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ORIGINAL ARTICLE

Biorefinery based on discarded red beetroot: production of bioactive compounds and 2,3-butanediol

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Abstract

Discarded red beetroot (DRB) is an organic waste produced in the food processing industry, rich in phytochemicals and sugars. This study compares biorefinery schemes for the valorisation of DRB. Two different alternatives were compared to select the most favourable considering global yields and production costs. In scenario 1 (multi-product biorefinery), 0.9 g of phenolics and 0.8 g of betalains were recovered from 100 g of DRB (on dry basis). After extraction, the solid fraction was fed to enzymatic hydrolysis and fermentation to obtain 2,3-butanediol (2,3-BDO) with *Paenibacillus polymyxa*, achieving a global yield of 9.3 g/100 g DRB. In scenario 2, all the DRB was subjected to enzymatic hydrolysis and subsequent fermentation with *P. polymyxa*, obtaining a 2,3-BDO global yield of 25.5 g from 100 g DRB. The economic evaluation indicated that a multi-product biorefinery could be the most cost-effective alternative for DRB valorisation, leading to minimum selling prices competitive with the petrochemical route. Thus, the potential for the efficient use of DRB in an integrated biorefinery for the production of high value-added products was demonstrated.

Keywords Red beetroot · 2,3-Butanediol · Phenolics · Betalains · *Paenibacillus polymyxa* · Biorefinery

1 Introduction

Fruit and vegetable waste (FVW) consists mostly of inedible parts (stems, seeds and peel), but also rotten, damaged or poor-quality edible parts [1]. It is estimated that 857 million tons (Mt) of FVW are discarded annually, which corresponds to 40–50% of the total world production of fruit and vegetables [2]. FVW is traditionally used for animal feed [3]. However, about 60% of the generated FVW is dumped in landfills [2]. The decomposition of organic matter produces methane as a primary product, a greenhouse gas (GHG) with a global warming potential 25 times higher than CO_2 [3]. As a result, FVW is responsible for about 11% of the annual global methane emissions into the atmosphere [2]. Considering that Europe generates over 100 Mt of food processing waste every year, the use of by-products as raw material to produce bioproducts and biofuels has a great potential [4]. FVW can be used as a carbon source for bacterial fermentation, as it contains free sugars and polysaccharides. Prior to the fermentation of the carbohydrate fraction, high value-added compounds can be recovered [5].

World red beet production has reached 270 Mt per year [6]. In 2017, around 208 Mt of beetroot were produced in Europe [7]. Huge amounts of residue, such as discarded red beetroot (DRB), are generated each year from the processing industries, accounting for as much as 50% of the total by-products [6]. Beetroot is rich in bioactive compounds such as polyphenols [5]. Phenolic compounds are phytochemicals produced by plants as an integral part of their structure [1]. Phenolic compounds are excellent bioproducts for the development of natural ingredients that can replace synthetic preservatives. They can be used in functional and nutraceutical preparations due to their antioxidant and

Highlights
- Comparison of biorefinery scenarios for discarded red beetroot.
- In a multi-product biorefinery, phenolics, betalains and 2,3-BDO were produced.
- High-value co-products improve the economic profitability.
- The multi-product biorefinery could compete with the petrochemical 2,3-BDO pathway.

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antimicrobial capacity, as well as their anti-inflammatory, anti-carcinogenic, anti-diabetic, anti-allergic and other biological properties [1]. On the other hand, beetroot is the main source of betalains, which are water-soluble pigments. There are two types of betalains depending on the chemical structure: betaxanthins and betacyanins [8]. Betalains present antioxidant activity, anti-inflammatory, antimicrobial, anticancer and antiviral effects [9]. Betalains are one of the most widely used natural plant-derived pigments in the food industry [10].

Growing consumer demand for sustainable products has led to the interest of researchers in the production of natural food colorants. According to Popa et al. [9], the main method used to extract colorants is solid–liquid extraction. Since betalains are water-soluble pigments, they can be extracted using acidified water, a water-alcohol mixture, or an acidified alcoholic solution [9]. The main parameters that influence the efficiency of the extraction are the solvent, temperature, extraction time and solid-to-solvent ratio [9]. Ethanol–water mixtures as an extraction solvent to recover polar compounds are interesting as they are generally recognised as safe solvents [7].

2,3-Butanediol (2,3-BDO) is an industrial chemical essential in the production of plastics, solvents, cosmetics, foods and fuels [11], as well as in agriculture and pharmaceuticals for producing various polymers and derivatives [12]. Specifically, it is a forerunner in the production of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticisers, foods and pharmaceuticals [13]. 2,3-BDO can be used as an antifreeze agent as it has a freezing point of − 60 °C [14]. It has a high heating value of 27.2 kJ/g, which compares favourably with other liquid fuels such as methanol (22.1 kJ/g) or ethanol (29.1 kJ/g) [14]. 2,3-BDO can also be used as a platform chemical to manufacture methyl ethyl ketone (MEK) [15]. It can also be transformed into 1,3-butadiene, a synthetic rubber monomer, or acetoin, which can be oxidised to diacetyl, a valued product used as a flavouring agent in food products [14]. The 2,3-BDO market is estimated to grow at a gradual increase of 3%, reaching a value of approximately USD 220 million by 2027 [12]. 2,3-BDO is currently obtained from fossil resources. As fossil resources are limited, the production of 2,3-BDO from renewable biomass has recently been gaining interest [11]. Different microorganisms are capable of producing 2,3-BDO under both aerobic and anaerobic conditions. However, *Paenibacillus* stands out for its ability to achieve high yields in a biosecure manner [16]. *P. polymyxa* is a non-pathogenic strain (class 1) [17] and is generally recognised as safe (GRAS) [16]. It is therefore suitable for use on an industrial scale without the need for additional biosafety standards [17]. Moreover, *P. polymyxa* can produce the *levo*−2,3-BDO isomer with applications of industrial interest as an antifreeze agent and chemical synthesis [13]. *Paenibacillus* strains can consume various carbon sources (but not complex carbohydrates) from renewable resources. Lignocellulosic biomass such as poplar wood [18] and wheat straw [19] can be used as feedstocks to produce 2,3-BDO. Other by-products, such as crude glycerol and molasses, have also been used [19]. However, 2,3-BDO production from fruit and vegetable residues has hardly been investigated. In addition, most studies focus mainly on the extraction of phenolics from DRB, but do not address the valorisation of the extracted solid to produce biofuels or value-added compounds in a biorefinery scheme.

This study compares two strategies for obtaining bioproducts of significant industrial value from DRB, aiming to select the most economically favourable option. The first strategy (multi-product biorefinery) involves the production of phenolics, betalains and 2,3-BDO from DRB, while the second (single-product biorefinery) uses the entire raw DRB to produce 2,3-BDO. To achieve this, betalains and phenolics were extracted using ethanol–water mixtures as solvents. After optimising the extraction conditions, the solid fraction was used as a substrate for 2,3-BDO production through enzymatic hydrolysis and fermentation with *P. polymyxa*. In the single-product biorefinery, DRB was directly subjected to enzymatic hydrolysis and fermentation to convert fermentable sugars into 2,3-BDO. A preliminary economic feasibility analysis was then conducted to compare production costs. This research represents the first attempt to propose a biorefinery based on DRB, comparing the standalone production of 2,3-BDO with multi-product approaches (phenolics, betalains and 2,3-BDO). As a result, DRB, an organic fruit waste, can be valorised to produce high value-added bioproducts, contributing to the achievement of Sustainable Development Goal 12: Sustainable consumption and production patterns.

2 Materials and methods

2.1 Raw material

DRB was kindly supplied by Huercasa (Sanchonuño, Spain) and stored frozen at − 20 °C. The DRB was washed, dried at 60 °C, and ground (particle size 1–4 mm) before use.

2.2 Extraction of phenolic compounds and betalains

Extraction was carried out using mixtures of ethanol:water as solvent. Specifically, 5 g of dry DRB was transferred to

Erlenmeyer flasks, and the appropriate volume of the extraction solvent was added to reach a solid/liquid (S/L) ratio of 5% (w/v). The mixture was stirred at 150 rpm at 25 °C in an orbital shaker (Incubator Shaker ES-60, Miulab, China) under the conditions established by the CCD. After extraction, vacuum filtration was used to separate the solid and liquid fractions. The liquid fraction was stored at 4 °C for subsequent analysis of betalains and phenolic compounds.

In order to select the optimal conditions for phenolic compounds and betalain extraction, a central composite design (CCD) was planned. The experimental factors were the extraction time (15–60 min) and the ethanol concentration (0–20% v/v). The CCD consists of 13 experiments (Table 1), of which 5 were central points and 4 were star points, 2 for each factor of the design. Two responses were analysed: the concentration of betalains and the concentration of total phenolic compounds. The data were analysed with the software Statgraphics Centurion XVIII. A confirmatory run was carried out under the optimal extraction conditions to verify the results predicted by the model. The compositions of the solid and liquid fractions, as well as the antioxidant capacity of the liquid, were determined. The solid fraction was washed and dried at 50 °C for 48 h to calculate the gravimetric recovery and was further used in the fermentation stage to produce 2,3-BDO. Analytical determinations were performed in triplicate, and the average results are shown.

Table 1 Central composite design. Experimental conditions

Run	Time (min)	Ethanol concentration (% v/v)
1	6	10
2	38	10
3	38	24
4	60	0
5	60	20
6	38	10
7	15	0
8	70	10
9	38	10
10	38	0
11	38	10
12	15	20
13	38	10

2.3 Fermentation to 2,3-BDO

2.3.1 Enzymatic hydrolysis

The enzymatic hydrolysis of DRB was carried out in 250-mL Erlenmeyer flasks under the following conditions: 10% w/v loading, 50 °C, 150 rpm, 24 h and pH 4.8, employing an orbital shaker (Optic Ivymen Systems, Comecta, Barcelona, Spain). The pH was adjusted using sodium hydroxide (NaOH) at the beginning, and water was used as the solvent. The enzymes added were a blend of Cellic CTec 2 and Viscozyme L (Novozymes A/S, Bagsvaerd, Denmark). An enzyme load of 10 FPU/g dry matter (DM) was used for both enzymes. After 24 h of enzymatic hydrolysis, the hydrolysate was vacuum filtrated to be further used in the fermentation step.

2.3.2 Microorganism and inoculum

The microorganism, *P. polymyxa DSM 365*, was obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). The strain was maintained on 50% glycerol and stored at − 70 °C in 2-mL cryogenic vials. The inoculum was grown in 250-mL Erlenmeyer flasks with 100 mL of Häßler medium [20] under aerobic conditions in an orbital shaker (Comecta Optic Ivymen system) at 37 °C and 200 rpm for 24 h. A more detailed description of the culture medium and cultivation conditions can be found in the literature [21].

2.3.3 Fermentation step

Fermentation assays were carried out in duplicate in 250-mL Erlenmeyer flasks with 100 mL of medium at 37 °C, 200 rpm and pH 6. The pH was adjusted at the start of fermentation. The inoculum loading was 10% (v/v) with an optical density of 0.6. Samples were taken every 24 h to measure the concentration of monosaccharides, products (2,3-BDO) and by-products (acetoin and ethanol). The samples were centrifuged for 5 min at 10,000 g (MiniSpin, Eppendorf). A part of the supernatant was filtered through a 0.22 µm filter and then stored at 4 °C until analysis. Fermentation assays were performed at least in duplicate. The fermentation yield (g/g) was calculated as the relation between the 2,3-BDO concentration (g/L) and the concentration of sugars (g/L) consumed during fermentation. On the other hand, 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 the 2,3-BDO concentration was higher.

2.4 Evaluation of alternatives for DRB valorisation

2.4.1 DRB valorisation scenarios

Figure 1 depicts the two alternatives evaluated for the valorisation of DRB to produce phenolic compounds, betalains and 2,3-BDO in a multi-product biorefinery approach, or

2,3-BDO in a single-product biorefinery. The two scenarios were proposed to determine global yields and estimate production costs.

Namely, for scenario 1, the different stages were as follows: firstly, S/L conventional extraction, obtaining a liquid and a solid phase. The liquid stream is rich in phenolic compounds and betalains, while the solid stream is rich in carbohydrates. The solid phase is fed to an enzymatic hydrolysis step prior to fermentation to obtain 2,3-BDO. Scenario 2 included the production of 2,3-BDO in two steps: enzymatic hydrolysis and fermentation. In this way, all the sugars in the DRB can be converted into 2,3-BDO.

2.4.2 Economic evaluation

A preliminary economic study was carried out to analyse the feasibility of the proposed scenarios for the valorisation of DRB. A treatment capacity of 100 kg/h of DRB on a dry basis was assumed, and the mass balances of the proposed scenarios (Fig. 1) were solved from the experimental data for the upstream processes (extraction in scenario 1, enzymatic hydrolysis and fermentation). The purchased equipment cost (PCE) was estimated using Matches' online cost estimation tool. This database provides values for the year 2014, which were updated for the current year using the consumer price index (CPI). Based on the upstream section, the downstream was assumed to account for 70% of the total plant costs [17]. Lang's factor method was applied to calculate the total investment cost (TIC). This method is described in detail in the literature [22]. The raw material costs were as follows: 3.16 €/m^3 for process water, 0.9 €/kg for enzymes [17] and 1.1 €/kg for ethanol [23]. On the other hand, the cost of the DRB used as raw material was assumed to be negligible. Furthermore, the equipment amortisation time was assumed for a plant life of 10 years, considering that the plant operates for 8000 h/year. With all the above, the production cost of 2,3-BDO was estimated. Finally, the minimum selling price for considering the plant to be profitable was calculated, taking into account a net present value (NPV) of 0 € and an internal rate of return (IRR) of 10%.

2.5 Analytical methods for liquid extract characterisation

2.5.1 Total phenolic content

The Folin-Ciocalteu reagent was used to determine the content of total phenolic compounds (TPC), according to Ozturk et al. [24]. In brief, 40 µL of a sample was mixed with 3 mL of distilled water and 200 µL of Folin-Ciocalteu reagent. After 5 min, 600 µL of sodium carbonate solution (20%) was added. After incubation at 40 °C for 30 min, the mixture was allowed to cool, and absorbance was measured at 765 nm in a UV–Vis spectrophotometer (Shimadzu, Japan). The TPC is expressed as mg of gallic acid equivalents (GAE)/L.

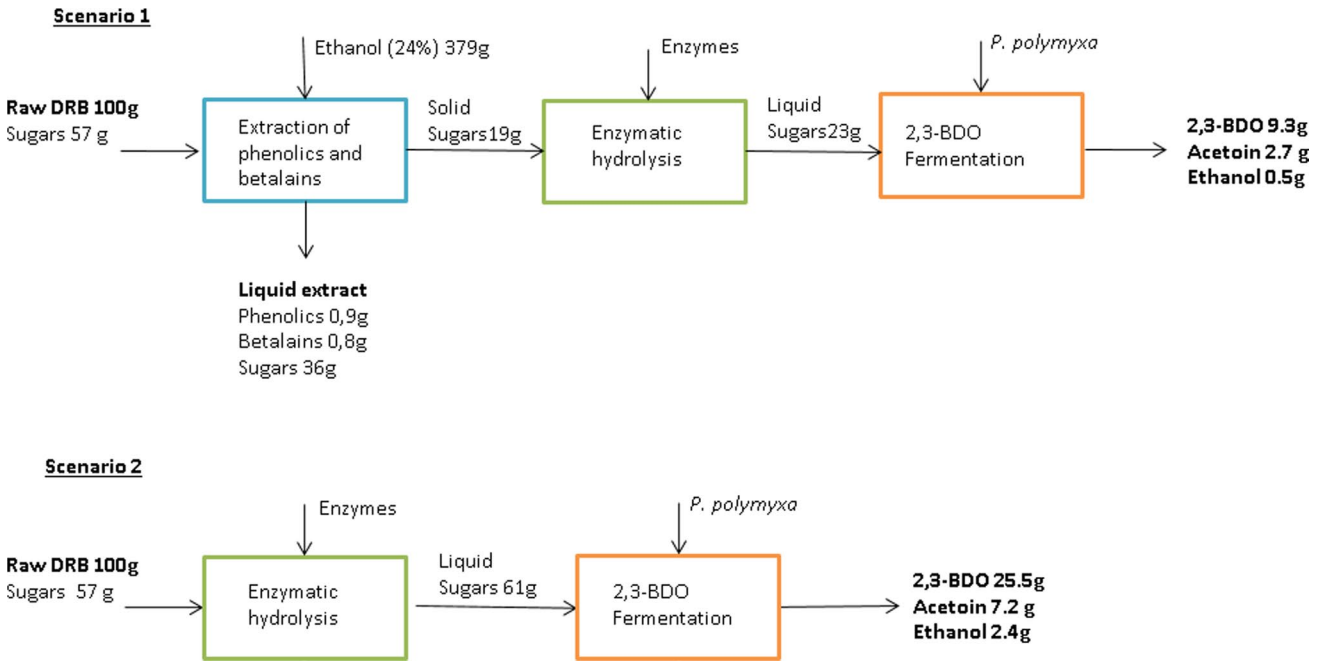

Fig. 1 Schematic diagrams for DRB valorisation to produce bioactive compounds and 2,3-BDO (scenario 1) and 2,3-BDO (scenario 2)

2.5.2 Betalain content

The betalain content was quantified as the sum of betacyanin (BC) and betaxanthin (BX) concentrations, in accordance with Amo-Mateos et al. [25]. The betalain content in the aqueous extracts was determined using optical spectroscopy (Shimadzu, Japan), measuring at 538, 476 and 600 nm to determine the concentration of betacyanin, betaxanthin and impurities, respectively.

2.5.3 Total flavonoid content

The flavonoid content (TFC) was quantified using the colorimetric method of aluminium trichloride according to Zhishen et al. [26]. Briefly, 300 µL of sodium nitrite (5%) was mixed with 1 mL of the sample. The mixture was allowed to stand for 5 min at room temperature, and then 500 µL of aluminium chloride (2%) was added. After 6 min, 500 µL of sodium hydroxide (1 M) was mixed with the samples. Finally, after 8 min, 10 mL of distilled water was added, and the absorbance was measured at 510 nm in a UV–Vis spectrophotometer (Shimadzu, Japan). The TFC was expressed as mg of catechin equivalents (CE)/L.

2.5.4 Antioxidant capacity

To evaluate the properties of the extract obtained under optimum conditions, the antioxidant capacity was assessed by the ferric antioxidant power reduction (FRAP) and the ABTS radical scavenging assays.

The FRAP assay was performed as described by Benzie and Strain [27]. In brief, a 100 µL sample was mixed with 3 mL of FRAP reagent. Then, the absorbance was measured after 6 min in darkness at 593 nm in a spectrophotometer (Shimadzu, Japan). The ABTS assay was done according to Re et al. [28]. A 30 µL sample was mixed with 3 mL of diluted ABTS solution, and the absorbance was measured (Shimadzu, Japan) after 6 min in darkness at 734 nm. The results were expressed as Trolox equivalents (TE) in mg/L.

2.5.5 Analysis of the monosaccharides, 2,3-BDO and fermentation by-products

The monosaccharide composition (glucose, galactose and arabinose), fermentation products (2,3-BDO) and by-products (acetoin and ethanol) were analysed by high-performance liquid chromatography (HPLC). The analysis was performed using an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Spain) at 60 °C, 0.01N H_2SO_4 at a flow of 0.6 mL/min as the mobile phase and a refractive index detector (Waters 2414). The injection volume was 20 µL. It should be noted that the column Aminex 87H is not able to separate the monosaccharides xylose, fructose, mannose and galactose. The levo-2,3-BDO isomer (Sigma-Aldrich) was used as the standard for quantification. The content of monosaccharides in the water extractives was quantified using a refractive index detector (Waters 2414), an Aminex HPX-87C column (300 × 7.8 mm, Bio-Rad, Spain) at 80 °C, water at a flow of 0.6 mL/min as the mobile phase and an injection volume of 20 µL.

2.6 Analytical methods for solid characterisation

The composition of the solid raw material, i.e., structural carbohydrates (cellulose and hemicellulose), lignin and ash content, was determined using the analytical methodology of the National Renewable Energy Laboratory (NREL) [29]. The extractives (in water and ethanol) were also measured according to the NREL procedures [30]. The composition of monosaccharides was measured as detailed in Section. The determinations were carried out in triplicate, and the average of the results is shown.

2.7 Data analysis

To determine statistical differences, an ANOVA test was carried out, at a confidence level of 95% ($p < 0.05$), using Statgraphics Centurion XVIII.

3 Results and discussion

3.1 Characterisation of DRB

The composition for DRB was the following (% w/w DM): glucan, 5.8 ± 0.0; arabinan, 7.0 ± 0.0; galactan, 2.3 ± 0.0; galacturonan, 4.4 ± 0.0; acid-insoluble lignin, 1.8 ± 0.2; acid-soluble lignin, 2.4 ± 0.1; extractives, 66.1 ± 1.9, of which extractives in ethanol, 18.6 ± 2.0 and extractives in water, 47.5 ± 1.2 (sucrose in extractives, 31.3 ± 3.4; glucose in extractives, 3.8 ± 0.5; fructose in extractives, 4.7 ± 1.1); ash, 3.3 ± 0.4.

Considering the above composition, the extractive content of this raw material is much higher than that found in other fruit and vegetable wastes. For example, carrot discard presented 43.1% of extractives [31], kale stems 46.9% [4] or apple pomace 3.12% [32]. In addition, it is important to highlight the low lignin content (insoluble and soluble), compared to other fruit and vegetable wastes, such as the 20% reported in apple pomace [33]. The low lignin content could eliminate the need for a pretreatment, which is a great advantage for the overall 2,3-BDO production process from DRB.

On the other hand, the total carbohydrate content was close to 55% (w/w). DRB presents 15.1% of structural

carbohydrates and 39.8% of non-structural carbohydrates in the extractives, mainly sucrose. These data agree with Costa et al. [34], who reported a similar composition (20.83% structural carbohydrate content) in a beet residue.

3.2 Optimisation of the extraction conditions

The results of the CCD were analysed to evaluate the effect of the experimental factors (time and ethanol concentration) on the co-extraction of phenolics and betalains.

As can be seen in Fig. 2, the TPC content ranged from 249.0 mg/L (run 4) to 435.2 mg/L (run 3), whereas the betalain concentration varied from 198.8 mg/L (run 4) to 385.0 mg/L (run 3). The lowest values for both responses were observed in the experiment (run 4) in which the extraction was carried out with water for a longer time (60 min). On the contrary, the highest values for both responses corresponded to the experiment carried out at the highest ethanol concentration (24% v/v) and an intermediate time (run 3). The data provide information on the influence of the experimental factors on the extraction of phenolics and betalains. As for the ethanol concentration, when the concentration was higher, higher concentrations of both phenolics and betalains were found in the extracts. However, when water was used as a solvent, a longer extraction time did not increase the concentrations of phenols and betalains.

Comparing the results obtained in run 3 (38 min, 24% ethanol) with those observed at the central point (runs 2, 6, 9, 11 and 13), a clear improvement in the concentrations of both phenolics and betalains can be observed. The concentrations of phenolic compounds increased from 288.9–323.6 mg/L at the central point to 435.2 mg/L when the ethanol concentration was 24% v/v. For betalains, the concentration rose from 237.9–280.2 mg/L at the central point to 385.0 mg/L when the ethanol concentration was 24% v/v. These results indicate that the higher the ethanol concentration, the higher the concentrations of bioactive compounds in the extract. Analysing the experiments carried out at an ethanol concentration of 10% (runs 1, 2, 6, 8, 9, 11 and 13) at different extraction times, it can be observed that, when the time was 6 min (run 1), both phenolic (272.8 mg/L) and betalain (244.9 mg/L) concentrations were lower than those corresponding to the central point (288.9–323.6 mg/L for phenolic compounds and 237.9–280.2 mg/L for betalains). However, if the time was increased from 38 to 70 min (run 8), then the concentrations of phenolics and betalains were lower (316.2 mg/L and 257.9 mg/L for phenolic compounds and betalains, respectively) than some of the values found in the central point. This could indicate that there was an optimal extraction time. Lazăr et al. [35] stated that ethanol concentration and extraction time exert a positive influence on the extraction of betalains from beetroot by-products. Popa et al. [9] indicated that the betalain concentration in extracts

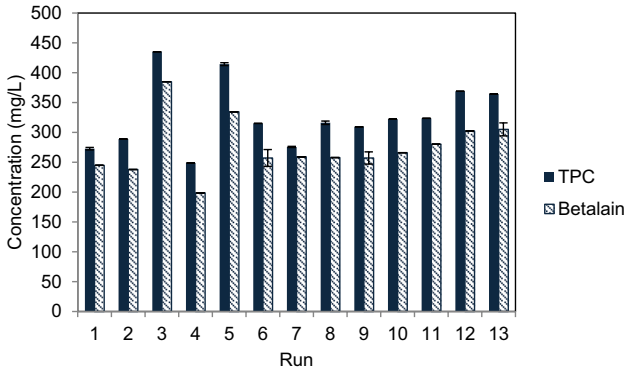

Fig. 2 Central composite design: TPC and betalain concentrations in the liquid fractions

increased with the extraction time, but at times higher than 75 min, the pigment concentration remained constant. In the same way, Lazăr et al. [35] reported that the extraction time negatively affected betalain concentration when the values were higher than 50 min and lower than 15 min.

Figure 3a, b shows the interactions between time and ethanol concentration on TPC and betalain extraction. As can be seen, higher TPC and betalain concentrations were obtained at longer times and higher ethanol concentrations. If the response surface is observed, when the ethanol concentration was increased, the concentrations of phenolic compounds and betalains presented a clear upward trend. However, when the extraction time was increased, there was an optimal point (extraction time) at which the responses of the phenolic and betalain concentrations started to decrease.

Two second-order polynomial equations for TPC (Eq. 1) and betalain (Eq. 2) concentrations were obtained to predict the responses from the experimental data.

$$TPC\left(\frac{mg}{L}\right) = 316.047 + 104.605 \cdot C + 57.8548 \cdot C^2 \quad R^2 = 98.8048; R^2 adjust = 89.6097 \tag{1}$$

$$Betalain\left(\frac{mg}{L}\right) = 264.867 + 86.8434 \cdot C + 53.321 \cdot C^2 + 46.285 \cdot C \cdot t \quad R^2 = 95.6015; R^2 adjust = 91.203 \tag{2}$$

where t is the time (min) and C is the ethanol concentration (%v/v). In the modelling, TPC and betalain concentrations were adjusted with confidence levels (90%, $p < 0.05$) between the experimental and predicted data. As observed in Eq. 1 and Eq. 2, the most significant effect was the ethanol concentration. Based on Eq. 1, the ethanol concentration and the quadratic ethanol concentration have a positive impact on TPC extraction, while the extraction time was not significant ($p > 0.05$). In the case of betalain concentration (Eq. 2), the ethanol concentration, the quadratic ethanol concentration and the combined effect of the ethanol concentration

and time were positive and significant effects ($p < 0.05$). Therefore, the effect of time was different for the extraction of TPC and betalains, showing a greater influence on the extraction of betalains, according to the experimental results.

The optimisation of the hydroalcoholic extraction was carried out, maximising both responses: the TPC and betalain concentrations. Based on the models, the optimal extraction conditions were 44 min and 24% v/v of ethanol concentration. Under these conditions, optimal values of 454.9 mg/L for TPC and 385.9 mg/L for betalains were predicted. The model was validated by an experimental run under the optimal conditions. The experimental values were 451.4 mg/L of TPC and 406.7 mg/L of betalains, with a solid recovery of 42.2%. Thus, a good agreement between the experimental and predicted values was found. Performing the global mass balances, these concentrations corresponded to 0.9 g of phenolic compound and 0.8 g of betalains per 100 g of DRB (Fig. 1).

Higher values of ethanol concentration would lead to higher concentrations of both TPC and betalains, due to the higher solubility of TPC in less polar organic solvent [36]. The optimum value found (24% v/v) corresponds to the highest ethanol concentration, which was the star value of the CCD. Therefore, additional experiments were performed, using higher ethanol concentrations (30–40–50% v/v), at the optimum extraction time (44 min). It was found (Fig. 4) that, above 24% of ethanol concentration, the concentrations of TPC decreased significantly ($p < 0.05$). The same trend was observed for betalains. This could be due to the solubility of these pigments, primarily in water, so the addition of ethanol to the water may enhance the extraction of other compounds present in DRB [37].

The results are slightly better than those found by Iahtisham-Ul-haq et al. [37], who reported optimal extraction conditions for betalains from red beetroot (107.71 mg/g of extract) at 35.34% ethanol, 43 min and an S/L ratio of 5%. Nouairi et al. [8] applied mixtures of ethanol and water at different ratios, concluding that the solvents with higher water content can extract a considerable amount of betalains from red beetroot (0.342 mg/g). The study by Prieto-Santiago et al. [38] obtained 331.8 mg/kg DRB, using ethanol:water 50:50, an S/L ratio of 10%, 40 min extraction time and 120 °C. This showed that high temperatures led to lower concentrations, probably due to the degradation of the pigments. As for the extraction time, the results obtained agree with those found by Iahtisham-Ul-haq et al. [37], who pointed out that these compounds degrade when the extraction time was prolonged from 15 to 45 min. Red beetroot is among the top 10 vegetables with the highest total phenolic density. In addition to betalains, the extracts can also contain several phenolic acids, such as p-coumaric, ferulic, vanillic, protocatechuic, syringic and p-hydroxybenzoic acids [37].

3.3 Characterisation of the solid and liquid fractions obtained under optimal extraction conditions from DRB

The composition of the solid fraction obtained from DRB after extraction under optimal conditions is given in Table 2. Comparing this with the composition of raw DRB, the glucan content increased from 5.8 to 20.0%. Due to the high carbohydrate content (40.2%), this stream can be used to produce fermentation-based intermediates, e.g., 2,3-BDO. The liquid fraction was also rich in monosaccharides, with up to 18.1 g/L total sugars (Table 2). It is important to highlight the high glucose content (12.3 g/L).

The antioxidant content of food products has become an important aspect of their quality and functionality [39]. Although betalains and phenolic compounds are the two bioactive compounds that explain the total antioxidant capacity of red beetroot [40], other methods can be used to measure the antioxidant capacity of the liquid extract, such as TFC, ABTS and FRAP. The TFC was 90 mg CE/L, which is equivalent to 1.8 mg CE/g of DRB. The TFC of ethanol

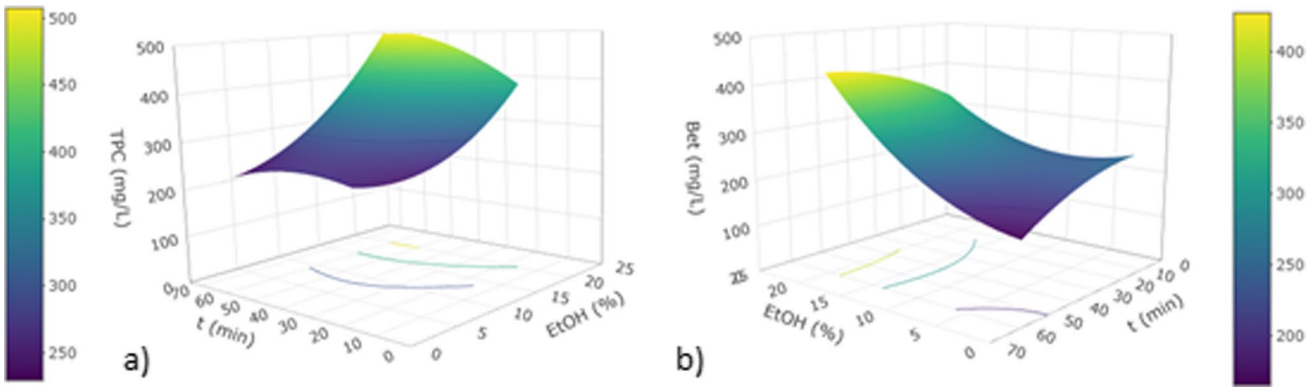

Fig. 3 Central composite design. Response surfaces. Influence of time and ethanol concentration on TPC (**a**) and betalain (**b**) concentrations in the liquid extract

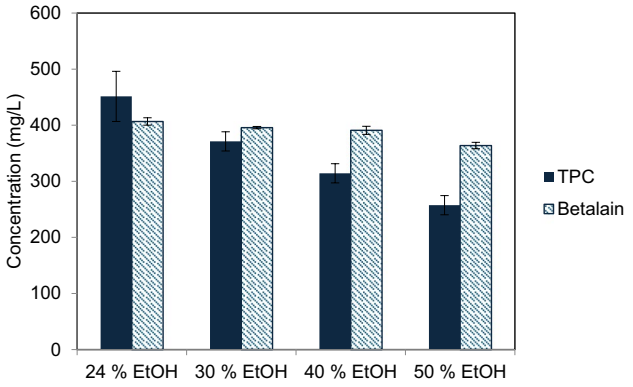

Fig. 4 TPC and betalain content in the liquid extracts obtained under optimal extraction conditions (44 min, 24% ethanol) and after increasing the ethanol concentration (% v/v)

extracts (10% S/L ratio, 100% ethanol and reflux technique for 4 h) from beetroot reported by Rangani and Ranaweera [41] was 1.90 mg CE/g. In this work, an extract with the same concentration of flavonoids was obtained using a lower concentration of ethanol (5% S/L ratio, 24% v/v ethanol and 44 min). In terms of antioxidant capacity, ABTS and FRAP assays were applied, reaching 1240.1 mg TE/L and 719.6 mg TE/L, respectively. These concentrations corresponded to 24.8 and 14.4 g TE/kg of DRB, respectively. Fernando et al. [42] analysed the antioxidant capacity of the liquid extracts obtained from red beetroot with 30% v/v ethanol–water and ultrasound for 30 min at 30 °C. The antioxidant capacity with FRAP was about 6.5 mg TE/g DRB, lower than the value found in our study [42]. Similar results for ABTS (25.06 mg TE/g DRB) were reported for red beet extracts after 100% ethanol extraction, 5% S/L ratio and 35 °C for 24 h [43]. Other emerging methods have been proposed as alternatives to conventional solid–liquid extraction for the recovery of phytochemicals, such as microwave-assisted extraction or pressurised liquid extraction. However, these processes may require more expensive equipment, which could lead to increased costs [43]. Therefore, the DRB extract obtained in this study through conventional extraction and using a low-toxicity solvent is rich in bioactive compounds and holds great potential for applications in the food and pharmaceutical industries [44].

3.4 3-Butanediol fermentation by P. polymyxa

As explained before, two scenarios have been compared for the valorisation of DRB. In scenario 1, the solid obtained after DRB extraction under optimal conditions, which is rich in glucan (Table 2), was subjected to enzymatic hydrolysis and fermentation with *P. polymyxa*. Scenario 2 used all the raw DRB to obtain 2,3-BDO in two stages: enzymatic hydrolysis and fermentation.

Table 2 Composition of the solid (% w/w DM) and liquid fractions after DRB extraction under optimal conditions

Component		Value
Solid fraction (% w/w)		
Glucan		20.0 ± 0.7
Arabinan		14.7 ± 0.8
Galactan		5.5 ± 0.2
Galacturonan		13.6 ± 0.4
Lignin	Acid-soluble lignin	5.3 ± 0.1
	Acid-insoluble lignin	5.6 ± 0.4
Ash		4.6 ± 0.1
Liquid extract		
Glucose (g/L)		12.3 ± 1.3
Galactose (g/L)		3.0 ± 0.6
Arabinose (g/L)		2.8 ± 0.4
Galacturonic acid (g/L)		0.2 ± 0.0
TPC (mg GAE/L)[a]		451.4 ± 44.7
Betalain (mg/L)		406.7 ± 6.6
TFC (mg CE/L)[b]		90.1 ± 3.2
ABTS (mg TE/L)[c]		1240.1 ± 88.1
FRAP (mg TE/L)[c]		719.6 ± 65.9

[a]*GAE* gallic acid equivalent

[b]*CE* catechin equivalent

[c]*TE* trolox equivalent

The use of agro-industrial residues containing complex sugars to produce 2,3-BDO may be hampered by the fact that carbohydrates cannot be directly assimilated by *P. polymyxa*. This may require a pretreatment step prior to enzymatic hydrolysis and fermentation. In this study, enzymatic hydrolysis proved to be an efficient alternative for the recovery of sugars. Total sugar concentrations of 55.3 g/L (recovery about 96%) and 61.4 g/L (recovery about 100%) were achieved, respectively, for scenarios 1 and 2 after enzymatic hydrolysis, making pretreatment unnecessary. This could already be expected, since DRB presented a low concentration of lignin.

During fermentation, the sugar consumption (Fig. 5a) was almost 100% after 48 h (98.9% and 98.3% for scenarios 1 and 2, respectively). At this time, the maximum concentrations of 2,3-BDO were reached (17.6 g/L and 20.4 g/L for scenarios 1 and 2, respectively). The concentrations of by-products in scenario 1 were 5.0 g/L and 1.0 g/L of acetoin and ethanol, respectively, and 5.8 g/L and 2.0 g/L in scenario 2 (Fig. 5b). The concentration of 2,3-BDO started to decrease after 48 h of fermentation. This is due to the metabolic pathway of *P. polymyxa*, which, once it had exhausted the main source of sugars (glucose) [45], started to convert 2,3-BDO into acetoin, regenerating the NADH and maintaining a state of continual oxidation–reduction [46].

The 2,3-BDO yield at 48 h was 0.40 g/g for scenario 1 and 0.42 g/g for scenario 2. In addition, the 2,3-BDO

productivities were 0.37 g/(L·h) and 0.42 g/(L·h) for scenarios 1 and 2, respectively. The concentrations achieved in the fermentation are in accordance with values previously reported. For instance, *P. polymyxa* produced 13.5 g/L of 2,3-BDO from a poplar wood hydrolysate containing 42 g/L of sugars [18].

The 2,3-BDO concentration obtained in scenario 2 was higher, as the initial concentration of monosaccharides in the enzymatic hydrolysate was also higher (Fig. 5). In scenario 1, free sugars are recovered in the extract together with phenolics and betalains, so they were not converted into 2,3-BDO. According to Jiang et al. [47], polysaccharides and phenolics can be separated using dialysis membranes in combination with ionic liquid extraction.

In addition to these experimental runs, the solid fraction obtained after DRB extraction and the raw DRB were used directly in fermentation without previous enzymatic hydrolysis to evaluate the production of 2,3-BDO by direct fermentation with *P. polymyxa*. In the first case, no 2,3-BDO was produced. In the second, a low 2,3-BDO concentration of 2.5 g/L was obtained. The explanation for these results lies in the monosaccharides present in the extractives. When using the extracted solid fraction directly in fermentation, free sugars were no longer available, and the microorganism was not able to consume carbohydrates; when using raw DRB, the microorganism was able to consume simple sugars and produced a low amount of 2,3-BDO. This corroborated the hypothesis that, for this raw material, the enzymatic hydrolysis stage is necessary to release sugars so that they can be used in the fermentation stage.

3.5 Mass balance of the biorefinery processes

The proposed biorefinery process consists of three consecutive stages (extraction, enzymatic hydrolysis and fermentation) for scenario 1 and two stages (enzymatic hydrolysis and fermentation) for scenario 2. The overall mass balances were solved, and global yields corresponding to 48 h of fermentation are depicted in Fig. 1 and Table 3.

In scenario 1, the initial extraction stage yielded an extract rich in phenolic compounds and pigments containing (g/100 g of DRB) 0.8 g of betalains, 0.9 g of phenolic compounds and 36.2 g of monosaccharides. This stage allows simple sugars to be recovered in the liquid extract. It therefore reduces the quantity of fermentable sugars contained in the solid fraction, which is then taken to enzymatic

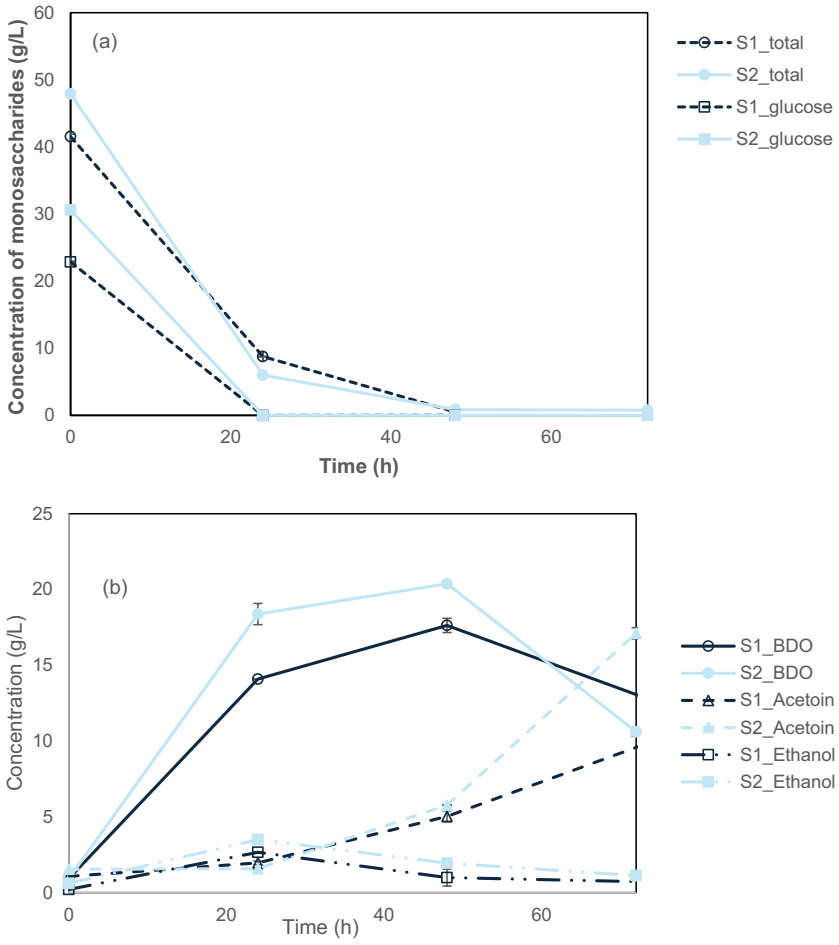

Fig. 5 Time courses of the concentrations of monosaccharides (**a**) and fermentation products (**b**) for the different scenarios evaluated. Total sugars are the sum of glucose, galactose and arabinose

hydrolysis and fermentation. The global yields obtained in terms of 2,3-BDO were 9.3 g/100 g DRB for scenario 1 and 25.5 g/100 g DRB for scenario 2. As by-products, in scenario 1, 2.7 g of acetoin and 0.5 g of ethanol were obtained (g/100 g DRB), while in scenario 2, 7.2 g/100 g and 2.4 g/100 g of acetoin and ethanol were produced, respectively. The results obtained in scenario 2 are favourable with respect to those obtained in other works with *P. polymyxa*. López-Linares et al. [21] reported 18.8 g of 2,3-BDO, 2.1 g of acetoin and 2.5 g of ethanol per 100 g carrot residue.

The global market for natural dyes was estimated to be between 1.5 and 1.75 billion USD in 2022 [48]. Considering that 10% of the world's natural dye production is obtained from beetroot, the production of natural dyes from DRB is expected to become increasingly important [49]. The price of red dye was estimated at 300 US/kg in 2022 [50]. At the same time, the significant quantity of solid waste generated after the extraction of colorants could be utilised for 2,3-BDO production. The market size of 2,3-BDO is estimated at 32 Mt/year and is expected to increase, with a price of 2.63 $/kg [51]. These results show the potential of DRB as a raw material to produce high added-value products in the framework of the circular economy.

3.6 Preliminary economic evaluation for DRB valorisation scenarios

A preliminary economic assessment of the two proposed scenarios has been carried out to compare the production of 2,3-BDO, phenolics and betalains (scenario 1) and the production of 2,3-BDO (scenario 2). The basis of the calculation was 100 kg/h of DRB with a moisture content of 80% (w/w). 2,3-BDO and betalains were considered products for scenario 1 and only 2,3-BDO for scenario 2. The results shown below were obtained using the simplifications detailed in Sect. 2.4.2 (Economic evaluation).

Table 4 shows the results of applying Lang's factor method. The PCE was higher in scenario 1 (1.09 M€) due to the need for equipment for the extraction stage. While scenario 1 requires three reactors (for extraction, enzymatic hydrolysis and fermentation), scenario 2 only requires two reactors, assuming a lower PCE (0.65 M€). According to Lang's factor method, the PCE directly affects the TIC, as was observed in Table 4. The TIC for scenario 1 (5.1 M€) was higher than that calculated for scenario 2 (3 M€). The production costs take into account the energy needed in the different stages and also the chemicals. Scenario 1 requires a higher amount of reagents (water and ethanol) in the extraction stage, which raises the production cost to 3.1 M€. In scenario 2, this value was lower (2.2 M€), and the main contribution was due to enzymes. On the other hand, the production costs per kg of 2,3-BDO have been calculated considering the production costs (M€/year) and the global yield. Thus, scenario 2 presents production costs (10.8 €/kg 2,3-BDO) four times lower than scenario 1 (42.1 €/kg 2,3-BDO), mainly due to the fact that scenario 2 has a higher production of 2,3-BDO, as it uses all the DRB. Therefore, scenario 2 emerges as the most cost-effective scenario. However, this is still far from being able to compete with the market prices for 2,3-BDO. In this context, the idea of incorporating the profits from the sale of the natural dye arises. The selling price of natural dyes varies widely, from 300 US/kg [50] to 2400 US/kg [52]. For the preliminary economic assessment, an intermediate selling price for betalains of 800 €/kg has been assumed. The sale of this natural dye can generate a high profit due to its high selling price, which significantly reduces the production costs per kg of 2,3-BDO as the main product. As can be seen, the minimum selling price of 2,3-BDO for scenario 1 (1.4 €/kg) is much lower than that of scenario 2 (17.3 €/kg). Therefore, from a technical and economic point of view, the best scenario would be scenario 1, in which several products are obtained from DRB. Moreover, the minimum estimated selling price of 2,3-BDO was similar to the price of 2,3-BDO obtained by petrochemical methods [17]. Future studies should be focused on improving the 2,3-BDO titre by optimising fermentation parameters at a larger scale bioreactor.

Table 3 Mass balances for the different scenarios (g per 100 g of DRB on dry basis). Global yields for fermentation products were calculated at 48 h of fermentation

	Scenario 1	Scenario 2
Fermentation end products (g/100 g DRB)		
2,3-BDO	9.3	25.5
Acetoin	2.7	7.2
Ethanol	0.5	2.4
Liquid extract (g/100 g DRB)		
Sugars	36.2	-
Betalain	0.8	-
Phenolics	0.9	-

4 Conclusions

The results presented in this work show the possibility of obtaining high value-added products such as 2,3-BDO, phenolics and betalains through integrated bio-based processes using DRB as a feedstock. This study evaluated two scenarios for the valorisation of DRB to determine the most favourable alternative from a technical and economic point of view. First, the characterisation of DRB revealed substantial amounts of monosaccharides and complex carbohydrates. Enzymatic hydrolysis was necessary for the saccharification of DRB, but no pretreatment was required

Table 4 Preliminary economic evaluation of the two scenarios proposed for DRB valorisation

	Global yield (kg 2,3-BDO/100 kg DRB)	PCE (M€)	TIC (M€)	Production cost (M€/year)	Production cost (€/kg 2,3-BDO)	Minimum selling price (€/kg 2,3-BDO)
Scenario 1	9.3	1.09	5.1	3.1	42.1	1.4
Scenario 2	25.5	0.65	3.0	2.2	10.8	17.3

due to the low lignin content. In scenario 1 (multi-product biorefinery), 0.9 g of phenolics and 0.8 g of betalains were recovered from 100 g of DRB under the optimal extraction conditions (24% v/v ethanol, 44 min). The subsequent enzymatic hydrolysis and fermentation of the extracted solid fraction led to a global yield of 9.3 g 2,3-BDO/100 g DRB. In scenario 2, all the DRB was subjected to enzymatic hydrolysis and subsequent fermentation to obtain 2,3-BDO with a global yield of 25.5 g/100 g DRB. A preliminary economic analysis considering investment and production costs revealed a higher production cost for 2,3-BDO production in scenario 1. However, if the sale of a co-product (extract rich in betalains and phenolics) is considered, scenario 1 could be the most competitive and economically profitable option. The process could be improved by selling or valorising other co-products (sugars present in the extract) or by optimising the production of 2,3-BDO to reduce by-products such as ethanol or acetoin. The results demonstrate that multi-product biorefineries have the advantage of improving resource efficiency by valorising different streams, increasing the economic viability of the biorefinery.

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Data availability The datasets used in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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