Biobutanol production from pruned vine shoots

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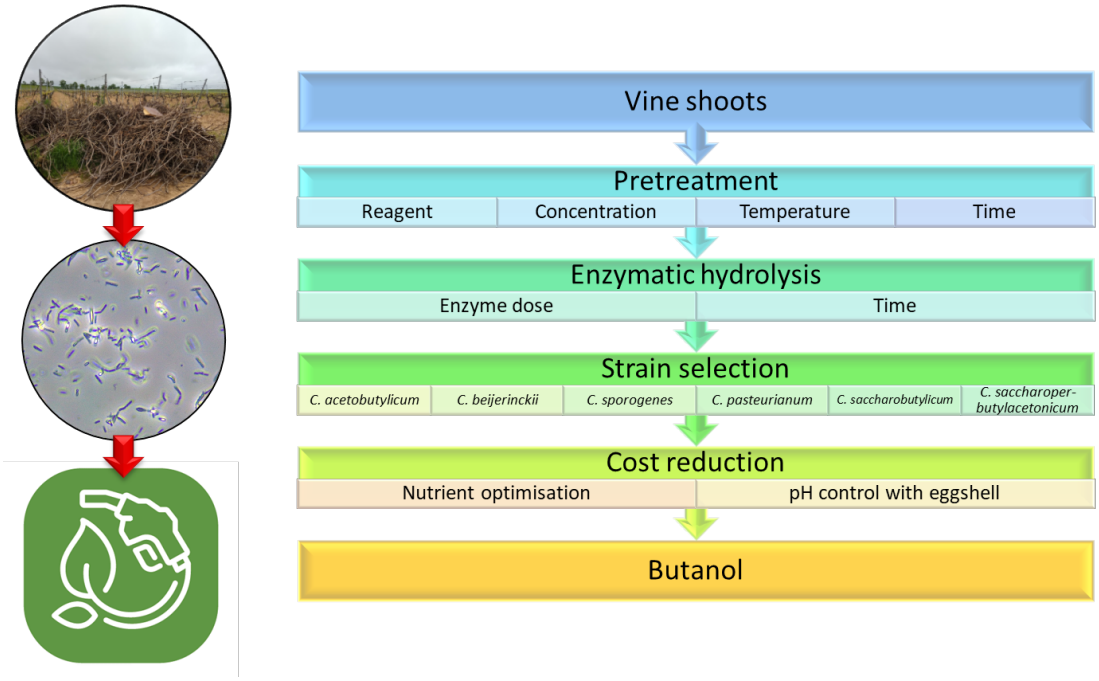


Abstract: This research aimed to demonstrate the feasibility of producing biobutanol by acetone-butanol-ethanol (ABE) fermentation on vine shoots at laboratory scale. In order to avoid a detoxification process prior to fermentation, an alkaline pretreatment and its posterior enzymatic hydrolysis were optimised through a response surface methodology (RSM) approach to minimize the concentration of phenolic inhibitors and maximize the amount of fermentable sugars. This vine shoots hydrolysate was directly fermented by 11 solventogenic *Clostridium* strains, reaching butanol concentrations over 8.0 g/L with *C. beijerinckii* CECT 508 and DSM 6423. The strain CECT 508 was selected to tune up a fermentation medium with the minimum possible amount of nutrients through a Plackett-Burman experimental design, thus reducing the cost of the fermentation broth while ensuring a high ABE solvent production. Finally, it was also shown that $CaCO_3$ could be substituted by eggshell powder as buffering agent, maintaining a biobutanol production over 7 g/L. This study demonstrates, for the first time, that biobutanol production from vine shoots is possible and that cost reduction based on alternative strategies of nutrient supplementation is viable.

Keywords: Biobutanol, vine shoots, lignocellulosic biomass, alkali pretreatment, eggshell, *Clostridium*.

1. Introduction

Among the worldwide beverage industries associated with the agrarian sector, the wine industry is one of the most powerful as well as one of those to which more productive land is dedicated. According to the International Organisation of Vine and Wine (OIV), in 2018, about 7,450,000 ha were under vines, and Spain (13%), China (12%), France (11%), Italy (9%) and Turkey (6%) comprised half of the world vineyards. In terms of grape production (77.8 Mt), China is the first producer (11.7 Mt) followed by Italy (8.6 Mt), USA (6.9 Mt), Spain (6.9 Mt) and France (6.2 Mt). During this period, grapes harvested worldwide were destined to produce 292 million of hectolitres of wine, 1,348 Mt of table grapes and 27 Mt of dried grapes [1].

By-products and residues generated by agro-industries open up the potential to generate sustainable bioproducts and bioenergy. To cope with this objective, the development of the biorefinery concept is presented as a promising solution [2]. Grape production and processing in the wine-making industry leads to a seasonal generation of a huge number of solid by-products which comprise up to 30% w/w of the material used. Among them, organic wastes mainly comprise leaves, stems, and pomace during the wine-making process and the pruned vine shoots obtained during the agronomic tasks devoted to vineyard maintenance [3–5]. As in other industries, the wine-making industry needs to incorporate technologies to minimize environmental damage as well as to recover and generate several valuable products such as fermentable sugars, polyphenols, pigments and tannins, among others.

Vine shoots represent up to 93% of the residues generated in the winery industry. They are highly rich in lignocellulosic compounds (34% cellulose, 27% lignin and 19% hemicellulose) and their production in Spain is estimated between 1.4 and 2.0 t/ha, depending on the vineyard structure and technological level [3,6]. In most cases, vine shoots have been considered as a residue without economic value and, according to this, they have been directly burned or left in fields in order to use them as a natural fertilizer [7]. Nowadays, due to the high amount of vine shoots produced in countries devoted to viticulture and the technological advances in the treatment of lignocellulosic material, this by-product is attracting attention to implement its valorisation by using a biorefinery approach for the obtention of value-added bioproducts that could render economic and environmental gains by potentiating a zero-waste wine industry [8,9].

Recently, Bharathiraja et al. [10] reviewed the approaches that have been explored for the bioconversion of vine shoots in value-added products such as lactic acid, phenyllactic acid, xylitol and biosurfactants; but this biomass has also been assessed for the production of second-generation bioethanol [4]. Regarding biobutanol, to the best of our knowledge, there is a lack of information on the utilisation of vine shoots for the bioproduction of this biofuel through the acetone-butanol-ethanol (ABE) fermentation process. Solely two studies have been performed for the obtention of this biofuel from grape pomace, reaching a maximum production of 9.84 g/L ABE with a yield of 0.32 g/g [11,12].

Biobutanol generation from lignocellulosic biomass still has drawbacks that made its production not competitive in comparison with other fossil fuels, such as gasoline. Therefore, in order to use the ABE process at industrial scale, it is required to reduce its production cost from 1.8 $/L (~ 1.53 €/L) to 0.6 $/L [13]. Several strategies are being developed from a multidisciplinary view to improve ABE fermentation proficiency. During the lignocellulosic pretreatment process, it is necessary to release hydrolysable and fermentable sugars avoiding the generation of inhibitors of solventogenic clostridia, reducing the requirement of an additional detoxification step which is impractical in an industrial environment [14]. In the same way, approaches focused on the selection of an appropriate

Clostridium strain for each lignocellulosic material utilised, as well as on the development of specifically adapted fermentable media to each strain and substrate, contribute to increase fermentation yield and productivity by reducing the costs of butanol production [15]. For instance, the use of agri-food industry by-products as buffering reagents or nitrogen sources has been proven efficient in different bioconversion systems [10,16].

The objective of this work was to develop an appropriate pretreatment for vine shoots (consisting of a physicochemical stage followed by an enzymatic hydrolysis) in order to obtain a readily fermentable hydrolysate for the ABE process. Thirty-one Clostridium strains were assessed to select the most efficient one for ABE production from this feedstock. In addition, a strategy for cost reduction on reagents was evaluated by optimising nutrient addition and replacing $CaCO_3$ by eggshell powder as buffering agent during the fermentation.

2. Material and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade. The enzyme Cellic-CTec2 (enzymatic activity 113 FPU/mL) was kindly provided by Novozymes (Bagsværd, Denmark). Eggshells were collected from domestic wastes.

2.2. Biomass description

Vine shoots were obtained from the experimental plots of the Department of Agriculture Research (ITACyL, Finca Zamadueñas, Valladolid, Spain) in May 2019. These viticulture by-products were dried in an oven at 45 °C during 48 h (until constant weight), ground in a rotary mill SM100 Comfort (Retsch GmbH, Haan, Germany) and sieved to 0.5-1.0 mm particle size. The procedures of the National Renewable Energy Laboratory (NREL) were utilised to determine moisture, ash, structural carbohydrates (cellulose and hemicellulose) and Klason lignin [17,18]. Galacturonic acid was determined by a two-stage sulfuric acid hydrolysis procedure [18]. Total carbohydrate content was calculated as the sum of monomeric sugars. Protein content was determined by the Kjeldahl method using a conversion factor of 6.25. Fat content was determined with the ANKOM XT15 Extractor (Ankom Technology) according to the manufacturer's procedure. Total phenolic compounds were extracted and analysed according to Hijosa-Valsero et al. [19]. The vine shoots utilised were chemically composed of 49.27% total carbohydrates, 32.77% glucan/cellulose, 11.31% hemicellulose, 2.82% galacturonic acid, 21.3% Klason lignin, 4.34% protein, 0.52% fat, 2.4% ashes, 7.91% moisture and 13.3 mg/g total phenolic compounds.

2.3. Preliminary physicochemical pretreatment and enzymatic hydrolysis

Vine shoots were initially subjected to an acidic pretreatment using H_2SO_4 (1.72% (w/w) of H_2SO_4, 134 °C, 17 min and 10% (w/w) biomass-to-solvent ratio). After the pretreatment, the solid/liquid mixture was subjected to enzymatic hydrolysis with 36 µL/g biomass (equivalent to 4.07 FPU/g biomass) [19]. This approach, as has been observed in other woody biomasses [20], generated a low cellulose hydrolysis yield (28.25%), with a total sugar recovery of 44.46% and a high concentration of total inhibitors (5.58 g/L). In order to delignify and increase the exposition of cellulose to the hydrolytic enzyme, a preliminary alkaline pretreatment with NaOH was prepared (2% (w/w) of NaOH concentration at 121 °C during 60 min, with a solid loading of 10% (w/w)), as well as one acetone organosolv pretreatment with sulfuric acid catalyst (40% (w/w) of acetone concentration, and the

optimal operational parameters used in the acidic pretreatment described above) followed by an enzymatic hydrolysis with the enzyme dosage aforementioned. The highest cellulose hydrolysis yield (63.16%) was achieved with the alkaline treatment with a lower concentration of total inhibitors (3.41 g/L). Nevertheless, the total sugar recovery was 41.46% because of lower hydrolysis of hemicellulose.

Finally, the alkaline pretreatment was selected and conducted at 121 °C during 60 min in a 2-L high-pressure reactor made of alloy Carpenter-20 (Parr Instrument Company, Moline, IL, USA) with 40 g of dry biomass mixed with 360 g of aqueous solutions containing NaOH (2% w/w). After cooling, the solid biomass was separated by vacuum filtration and the liquid phase was substituted by the same volume of distilled water. With the aim of achieving greater hydrolysis of total sugars, and based on other previous works of vine shoots hydrolysis [21,22], a higher enzyme dose (130 µL/g biomass) was tested. Hydrolysis conditions were performed according to Hijosa-Valsero et al. [19]. Finally, quantification of total sugars (arabinose, cellobiose, glucose, rhamnose and xylose) and total inhibitors (acetic acid, formic acid, levulinic acid, furfural, 5-hydroxymethylfurfural (5-HMF) and phenolic compounds) present in the hydrolysate was performed after enzymatic hydrolysis according to section 2.8.

2.4. Pretreatment and hydrolysis optimization

In order to obtain a fermentable hydrolysate with the highest concentration of sugars and the lowest concentration of inhibitors (response variables), the preliminary alkaline physicochemical pretreatment and the subsequent enzymatic hydrolysis conditions (NaOH concentration, temperature, time and enzyme load) were optimized by using a response surface methodology (RSM) approach consisting of 4 factors, 1 replicate, 30 runs, 2 blocks and 6 central points (Supplementary Tables 1 and 2). After calculating response surfaces, the resulting equations were used to estimate the optimal values for each independent variable (NaOH concentration, temperature, time and enzyme load) and all the estimated optimal points were validated experimentally. General conditions for vine-shoots pretreatment and enzymatic hydrolysis were performed as mentioned in section 2.3. The analyses of hydrolysed sugars and total inhibitors were performed after enzymatic hydrolysis as described in section 2.8.

2.5. Strain cultivation and preliminary strains selection

Thirty-one solventogenic clostridia strains from the species *C. acetobutylicum* (9 strains), *C. beijerinckii* (9), *C. butyricum* (2), *C. pasteurianum* (2), *C. saccharobutylicum* (1), *C. saccharoperbutylacetonicum* (2), *C. sporogenes* (5) and *Clostridium* sp. (1) were initially tested to determine their potential as ABE producers (Supplementary Table 8). All the strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the Spanish Type Culture Collection (CECT, Paterna, Spain) and the Agricultural Research Service Culture Collection (NRRL, Peoria, IL, USA). Bacterial spores or cryopreserved cells (as in the asporogenous strain DSM 6228) were used for inocula preparation in Reinforced Clostridial Medium (RCM, Oxoid) or in a potato supplemented medium (strains DSM 792 and DSM 2152) as explained in previous works [23,24]. Bacterial cultures were kept at 35 °C until obtaining a density of $5 \cdot 10^8$ cells/mL (24-48 h) as determined by counting in a Bürker chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). These cultures were used as inocula for fermentation tests conducted in an aqueous solution, routinely used as a fermentation control in our laboratory, composed of glucose (23 g/L) and xylose (17 g/L) supplemented with yeast extract (5 g/L), KH_2PO_4 (1 g/L), NH_4Cl (2.1 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), cysteine (0.5 g/L) and $CaCO_3$ (5

g/L). The pH was adjusted to 6.0 with NaOH 50% (w/w) and 1.5 mL of bacterial inoculum was added to 48.5 mL of fermentation medium contained in rubber-capped bottles. An anaerobic environment was produced by injecting gaseous N_2 into the bottom of the closed fermentation bottles during 5 min. Fermentation conditions were set at 35 °C, 100 rpm and 96 h in an orbital shaker (Infors HT Minitron; Bottmingen, Switzerland). All the experiments were performed in triplicate and the criterion for strain selection was a butanol production over 5.0 g/L.

2.6. Clostridia strain screening

Vine shoots hydrolysates were generated after an optimised alkaline pretreatment (1.16% NaOH (w/w), 125 °C and 110 min) followed by an enzymatic hydrolysis (154 µL of enzyme Cellic CTec2 per gram of dry biomass, 50°C, 48 h). For fermentation tests, vine shoots hydrolysates were vacuum filtered (Filter paper No. 1305, 73 g/m^2, Filtros Anoia SA, Barcelona, Spain) and supplemented with yeast extract (5 g/L), KH_2PO_4 (1 g/L), NH_4Cl (2.1 g/L), $CaCO_3$ (10 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L) and cysteine (0.5 g/L). Medium preparation, bacterial inoculation and fermentation were prepared as described in section 2.5, by using the eleven selected strains from the species *C. acetobutylicum* (strains DSM 792, DSM 6228), *C. beijerinckii* (DSM 51, CECT 508, DSM 791, DSM 1820, DSM 6423), *C. pasteurianum* (DSM 526), *C. saccharobutylicum* (DSM 13864) and *C. saccharoperbutylacetonicum* (DSM 2152, DSM 14923), according to the conditions described in section 2.5. All experiments were performed in triplicate. The most efficient strain, in terms of butanol production and total sugars consumption, was selected for the next experiments.

2.7. Development of a minimal nutrient fermentation medium

After the selection of *C. beijerinckii* CECT 508, a Plackett-Burman experiment was designed to determine the minimum nutrient supplementation required in the broth to perform an efficient ABE fermentation (minimal fermentation medium, MFM). Vine shoots hydrolysate was prepared using the pretreatment and hydrolysis combined process optimized in section 2.4. The Plackett-Burman design consisted of 12 experimental runs combining the presence and absence of the 7 constituent nutrients of the culture medium (yeast extract, KH_2PO_4, NH_4Cl, $CaCO_3$, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$ and cysteine) as independent variables. Fermentation conditions were described in section 2.6. The response variable was butanol titre in the broth. The design characteristics and results are shown in Supplementary Table 9.

In order to compare the differences in sugar consumption and solvent production during ABE fermentation of vine shoots hydrolysates, an analysis was performed on a fermentation broth with all the nutrients described in section 2.6 (rich fermentation medium, RFM) and also with the minimal nutrients selected for *C. beijerinckii* strain CECT 508 through the Plackett-Burman approach (MFM). All the fermentations were performed as aforementioned in section 2.6. Bacterial cell growth, solvent production and sugar consumption were followed from 0 to 120 h in 24-h intervals. Each assay was carried out in triplicate.

After the selection of the minimal required nutrient supplements in the fermentation broth (MFM), $CaCO_3$, as the supplement required in the highest quantity, was substituted by eggshell powder to test its buffering capacity [25]. Eggshells were washed with running tap water 5 min and then dried in an oven 2 h at 100 °C and milled to a powder. In average, it was assumed that eggshell powder contains 94% of $CaCO_3$ [26], therefore it was added to the vine shoot fermentation broth in a final concentration of 10.64 g/L. Fermentation experiments were performed with the selected strain (CECT 508) as described in section 2.6.

2.8. Chemical analyses

Aqueous samples obtained after the physicochemical pretreatment and the subsequent enzymatic hydrolysis were managed and analysed according to Hijosa-Valsero et al. [19]. In brief, fermentable sugars (cellobiose, glucose, xylose, rhamnose and arabinose) and inhibitors (formic acid, acetic acid, levulinic acid, furfural and 5-hydroxymethylfurfural (5-HMF)) were analysed by HPLC with an Agilent 1200 HPLC equipment (Agilent Technologies) provided with a 300 x 7.8 mm i.d. cation exchange column Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) and a refractive index detector (RID) G1362A (Agilent Technologies). In addition to the fermentation inhibitors mentioned above, the concentration of total phenolic compounds was determined by Folin-Denis' method and expressed as gallic acid equivalents (GAE).

Fermentation metabolites (acetone, butanol, ethanol, acetic acid and butyric acid) were determined by GC-FID with an Agilent 7890 GC equipped with a flame ionisation detector (FID) using an HP-INNOWax 30 m x 0.530 mm, 100 µm column (Agilent Technologies) [19]. Fermentation yields (Y_i/s, g/g), metabolite productivity rates (W_i, g/(L·h)) and sugar recovery or sugar conversion efficiency (%) were calculated as described elsewhere [19].

2.9. Statistical Analyses

Comparative analyses among treatments were performed with one-way ANOVA and Tukey's HSD test or with t-test when only two treatments were compared; all tests were computed with the software Statistica v.7 (StatSoft Inc., Tulsa, OK, USA). Response Surface Methodology (RSM) and Plackett-Burman experimental designs and analysis were made with Minitab 16 (Minitab Inc., State College, PA, USA). It was required a probability of $p < 0.05$ to be considered significant (unless stated otherwise).

3. Results and Discussion

3.1 Pretreatment and hydrolysis optimization

As mentioned in section in section 2.3, an initial experimental approach showed that the best conditions that can be used as a starting point for a subsequent optimization were provided by the alkaline pretreatment with NaOH 2% (w/w), 10% biomass load, 121 °C, 60 min and a hydrolysis enzyme dosage of 130 µL/g. Using these conditions, it was possible to obtain up to 42.43 g/L total sugars (0.20 g/L arabinose, 2.06 g/L cellobiose, 29.73 g/L glucose, 0.12 g/L rhamnose, 10.32 g/L xylose) as well as 3.66 g/L total inhibitory compounds (2.15 g/L acetic acid, 0.60 g/L formic acid, 0.87 g/L phenolic compounds, 0.04 g/L 5-HMF). On the other hand, when using an enzyme dosage of 36 µL/g, only 25.14 g/L of total sugars were obtained (0.06 g/L arabinose, 1.06 g/L cellobiose, 16.74 g/L glucose, 7.28 g/L xylose) and a total of 3.41 g/L inhibitory compounds were released (2.06 g/L acetic acid, 0.58 g/L formic acid, 0.77 g/L phenolic compounds). These results are in accordance with those obtained by other authors for vine shoots (summarized in Table 1) where alkaline pretreatment followed by an enzymatic hydrolysis permitted obtaining a sufficient amount of fermentable sugars to be biotransformed in other biocompounds such as biosurfactants and ethanol [21,27].

The optimisation of physicochemical conditions of biomass pretreatment (NaOH concentration, temperature and time), as well as the enzyme dosage used during the hydrolysis stage, were performed via RSM experimental design (Supplementary Tables 1-7). RSM analysis was performed taking the amount of total sugars and phenolic compounds as response variables. The amount of

phenolic compounds, generated from partial decomposition of lignin, is a key limiting factor for growth and fermentation performance of solventogenic clostridia [35,36]. Accordingly, when applying the optimisation functions to the RSM equations obtained, different importance factors to each response variable (concentration of phenolic compounds and concentration of sugars) were applied. Therefore, three different importance ratios were assessed, namely 3:1, 2:1 and 1:1 (importance of phenolic compounds: importance of sugars). The optimization criteria were to simultaneously minimize phenolic compounds concentrations and maximize sugar concentration. Based on these three scenarios, optimized conditions obtained for each ratio are shown in Table 2. Estimated values for each optimized condition were validated experimentally; in all the cases, the amount of total sugars released was slightly lower than estimated (1.03 – 2.03 g/L). Besides, experimental phenolic compounds concentrations were slightly higher (0.1 – 0.2 g/L), but in general the three modelled optimal estimations were acceptable (Table 2, Supplementary Tables 1-7).

Table 1. Comparison of pretreatment methods used for sugar obtention from vine shoots.

Pretreatment	Enzymatic hydrolisis	Detoxification	Obtained sugars	Bioproduct	Reference
Autohydrolysis (210 °C, liquid/dry-solid ratio 8:1) + Acid Posthydrolysis (130°C, 1% H_2SO_4, 120 min)	No	$CaCO_3$ + Activated charcoal	15 g/L xylose, 7 g/L glucose, 2 g/L arabinose	Lactic acid	[28]
Acid prehydrolysis (130 °C, 3% H_2SO_4, 15 min, liquid/dry-solid ratio 8 g/g)	No	$CaCO_3$	17.5-18 g/L xylose,10.3- 11 g/L glucose, 5 g/L arabinose	Lactic acid	[28,29]
Autohydrolysis (200 °C, liquid/dry-solid ratio 8 g/g)	No	No	8.64 g/L glucooligosaccharides, 12.2 g/L xylooligosaccharides	No	[30]
Acid prehydrolysis (130 °C, 2% H_2SO_4, 15 min, liquid/dry-solid ratio 8 g/g)	No	$CaCO_3$ + Activated charcoal	10 g/L xylose, 6 g/L glucose	Biosurfactants	[27]
Alkaline hydrolysis (8% NaOH, 130 °C, 120 min, liquid/dry-solid ratio 10 g/g)	Yes	No	21.57 g/L glucose	Biosurfactants	[27]
Autohydrolysis (210 °C, liquid/dry-solid ratio 8 g/g, severity 4.47-4.65)	Yes	No	14.33-14.32 g/L glucose	Ethanol	[4]
Acid prehydrolysis (130 °C, 3% H_2SO_4, 15 min, liquid/dry-solid ratio 8:1 g/g)	No	$CaCO_3$	18 g/L xylose, 11.1 g/L glucose, 4.3 g/L arabinose	Lactic acid, biosurfactants	[31]

Pretreatment	Enzymatic hydrolisis	Detoxification	Obtained sugars	Bioproduct	Reference
2-stage Autohydrolisis (1st stage: 180 °C, 60 min, liquid/dry-solid ratio 6 g/g; 2nd stage: 200 °C, 30 min, 10% solid load)	Yes (after 2nd stage)	No	1st stage autohydrolysis: 1.3 g/L glucose, 2.0 g/L xilose, 0.4 g/L arabinose, 5.0 g/L glucooligosaccharides, 13.2 g/L xylooligosaccharides. 2nd stage autohydrolysis + enzymatic hydrolysis: 40.5 g/L glucose, 4.80 g/L xylose	Ethanol	[22]
Acid prehydrolysis (130 °C, 3% H_2SO_4, 15 min, liquid-solid ratio 8 g/g)	No	$CaCO_3$ + Activated charcoal	7.4 g/L glucose, 14.6 g/L xylose, 3.0 g/L arabinose	Lactic acid, xylitol	[32]
Alkaline hydrolysis (2.5% NaOH, 120 °C, 40 min, 5% solid load)	Yes	No	202 g glucose/kg of raw material	Ethanol	[21]
Acid prehydrolysis (130 °C, 3% H_2SO_4, 15 min, liquid-solid ratio 8:1 g/g) + Alkaline delignification (130 °C, 12% NaOH, 75 min)	Yes (post alkaline delignifica-tion)	$CaCO_3$ (post acid prehydrolisis)	11.1 g/L glucose, 17.4 g/L xilose, 4.3 g/L arabinose (post acid prehydrolysis). Solid from alkaline delignification was directly used in fermentation via SSF	Lactic acid	[33]
Acid prehydrolysis (130 °C, 3% H_2SO_4, 15 min, liquid-solid ratio 8:1 g/g) + Alkaline delignification (130 °C, 8% NaOH, 120 min, liquid-solid ratio 10:1 g/g)	Yes (post alkaline delignifica-tion)	No	16.1 g/L xylose, 11.7 g/L glucose, 1.6 g/L arabinose	Phenyllactic acid and biosurfactants	[34]
Alkaline hydrolysis (125 °C, 1.16% NaOH, 110 min, 10% solid load)	Yes	No	0.11 g/L arabinose, 1.24 g/L cellobiose, 30.94 g/L glucose, 10.55 g/L xylose	Butanol	This work

The most efficient conditions to perform vine shoots pretreatment, in terms of total sugars and phenolic compounds released, were 1.16% NaOH (w/w), 125 °C and 110 min, followed by a hydrolysis stage performed with an enzymatic load of 154 µL/g of pretreated biomass at 50°C during 48 h. Under these conditions, 42.84 g/L total sugars (0.11 g/L arabinose, 1.24 g/L cellobiose, 30.94 g/L glucose and 10.55 g/L xylose) were obtained, which corresponded to a recovery rate of 65.21% of available fermentable sugars in the pretreated biomass (63.75% of cellulose hydrolysis), as well as 0.84 g/L of phenolic compounds which corresponded to 23% of total inhibitory compounds released (2.23 g/L acetic acid, 0.54 g/L formic acid and 0.84 g/L phenolic compounds). The other two potential scenarios explored, generated a similar amount of fermentable sugar but they were not useful for conducting a direct ABE fermentation (data not shown) (Table 2, Supplementary Table 7). Compared to previous studies [21,27], in which glucose was the unique fermented sugar, the hydrolysis process optimised in this paper enhanced the amount of glucose reached in the

hydrolysate by obtaining up to 10.74 g/L more. Besides, the concentration of xylose obtained in this work was lower than the amount reported in most of the previous studies conducting autohydrolysis or acid hydrolysis (10 to 18 g/L xylose) (Table 1), since these two pretreatments are known to be especially successful for hemicellulose hydrolysis [37]. As a whole, the pretreatment developed in this study produces a very appropriate amount of sugars for ABE fermentation.

Table 2. Optimal working conditions for pretreatment and hydrolysis, estimated responses (according to an RSM experimental design) and experimental validation for vine shoot biomass.

	Physicochemical treatment and hydrolysis conditions (RSM)				Estimated responses (RSM)		Experimental responses	
Sce-narios*	NaOH (% w/w)	T (°C)	t (min)	Enzymatic load (µl/g)¥	Total sugars (g/l)	Phenolic compounds (g/l)	Total sugars (g/l)	Phenolic compounds (g/l)
3:1	1.16	125	110	154	43.89	0.70	42.84	0.84
2:1	1.72	101	69	154	42.98	0.70	40.95	0.80
1:1	1.59	104	71	154	42.99	0.70	41.09	0.83

*Three different importance ratios of each response variable in RSM (importance of phenolic compounds: importance of sugars) were assessed.
¥ µl of enzyme (enzymatic activity 113 FPU/ml) per gram of dried vine shoot

3.2 Fermentation of vine shoots hydrolysates and solventogenic strain selection

Initial strain selection was performed on 31 strains from a bacterial collection of solventogenic clostridia by using a routine synthetic fermentation medium with glucose and xylose as carbon sources. Butanol was not produced by eight of the strains (NRRL B-530, DSM 1739, DSM 13821, DSM 2477, DSM 2478, DSM 13136, DSM 634 and DSM 46278), while strain DSM 13864 produced the highest butanol concentration with 9.55 ± 0.40 g/L (Supplementary Table 8). According to the selection criteria defined in Section 2.5 based on a mean butanol production ≥ 5.0 g/L, eleven clostridia strains (CECT 508, DSM 13864, DSM 14923, DSM 1820, DSM 2152, DSM 51, DSM 526, DSM 6228, DSM 6423, DSM 791 and DSM 792) were selected and employed for fermentation on vine shoots hydrolysate (Figure 1). All selected strains are well known as ABE producers and they have also been tested with different lignocellulosic agri-food by-products such as tomato pomace, corn stover and sugarcane straw [15,38–40].

Subsequently, the 11 selected strains were assessed to perform ABE fermentation with vine shoot hydrolysates supplemented with RFM nutrients. Strains DSM 6423 and DSM 792 transformed acetone into isopropanol (4.55 ± 0.07 and 3.68 ± 0.07 g/L, respectively); in accordance with previous studies and the metabolic pathways described for both strains [41–43]. ABE solvent production varied among the tested strains: acetone was produced in a range from 0.00 g/L in DSM 51 to 3.72 ± 0.05 g/L in DSM 2152; butanol was produced in a range from 0.41 ± 0.20 g/L to 9.33 ± 0.25 g/L for strains DSM 13864 and CECT 508 respectively, while ethanol was produced from 0.16 ± 0.00 g/L in DSM 791 to 0.51 ± 0.03 g/L in strain DSM 6228. As a whole, the best solvent production was achieved by *C. beijerinckii* CECT 508 (2.85 ± 0.45 g/L acetone; 9.33 ± 0.25 g/L butanol; 0.25 ± 0.02 g/L ethanol; 12.43 ± 0.70 g/L ABE) and *C. beijerinckii* DSM 6423 (0.23 ± 0.02 g/L acetone; 8.04 ± 0.03 g/L butanol; 0.34 ± 0.01 g/L ethanol; 4.55 ± 0.07 g/L isopropanol; 13.17 ± 0.05 g/L ABEI). Biobutanol production was not statistically different between these two strains ($p = 0.381$); besides, sugar consumption was higher in CECT 508 (97.67% ± 0.78) than in DSM 6423 (90.82% ± 1.83), but this

difference was not statistically significant (p = 0.173) (Figure 2; Supplementary Tables 11-16). Accordingly, *C. beijerinckii* CECT 508 was selected to perform the experiments to optimise the type and concentrations of nutrients added to vine shoot hydrolysates.

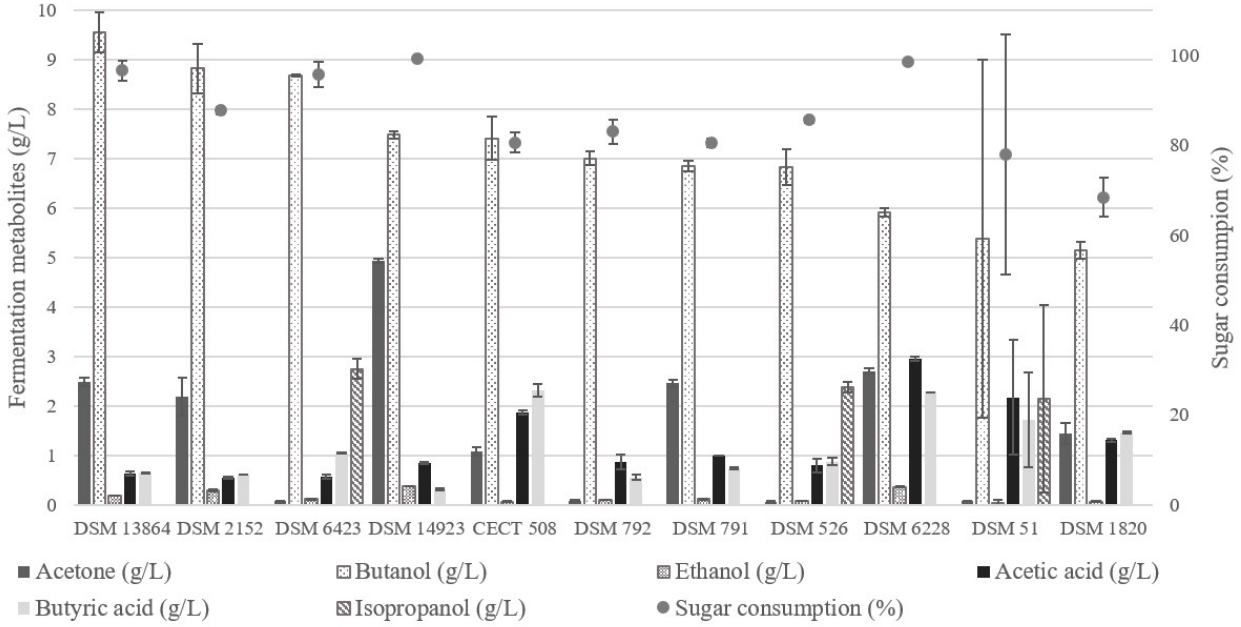


Figure 1. Solvent production and sugar consumption parameters for fermentation of 11 solventogenic clostridia strains with a mean butanol production higher than 5 g/L on a routine synthetic culture medium with 23 g/L glucose and 17 g/L xylose.

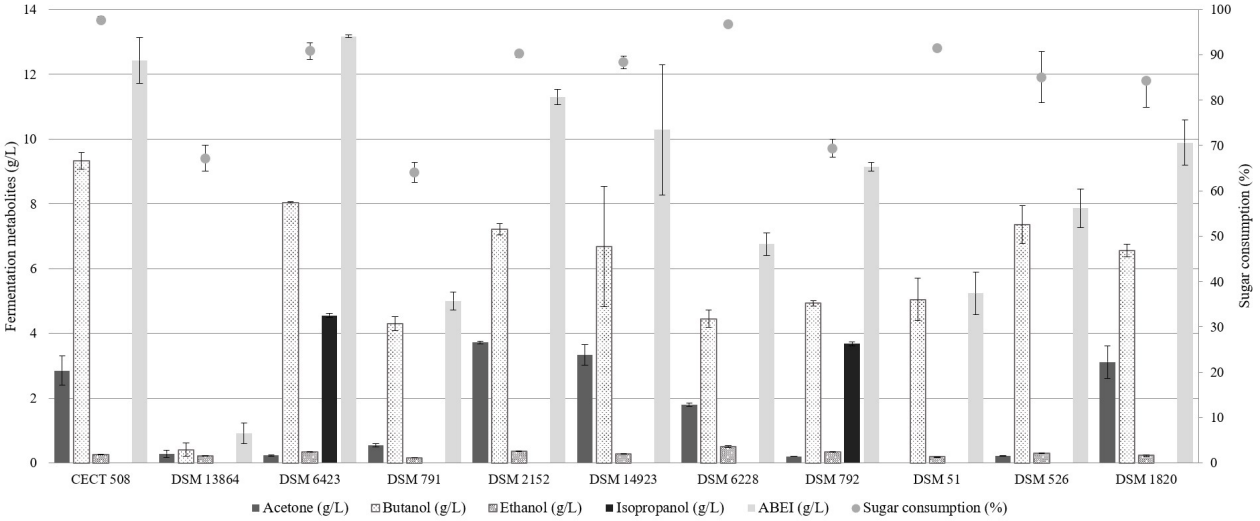


Figure 2. Parameters of ABE fermentation for vine shoot hydrolysate using eleven solventogenic clostridia strains. Different letters on butanol bars or sugar consumption dots represent statistical differences among strains for that parameter ($p < 0.05$). Strains DSM 6423 and DSM 792 also produced isopropanol. Complete data set is available in supplementary tables 11-16.

3.3 Development of a minimal nutrient fermentation medium

The high cost associated with nutrient supplementation in fermentation broths is one of the challenges to be addressed in biobutanol production [44]. To reduce the cost of using a rich fermentation medium (RFM) as the one described in section 3.2, the possibility of configuring a medium with minimal amount of nutrients (MFM) was explored, but guarantying the production of an acceptable amount of solvents. For this purpose, *C. beijerinckii* CECT 508 was selected by its high butanol production and total sugar consumption in vine shoots hydrolysate, but also since this is a well-known and a robust solventogenic strain with a good performance on several lignocellulosic feedstocks [45]. Through a Plackett-Burman experimental design, the essential nutrients for biobutanol production were determined. Briefly, acetone, butanol and ethanol titers ranged 0 – 4.30 g/L, 0.16 – 7.27 and 0 – 0.38 g/L respectively; while sugar consumption was in a range of 4.01 – 93.69% (Supplementary Tables 9-10).

According to the analysis of each nutrient on ABE fermentation performance (Supplementary Tables 9-10), $MgSO_4 \cdot 7H_2O$, KH_2PO_4 and yeast extract presented a negative effect, therefore they should be used in the minimal amount tested in the experimental design (0 g/L). Consequently, the best-fitting model was obtained when cysteine, $MgSO_4 \cdot 7H_2O$, KH_2PO_4 and yeast extract were removed from the fermentation broth. Besides, cysteine presented a positive but not significant effect ($p = 0.932$) and it was decided to set this nutrient at the lowest concentration, which also means the removal of this amino acid from the medium composition. On the other hand, $CaCO_3$ –the buffering agent that also enhances the tolerance to inhibitors and permits the concomitant utilisation of glucose and xylose [46] –, $FeSO_4$ – needed for ferredoxin production which is essential as an electron acceptor in *Clostridium* [47] – and NH_4Cl – as nitrogen source [48] – presented a positive and significant effect (considering $p < 0.06$), thus these three components were maintained in the fermentation broth and they were set at the highest concentration experimentally tested (10 g/L, 0.01 g/L and 2.1 g/L, respectively) (Supplementary Tables 9-10), which constitutes the MFM.

Finally, the Plackett-Burman experimental design model was validated experimentally employing vine shoots hydrolysate as substrate supplemented with the MFM cocktail. Under these conditions, strain CECT 508 produced 3.99 ± 0.20 g/L acetone, 7.44 ± 0.29 g/L butanol and 0.34 ± 0.01 g/L ethanol with an overall sugar consumption of 94.57 ± 1.57 % and a butanol productivity and yield of 0.06 ± 0.00 g/L·h and 0.19 ± 0.01 g/g, respectively. In a control run, employing a synthetic fermentation medium with the same carbon and nutrient content, the ABE solvent production was insufficient and not very repeatable (0.52 ± 0.47 g/L acetone, 1.32 ± 1.08 g/L butanol, 0.10 ± 0.08 g/L ethanol) with a low sugar consumption (17.16 ± 12.62%) and a butanol productivity and yield of 0.01 ± 0.01 g/L·h and 0.15 ± 0.03 g/g. This indicates that the presence of trace elements in vine shoots hydrolysate promoted cell growth as observed in other agrarian by-products [44]. These results also show that a low-cost MFM broth assured biobutanol production titers over 7.0 g/L, which can be considered above the average values obtained from most of lignocellulosic biomasses according to the available literature [45]. It was not possible to compare ABE fermentation performance in the present work with other studies conducted with vine shoots as feedstock, due to the apparent absence of previous studies on this subject. Regarding other winery residues, Jin *et al.* [12] reported a butanol and ABE yield of about 0.19 g/g and 0.32 g/g, respectively from grape marc hydrolysates, a higher-value byproduct, with similar results to those shown above using the minimally enriched MFM broth (0.19 ± 0.010 g/g butanol yield and 0.29 ± 0.004 g/g ABE yield).

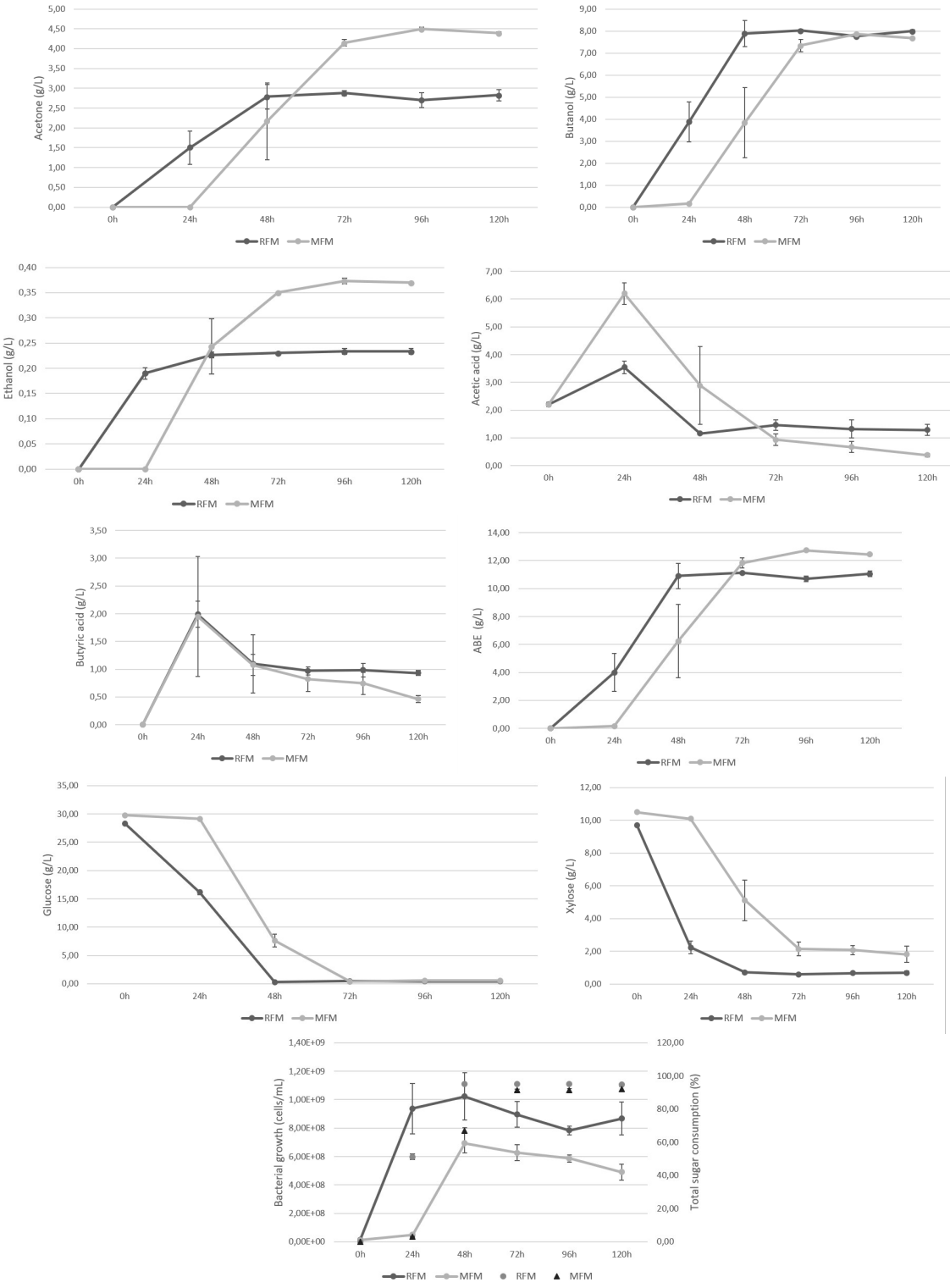


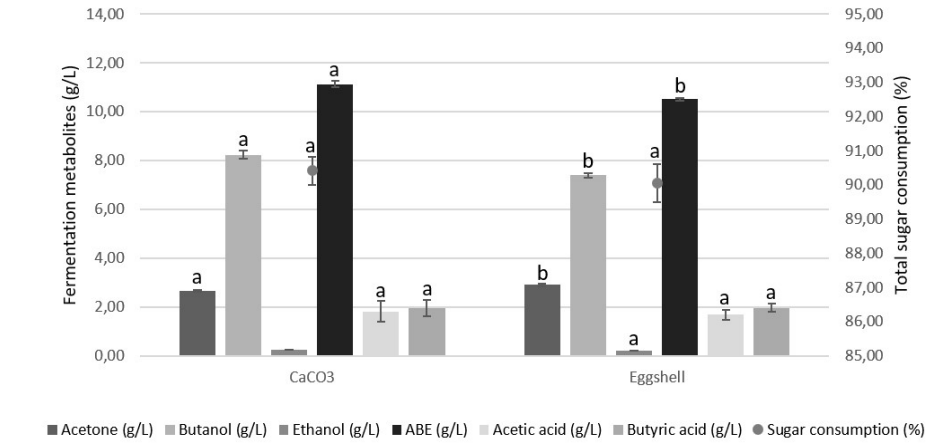
Figure 3. Parameters associated with ABE fermentation by *C. beijerinckii* CECT 508 from vine shoots hydrolysates supplemented with a rich nutrient content (RFM) or with a minimum nutrient content (MFM). Descriptions of both fermentation media are available in sections 2.6 (RFM) and 3.3 (MFM).

Finally, the evolution of ABE fermentation over time was assessed for *C. beijerinckii* CECT 508 in vine shoots hydrolysates with the two nutrient conditions MFM and RFM, over 120 h (Figure 3). Xylose and glucose in fermentation medium were 10.10 ± 0.56 g/L and 29.06 ± 1.01 g/L respectively. This

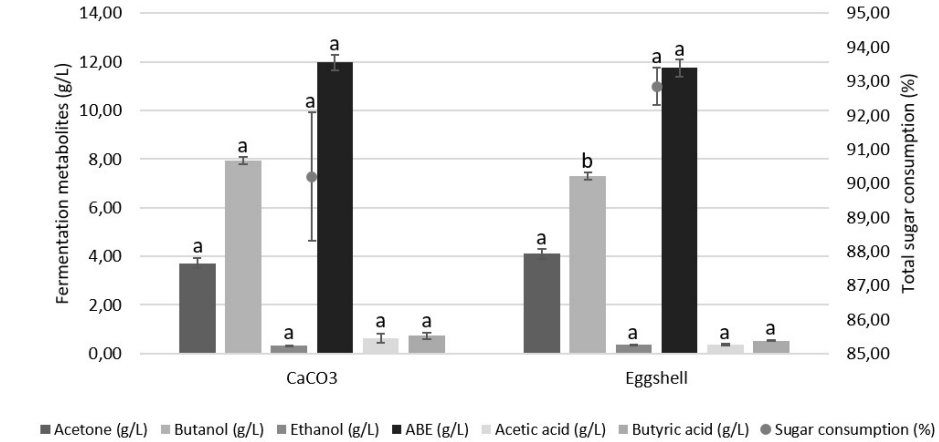
comparative analysis showed that the main difference between both fermentation media was the time required to reach the maximum butanol titer. In RFM, 7.89 ± 0.59 g/L butanol were produced at 48 h and no significant variation in butanol production was observed until 120 h ($p > 0.05$). In the case of MFM, 7.34 ± 0.28 g/L butanol were reached at 72 h and this concentration did not vary until the end of the experiment ($p > 0.05$) (Supplementary tables 17-18). This discrepancy was linked to a lag-phase in bacterial growth in MFM from 0 to 24 h (Figure 3). During this time, a high production of organic acids (6.20 ± 0.38 g/L acetic acid; 1.95 ± 1.08 g/L butyric acid) and a minimal sugar consumption (3.08 ± 0.25 %) was observed in MFM. In both fermentation media, the acidogenic to solventogenic shifting was done during exponential bacterial growth as described for several solventogenic clostridia [49] (Figure 3). In general, comparative analyses among both fermentation media at the end of the experiment, indicated that significant differences ($p < 0.05$) were observed in all the fermentation parameters (concentration of fermentation metabolites and sugar consumed), with a higher production of butanol, acetic acid, butyric acid and a total sugar consumption in RFM fermentation. However, ABE concentration was higher in MFM fermentation broth due to the higher amount of acetone and ethanol produced under this condition (Supplementary Tables 17-19).

3.4 Substitution of $CaCO_3$ by eggshell powder in the fermentation broth

The most abundant supplemented component in RMF and MFM broths was $CaCO_3$. Therefore, its replacement by eggshell powder – a non-value residue from the egg industry – was assessed. To this end, $CaCO_3$ was substituted with the equivalent amount of eggshell powder (assuming a $CaCO_3$ content of 94%), resulting in a significant reduction in biobutanol production (8.23 ± 0.47 butanol *vs* 7.38 ± 0.08 g/L butanol, $p = 0.001$), total solvent production (11.12 ± 0.13 g/L ABE vs 10.50 ± 0.06 g/L ABE, $p = 0.001$) when using eggshell powder in the RMF broth (Figure 4). This same trend was observed in the ABE fermentation performance when replacing $CaCO_3$ with eggshell powder in the MFM fermentation broth (7.93 ± 0.16 g/L butanol *vs* 7.30 ± 0.16 g/L butanol, $p = 0.008$), but no differences were found in total solvent production between these two conditions (11.97 ± 0.31 g/L ABE *vs* 11.75 ± 0.35 g/L ABE, $p= 0.446$) (Figure 4). Despite the slight reduction in butanol titre, these results confirmed the hypothesis that eggshell powder could be a suitable cheap substitute of $CaCO_3$ as buffering agent in ABE fermentation as it has been proven for the production of other bioproducts such as hydrogen, fumaric acid and polymalic acid [16,25,50]. In terms of cost reduction, under the laboratory conditions in which this study was performed, salts and nutrients required for biobutanol production from 1 L of vine shoots hydrolysate represents 1.56 € when using RFM as fermentation broth. This cost could be reduced by about 10% by supplementing RFM with eggshell instead of $CaCO_3$. However, the cost could be reduced up to 56% or 66% when using MFM as fermentation broth supplemented with $CaCO_3$ or eggshell, respectively (Supplementary Table 20). Therefore, this approach could be considered in future scalable assays in order to evaluate its potential in reducing industrial operational costs associated with the components of the fermentation broth, but also in environmental terms, using eggshells as a valuable material in ABE fermentation processes, thus fulfilling the European environmental requirements for this waste [51].



A



B

Figure 4. Fermentation of vine shoots hydrolysates performed in a rich nutrient (A) or a minimal nutrient (B) fermentation medium supplemented with $CaCO_3$ or eggshell powder as buffering agent with *C. beijerinckii* CECT 508. Different letters above bars and dots represent statistical differences among samples for a certain parameter ($p < 0.05$). Descriptions of both fermentation media are available in sections 2.6 and 3.3.

4. Conclusions

The optimisation of alkaline hydrolysis permitted obtaining the highest amount of glucose from vine shoots published to date, according to the studies available in this matter (Table 1), with a release of total sugars attractive for biobutanol production. This alkaline hydrolysate was directly fermented by eleven solventogenic clostridia strains, eliminating the need of performing any expensive and difficult detoxification process. Two *C. beijerinckii* strains (CECT 508 and DSM 6423) were able to produce a remarkable amount of ABE solvents (up to 12.43 g/L ABE by CECT 508) or ABEI (up to 13.17 g/L ABEI by DSM 6423) with a sugar consumption over 90% in both cases. As the highest butanol producer and the one with the highest sugar consumption, strain CECT 508 was selected for tuning up its fermentation medium by minimizing nutrient supplementation. Following this approach, it was possible to reduce from seven nutrients (described in section 2.6) to three ($CaCO_3$, $FeSO_4 \cdot 7H_2O$ and NH_4Cl), which implies a reduction of 56% in the operational cost, at laboratory scale, without a remarkable reduction in butanol production. However, the reduction of nutrients presented a drawback by increasing the fermentation time from 48 h to 72 h. Finally, it was demonstrated the possibility of substituting $CaCO_3$ by eggshell powder as buffering agent in ABE

fermentation, which implied a contribution to valorise a residue from the food industry in a circular economy approach, with a reduction of at least 10% in the operational cost, at laboratory scale. This buffer substitution implied a slight reduction in biobutanol production. However, in both cases, biobutanol production was over 7 g/L which is over the average obtained from most of the lignocellulosic biomasses according to the recent literature [45]. This study is the initial step for tuning up and test the valorisation of vine shoots at high scale, through a biorefinery process, such as those that are being currently boosted in Europe for this biomass that is massively produced and underused in those countries devoted to viticulture.

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Credit authors statement

Jerson Garita-Cambronero: Conceptualization, Investigation, Methodology, Formal analysis, Writing – Original Draft. Ana I. Paniagua-García: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Review and Editing, Investigation. María Hijosa-Valsero: Conceptualization, Methodology, Investigation, Writing – Review and Editing. Rebeca Díez-Antolínez: Conceptualization, Methodology, Writing – Review and Editing, Project administration.

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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