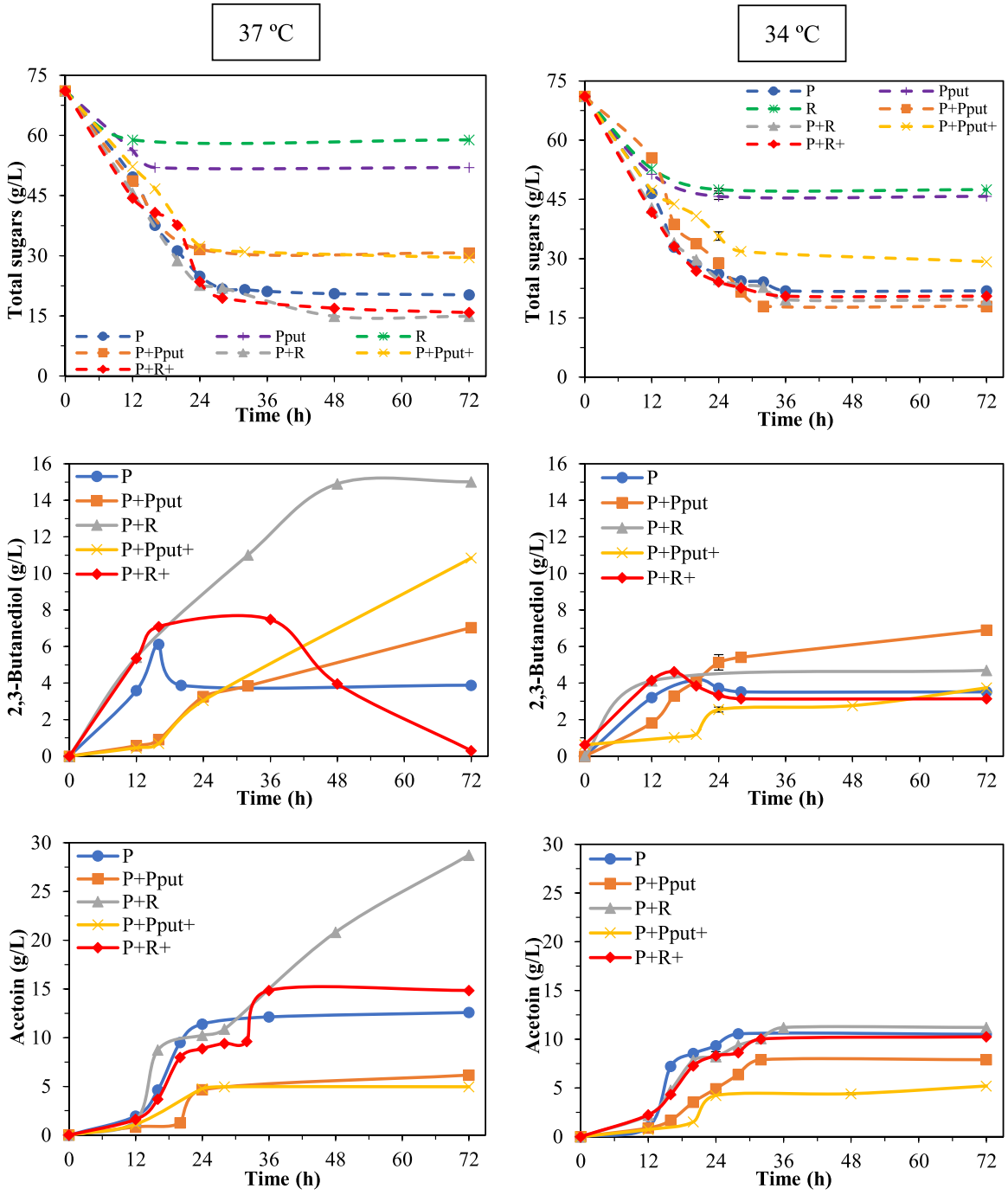

Fig. 3. Fermentation assays using concentrated mixed-sugars (C5 and C6) semi-defined medium (XAG-C-SM) at 37 °C and 34 °C by single cultures of *P. polymyxa* (P), *P. alloputida* (Pput), and *Rhodococcus* sp. (R), as well as co-cultures of *P. polymyxa* + *P. alloputida* (P + Pput) and *P. polymyxa* + *Rhodococcus* sp. (P + R). Inoculum loads of *P. alloputida* and *Rhodococcus* sp. in co-culture were 0.05 OD_{600} (P + Pput and P + R) and 0.1 OD_{600} (P + Pput^{+} and P + R^{+}). Average values and standard deviation for samples by triplicate are shown.

repression (CCR). According to Ma et al. [32], *P. polymyxa* has a preference for metabolizing glucose over xylose and arabinose due to the shorter time required for the synthesis of enzymes associated with glycolysis, in contrast to those required for xylose or arabinose metabolism (pentose-phosphate pathway).

Single cultures of *P. alloputida* and *Rhodococcus* sp. in XAG-C-SM presented relatively low sugar consumption (17 and 27 %, respectively) (Fig. 3).

P. putida M2 has been reported as being able to consume hexose and pentose sugars as well as aromatic compounds [33]. However, a recent study on the cultivation of this strain in a mixture of glucose and aromatic compounds (p-coumarate and ferulic acid) revealed an incomplete consumption of substrate [34]. The sugar consumption rates found in our study suggest that there was an excess of sugar mixture in the medium, which activated the carbon catabolite control (CCC). It is known that CCC inhibits the expression of the pathways for nonpreferred compounds but can also generate an important reorganization of the metabolism that requires the activation of several genes [35].

As for the production of 2,3-BDO and acetoin, distinct behavior was observed in co-cultures of *P. polymyxa* with *Rhodococcus* sp. or with *P. alloputida*. The utilization of the P + R co-culture at 37 °C notably boosted the levels of 2,3-BDO and acetoin, reaching 15 g/L and 28.7 g/L, respectively. This result represents a significant increase ($p < 0.05$) when compared to the production by monoculture of *P. polymyxa*, which

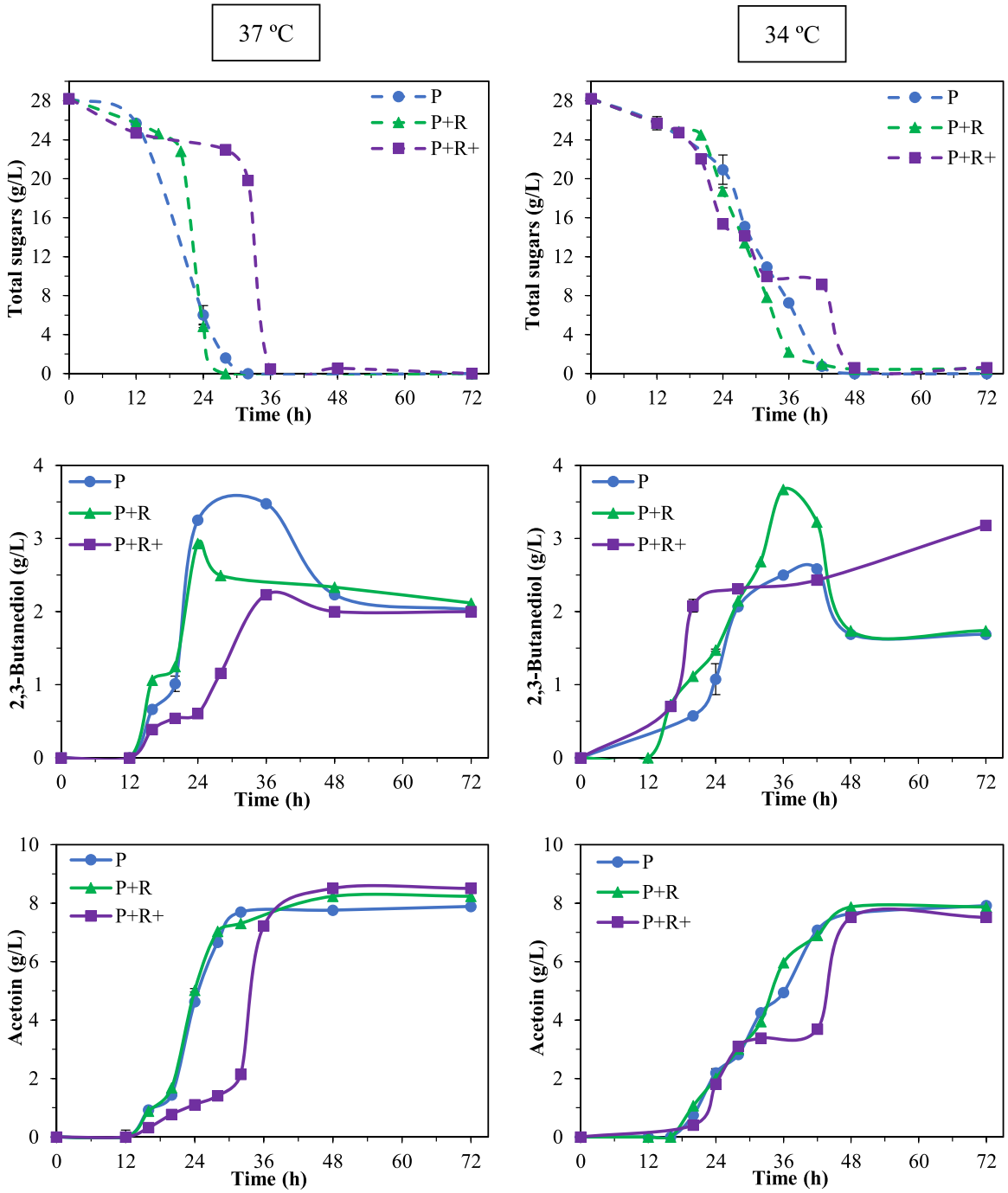

Fig. 4. Fermentation assays using BSG hemicellulosic hydrolysate (BSG-H) at 37 °C and 34 °C by single culture of *P. polymyxa* (P) and by co-culture of *P. polymyxa* with *Rhodococcus* sp. (P + R). Inoculum loads of *Rhodococcus* sp. in co-culture were 0.05 OD_{600} (P + R) and 0.1 OD_{600} (P + R^{+}). Average values and standard deviation for samples by triplicate are shown.

yielded 6.1 g/L of 2,3-BDO and 12.6 g/L of acetoin (Fig. 3). The results also showed a significant effect of the temperature ($p < 0.05$), with the highest concentrations of 2,3-BDO and acetoin being achieved at 37 °C compared to 34 °C (2,3-BDO: 6.1–15.0 vs 3.7–6.9 g/L; acetoin: 6.2–28.7 vs 5.2–11.2 g/L) (Fig. 3 and Table 1S - Supplementary material). This result is in line with the cultivation requirements of *P. polymyxa*, which has its optimal temperature at 37 °C, as discussed before.

3.4. Production 2,3-BDO and acetoin from BSG hemicellulosic hydrolysates

Two BSG hemicellulosic hydrolysates, concentrated (BSG-CH) and non-concentrated (BSG-H), were used for fermentation to produce 2,3-BDO and acetoin by monoculture and co-cultures at 30, 34 and 37 °C. Similar to what had been observed from synthetic media fermentation,

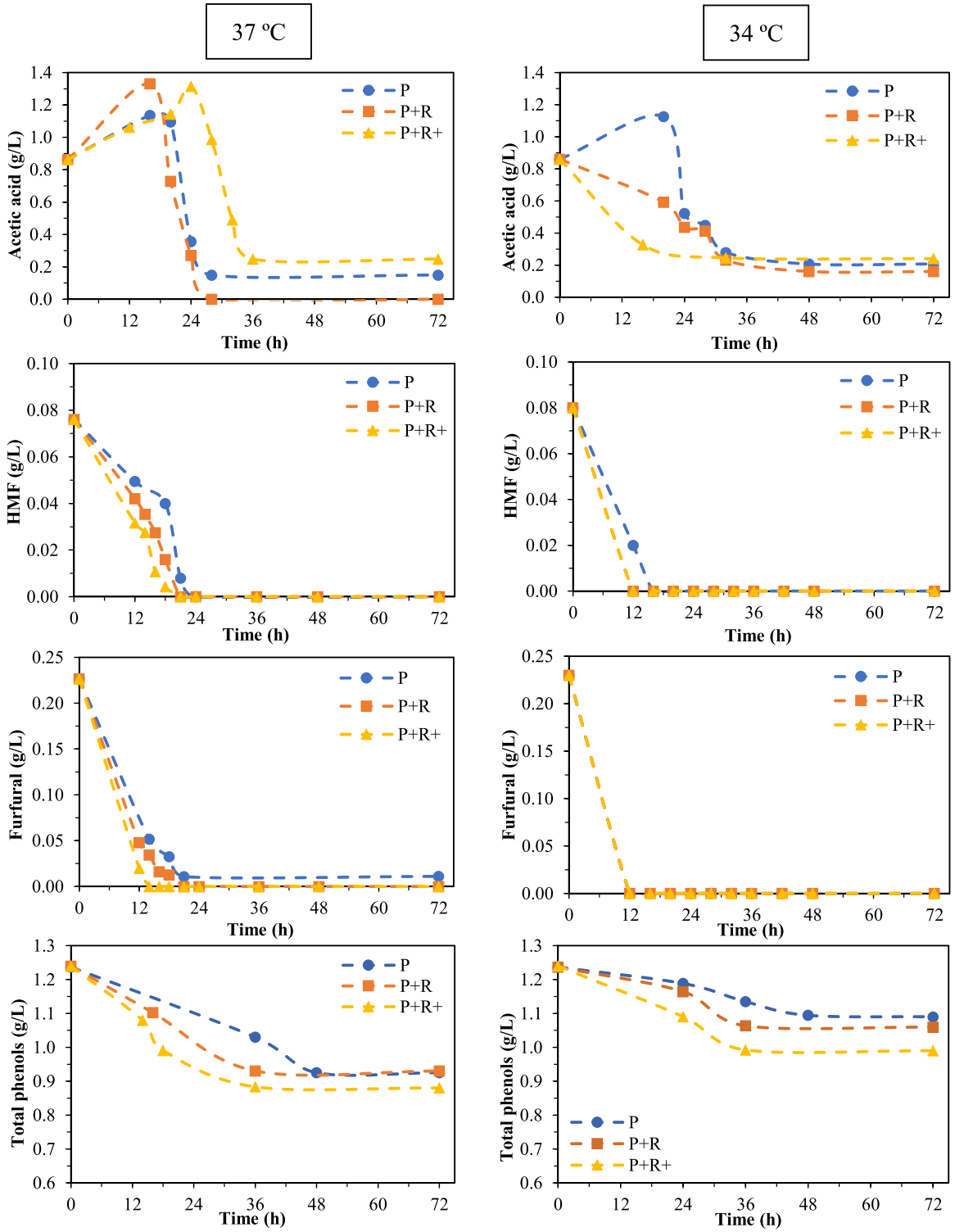

Fig. 5. Profiles of metabolites (acetic acid, HMF, furfural, and total phenols) in fermentation assays in BSG hemicellulosic hydrolysate (BSG-H): Fermentation assays were conducted at 37°C and 34°C using monoculture of *P. polymyxa* (P) and co-culture of *P. polymyxa* with *Rhodococcus* sp. (P + R). Inoculum loads of *Rhodococcus* sp. in co-culture were 0.05 OD_{600} (P + R) and 0.1 OD_{600} (P + R^{+}). Average values and standard deviation for samples by triplicate are shown.

no production of 2,3-BDO and acetoin was detected at 30 °C across all bacterial combinations tested in both BSG-H and BSG-CH. Moreover, when BSG-H hydrolysate was used for co-cultures with *P. alloputida*, 2,3-BDO and acetoin were also not produced at 34 and 37 °C. On the other hand, 2,3-BDO (2.2–3.7 g/L) and acetoin (7.5–8.5 g/L) were produced in co-cultures of *P. polymyxa* with *Rhodococcus* sp. achieving an even higher concentration of 2,3-BDO than the monoculture when the fermentation was carried out at 34 °C (3.7 vs 2.5 g/L) (P + R assay, Fig. 4).

When comparing the production of 2,3-BDO in BSG-H with that observed in XAG-SM, which had similar sugar composition but no inhibitory compounds, it was noted that 2,3-BDO concentrations (37 ºC: 2.2–3.5 vs 4.3–4.9 g/L; 34 ºC: 2.5–3.7 vs 3.5–5.2 g/L), yields (37 ºC: 0.08–0.11 vs 0.23–0.25 g/g; 34 ºC: 0.12–0.14 vs 0.19–0.21 g/g), and productivities (37 ºC: 0.062–0.080 vs 0.299–0.307 g/L·h; 34 ºC: 0.044–0.102 vs 0.293–0.348 g/L·h) in BSG-H were generally lower than in XAG-SM (Figs. 2 and 4, and Table 1S of the Supplementary material). This outcome can likely be attributed to the presence of the inhibitory compounds, mainly phenolic compounds, in BSG-H, which may have caused some toxicity for *P. polymyxa* [36].

In this sense, when concentrated BSG hydrolysate (BSG-CH) was used as fermentation medium, no microbial growth nor 2,3-BDO and acetoin production were observed. The high content of total phenolic compounds (3.24 g/L) in BSG-CH (Table 1) is likely responsible for its toxicity to *P. polymyxa*, *P. alloputida*, and *Rhodococcus* sp. The presence of phenolic compounds in lignocellulosic hydrolysates has been recognized as toxic to microorganisms, even at low concentrations, affecting cell growth and metabolism [16]. Concentrations of phenolic compounds exceeding 3 g/L are highly toxic to microorganisms such as *Debaryomyces hansenii* and *Candida guilliermondii* [37]. Furthermore, the combined effects of various inhibitory compounds can be even worse to microorganisms. So, the inhibition of *P. polymyxa*, *P. putida,* and *Rhodococcus* sp, could have been boosted by the synergistic effect of different inhibitory compounds in the hydrolysate [38].

Analyses of the results obtained by fermentation of BSG-H (non-concentrated) revealed that *P. polymyxa* partially consumed phenolic compounds (11.9–25.2 %), acetic acid (75.9–82.7 %), furfural (95.1–100 %), and HMF (100 %) during the fermentation. However, the concentration of these inhibitory compounds in the medium was even lower when co-culture with *Rhodococcus* sp. (especially (P + R^+)) was used, with an increase in the consumption of phenolic compounds (20.2–29.0 %) and acetic acid (81.4–100 %) (Fig. 5). HMF and furfural were rapidly consumed (between 12 and 24 h of fermentation) in all cases, with co-cultures exhibiting faster consumption. Interestingly, *P. polymyxa* demonstrated the ability to metabolize these molecules when their concentration was < 2 g/L. Similar to furfural and HMF, phenolic compounds were also faster consumed in co-cultures. Ljubas et al. [39] observed that *P. polymyxa* DSM 365 can extensively metabolize certain phenolic compounds, such as p-coumaric acid, while it shows a lower but still considerable consumption rate for others, like vanillic acid and vanillin.

In the case of acetic acid, an accumulation of this compound was observed during the first 18 h of fermentation, with a subsequent consumption of up to 80 % of its initial concentration, especially in co-culture P + R at 24 h. The biosynthesis of 2,3-BDO from pyruvate competes with the formation of various by-products, such as organic acids [40]. The accumulation of acid leads to acidification of the intracellular environment, and 2,3-BDO plays a crucial role in the neutralization of the pH derived from organic acid production [6]. According to Nakashimada et al. [41], acetate concentrations below 12 g/L can positively induce 2,3-BDO production and mitigate the inhibitory effect of other types of compounds in fermentation medium [42].

It is worth highlighting that despite *P. polymyxa* showed important abilities in reducing the concentration of several inhibitory compounds present in BSG hydrolysate, a substantial contribution was given by its co-cultivation with *Rhodococcus* sp. This outcome, together with the results obtained for 2,3-BDO and acetoin production, proved the effectiveness of the co-cultivation strategy in the fermentation of biomass-derived media.

4. Conclusion

In this study, BSG hemicellulosic hydrolysate was used as a fermentation medium for 2,3-BDO and acetoin production by single cultures of *P. polymyxa* and in co-culture with *P. alloputida* or *Rhodococcus* sp. *Rhodococcus* sp., in particular, presented great capacity to remove inhibitory compounds from the fermentation medium when used in co-culture with *P. polymyxa*. Co-cultures of *Rhodococcus* sp. with *P. polymyxa* exhibited higher sugar consumption rates, efficiently utilizing glucose, xylose, and arabinose at both 34 °C and 37 °C. In addition, 2,3-BDO production at 34 °C was enhanced in co-culture compared to monoculture of *P. polymyxa*. These findings provide valuable insights into the utilization of hemicellulosic hydrolysates for 2,3-BDO and acetoin production, highlighting the potential benefits of using co-cultures in complex substrate fermentations to overcome medium toxicity.

CRediT authorship contribution statement

Linares Juan Carlos L.: Writing – original draft, Investigation, Data curation. **Dragone Giuliano:** Writing – review & editing, Supervision. **Yamakawa Celina K:** Supervision. **Mussatto Solange I.:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **García-Cubero Maria Teresa:** Writing – review & editing, Supervision, Funding acquisition. **Rama Erlinda:** Writing – original draft, Investigation, Data curation. **Perez Caroline L:** Writing – original draft, Investigation. **Coca Mónica:** Writing – review & editing, Supervision.

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.nbt.2025.03.006.

Data availability

Data will be made available on request.

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