Journal of Cleaner Production 166 (2017) 706–716

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Development of a citrus peel-based biorefinery strategy for the production of succinic acid

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ARTICLE INFO

Article history:
Received 4 March 2017
Received in revised form
23 June 2017
Accepted 5 August 2017
Available online 5 August 2017

Keywords:
Citrus peel waste
Actinobacillus succinogenes
Succinic acid
Bioprocess
Biorefinery

ABSTRACT

A preliminary study has been performed for the valorization of citrus peel waste (CPW) through the biorefinery platform aiming to produce succinic acid. Following extraction of essential oils and pectin, different conditions of dilute acid hydrolysis were evaluated based on estimation of the sugars liberated and subsequent fermentation of hydrolyzates for production of succinic acid by *Actinobacillus succinogenes*. The most suitable pretreatment conditions involved 116 °C for 10 min using 5% (w/v) of dry raw material (drm). Thus, a total sugar (ts) yield of 0.21 $g_{ts}\ g_{drm}^{-1}$ and a succinic acid (sa) yield via microbial fermentations of 0.77 $g_{sa}\ g_{tsc}^{-1}$ was achieved, while the use of lower solid contents resulted in higher sugar yields. The residues from dilute acid hydrolysis were applied for subsequent enzyme hydrolysis using commercial enzymes and the most suitable combination of enzyme units included 30 IU cellulases and 25 BGL β-glucosidases achieving a yield of 0.58 $g_{ts}\ g_{drm}^{-1}$. Moreover, elemental analysis in hydrolyzates obtained from dilute acid hydrolysis and a combination of acid and enzyme hydrolysis indicated that during the combined treatment, high concentrations of Mg^{2+} and Ca^{2+} ions are liberated as compared to dilute acid hydrolysis, while the concentration of hydroxymethylfurfural was 0.038 g L^{-1} demonstrating low formation of inhibitors. The hydrolyzate generated through the combined pretreatment proposed was applied as feedstock for the production of succinic acid achieving a yield of 0.70 $g_{sa}\ g_{tsc}^{-1}$. However, although the combined hydrolysis approach could approximately double the sugars released in the hydrolyzate, the economic analysis performed confirmed that the use of the enzymatic treatment could not be competitive. The developed bioprocess constitutes a valuable alternative to the application of energy intensive chemical technologies for succinic acid production.

1. Introduction

Food waste (FW) constitutes a global environmental, economic and societal problem, that should be addressed by a combination of prevention and valorization approaches (Turon et al., 2014). The food manufacturing sector is generating 38% of the 90 million tons of FW produced by the European Union (Pfaltzgraff et al., 2013), while vegetables and fruits usually comprise the most-utilized items (Kosseva, 2013). Thus, the citrus worldwide production constitutes over 121 × 10^6 tons per year resulting in industrial generation of CPW that exceeds 25 × 10^6 tons (FAO, 2016). CPW formed during the processing of citrus for juice extraction, consist of peels, seeds and segment membranes, accounting for 50% of the whole fruit (Marín et al., 2007; Wilkins, 2009), while traditional management practices include the use of CPW as animal feed or disposal in landfills (Angel Siles López et al., 2010).

The need to replace the use of petroleum with new renewable resources for the production of fuels and chemicals and to identify novel practices for the reduction of biodegradable waste has led to the application of food waste as a feedstock for biorefineries (Lin et al., 2013). The valuable composition of the peel renders CPW a promising feedstock for biotechnological production of added-value commodities through the biorefinery platform. Specifically, CPW comprise a high content of cellulose, hemicellulose, soluble sugars, and pectin (Angel Siles López et al., 2010). Furthermore, CPW include 0.5% g $g_{wet\ mass}^{-1}$ of essential oils, consisting 90% of D-

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http://dx.doi.org/10.1016/j.jclepro.2017.08.039
0959-6526/

limonene (Li et al., 2010b) known to act as antimicrobial agent with numerous applications in the food and medical industries (Martín et al., 2010).

Pretreatment of CPW prior to the bioprocess usually requires the removal of essential oils due to the antimicrobial properties of D-limonene that may cause inhibition of the biosystem. However, pectin extraction is equally important comprising multiple functions in foods (jams, frozen foods, sugar replacer), pharmaceuticals (reduction of blood cholesterol levels, gastrointestinal disorders) and other applications such as paper substitute, edible films, foams and plasticizers (Thakur et al., 1997). Previous studies demonstrated that the generation of a hydrolyzate rich in carbohydrates through acid or enzymatic hydrolysis of CPW may serve as a valuable fermentation feedstock for the production of biofuels (e.g. bioethanol, biomethane), single cell protein or other products (Wilkins, 2009; Martín et al., 2010; Pourbafrani et al., 2010; Ruiz and Flotats, 2016; Koutinas et al., 2016). Thus, CPW could be employed through the biorefinery platform for the fermentative production of succinic acid, which is predicted to be one of the most important bio-based platform chemicals used for the manufacture of various added-value products. Succinic acid ($C_4H_6O_4$) is a dicarboxylic acid produced mainly by chemical routes such as catalytic hydrogenation, paraffin oxidation and electrolytic reduction of maleic acid or anhydride. Its conventional industrial applications include the production of polyester polyols, polybutylene succinate-terephthalate, resins, coatings and pigments as well as use in the pharmaceutical industry and in the food industry as flavorant and sweetener (Pateraki et al., 2016). However, the bio-based production of succinic acid entails a series of advantages, compared to its chemical production due to high theoretical yield and environmental friendly impact.

Various microorganisms such as *Mannheimia succiniciproducens* (Kim et al., 2004; Song et al., 2007) *Anaerobiospirillum succiniciproducens* (Lee et al., 1999, 2003), *Basfia succiniciproducens* (Scholten et al., 2009) and *Actinobacillus succinogenes* (Jiang et al., 2014; Li et al., 2010a) have been tested for succinic acid production in previous studies. Among the strains examined, *Actinobacillus succinogenes* is predicted to be one of the most promising industrial succinic acid-producing microorganisms based on the ability to utilize CO_2 and to produce high concentrations of succinic acid. *Actinobacillus succinogenes*, isolated from bovine rumen, is a capnophilic and mesophilic bacterium which is capable of valorizing monosaccharides under anaerobic conditions (Jiang et al., 2014).

The aim of the current work was to conduct a preliminary study for the development of a CPW-based biorefinery, as depicted on Fig. 1. The CPW biorefinery proposed was applied for valorization of the waste using *Actinobacillus succinogenes*. Specifically, the bioprocess presented targets estimating suitable dilute acid and enzyme hydrolysis conditions for enhancing the release of fermentable sugars from CPW in the hydrolyzate generated, following the removal of essential oils and pectin. The hydrolyzates formed were applied as fermentation feedstocks for the production of succinic acid, while the release of metal ions and fermentation inhibitors in the different hydrolysis approaches followed has been also evaluated.

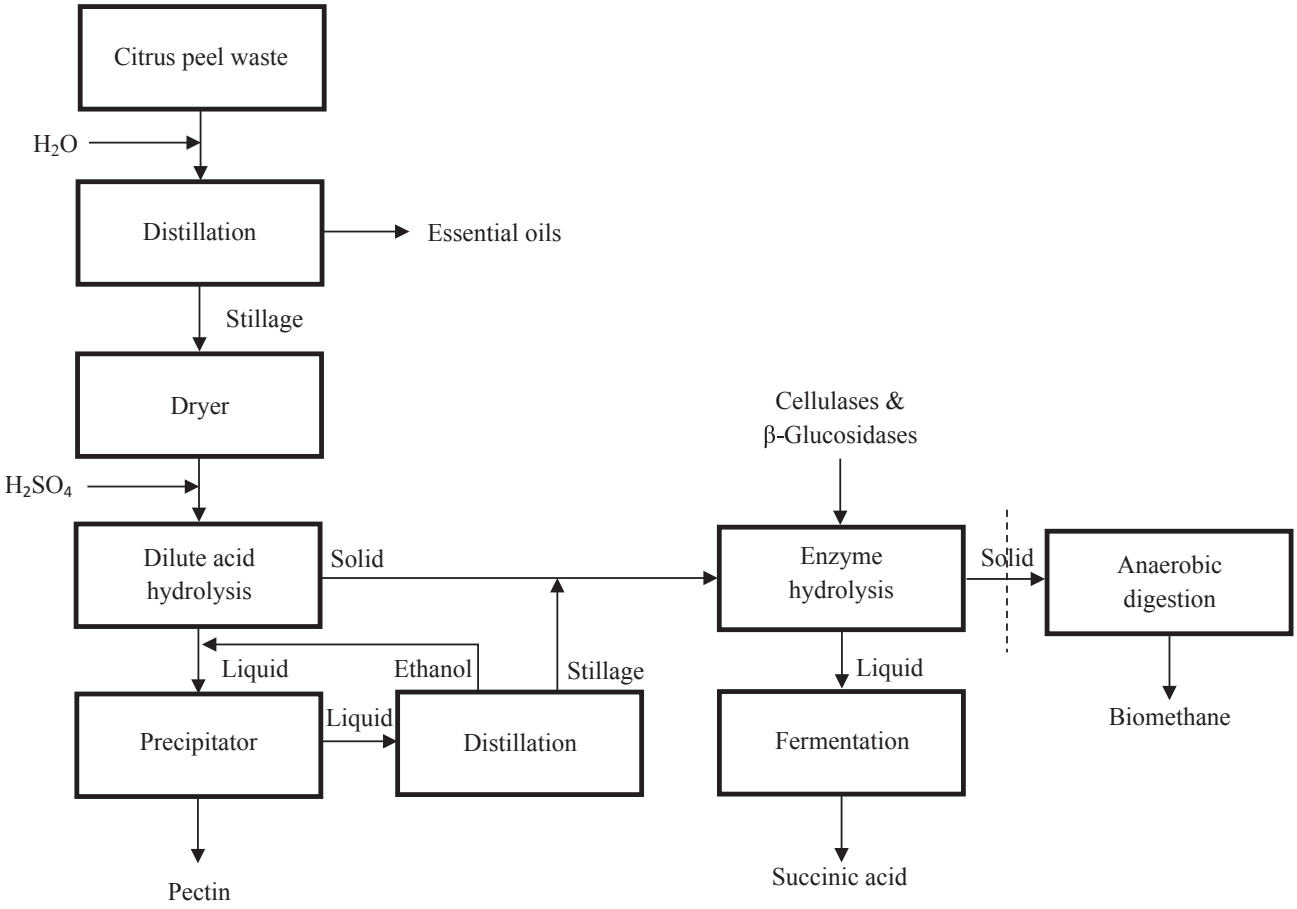

Fig. 1. Process flow sheet of the biorefinery used for CPW valorization. The dashed line denotes that anaerobic digestion was not examined in the present study.

Table 1
Conditions of acid hydrolysis.

Experiment	Temperature (°C)	Reaction time (min)	% (w/v) dry raw material
1	116	10	5
2	116	20	5
3	109	10	5
4	109	20	5
5	116	10	10
6	116	20	10
7	109	10	10
8	109	20	10

Table 2
Load of enzyme units employed in hydrolysis experiments (enzymatic treatment was performed in hydrolyzates generated through the conditions of acid treatment specified in experiment 1 - Table 1).

Experiment	Cellulases (IU g_{drm}^{-1})	β-Glucosidases (BGL g_{drm}^{-1})
A	20	25
B	30	25
C	20	35
D	30	35
E	20	50
F	30	50

2. Materials and methods

2.1. Citrus peel waste

The CPW used in the current work constituted citrus residues, which were obtained from a local juice factory (KEAN, Limassol, Cyprus) and stored at −20 °C until further use. CPW were thawed and ground to particles less than 2 mm in diameter using a laboratory blender (Waring Commercial, Texas, USA).

2.2. Isolation of D-limonene and pectin

The first step of CPW pretreatment required extraction and collection of essential oils through the addition of water to the raw material at a ratio of 6:1 (w/w) and boiling for 1 h. Essential oils were collected through distillation (Li et al., 2010b) and the residue was dried at 70 °C for 24 h (Wilkins et al., 2007a). An autoclave (SANYO MLS-3781L, Panasonic, Tottori, Japan) was used for dilute acid hydrolysis. Dry CPW was diluted with distilled water at 1:20 and 1:10 (w/v) ratios, while sulfuric acid was added to the mixture at a concentration of 0.5% (v/v) and hydrolysis proceeded at temperatures ranging between 109 °C and 116 °C, for 10 and 20 min (Table 1). Experiments were performed in duplicate. Centrifugation and filtration followed dilute acid hydrolysis in order to obtain the supernatant, which was mixed with an equal volume of ethanol (96% v/v) to precipitate pectin at room temperature for 4 h (Pourbafrani et al., 2010). Consequently, the mixture was centrifuged at 3000 rpm for 30 min. The precipitate was washed five times with ethanol (45% v/v) followed by drying at 50 °C to obtain pectin (Faravash and Ashtiani, 2007).

2.3. Enzyme hydrolysis

Following pectin extraction the hydrolyzate was mixed with solid residues from acid hydrolysis and it was subject to enzymatic treatment. The pH of the mixture was adjusted to 4.8 with the use of 1 M NaOH to ensure that the conditions for the process were within the optimal pH range 4.5–5.0 for the enzymes employed. Enzyme hydrolysis was performed in duplicate experiments for 48 h at 50 °C in shake flasks stirred at 100 rpm in a waterbath. Cellulases (Chem Cruz, Texas, USA) and β-glucosidases/pectinases (Oenozym FW, Lamothe-Abiet, Canejan/Bordeaux, France) from *Aspergillus niger* were employed in different ratios during the process based on Zheng et al. (2010). The enzyme ratios used in each experiment are provided on Table 2, while as soon as hydrolysis was completed the samples were heated in an oven at 105 °C for 15 min to inactivate the enzymes (Wilkins et al., 2005).

2.4. Microorganism and culture conditions

Actinobacillus succinogenes Z130 was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The strain was maintained at −80 °C in glycerol stock cultures and prior to the experiments the inoculum was cultured in 30 g L^{-1} of tryptic soy broth (TSB). TSB was sterilized at 121 °C for 20 min and *Actinobacillus succinogenes* was incubated at 37 °C for inoculum preparation in an orbital shaker stirred at 100 rpm for 14 h.

Succinic acid fermentations were performed in batch mode using 100 mL Duran bottles with a working volume of 130 ml. The reducing sugars obtained from dilute acid hydrolysis or combined acid and enzyme hydrolysis of CPW were used as carbon source for the experiments. The fermentation feedstock was additionally supplemented with 30 g L^{-1} $MgCO_3$ and 5 g L^{-1} yeast extract, while the inoculum volume applied was 13% (v/v) and continuous sparging of CO_2 was supplied with a flow rate of 0.5 vvm. Stirring was controlled at 100 rpm in a rotary shaking waterbath and temperature was maintained constant at 37 °C. All shake flask experiments were performed in duplicate, while two samples were analyzed for each replicate constituting analyses of a total of 4 samples in each experiment.

2.5. Analyses

2.5.1. Estimation of succinic acid and by-products concentration

The concentration of succinic acid during fermentation was determined through High Pressure Liquid Chromatography (HPLC). Culture samples were centrifuged at 13,000 × g for 5 min and filtered with 0.45 μm syringe filters. A Shimadzu LC-20AD liquid chromatograph (Shimadzu, UK) equipped with a Shimadzu SPD-20A UV/VIS detector, a Shimadzu SIL-20A HT auto sampler and a CTO-10AS VP column oven was used. The column was eluted isocratically at a rate of 0.6 mL min^{-1} from an organic analysis column (Rezex RHM-Monosaccharide H+ (8%) column, Phenomenex, USA) with 5 mM H_2SO_4 at 50 °C. The injection volume was 20 μL.

2.5.2. Estimation of reducing sugars' concentration

Depending on the requirements of each experiment two methods (NMR and phenol-sulfuric acid) were applied for determination of carbohydrates concentration. The content of reducing sugars in the CPW hydrolyzate obtained from the experiments that aimed in selecting the optimal conditions for dilute acid hydrolysis was measured through NMR analysis. Samples of each hydrolyzate (120 μL) were transferred to a 5 mm NMR tube and sodium acetate (50.0 μL, 5.00 mM) was added as internal quantification standard. Deuterium oxide was added up to a final volume of 500 μL, while all experiments were prepared twice. ^{1}H NMR experiments of freshly prepared solutions were recorded on a 300 MHz Bruker Avance spectrometer (UK), using a pre-saturating pulse for suppressing the water absorption peak, relaxation delay 5 s, 2925 Hz spectral window, and 128 scans. Data analysis was performed using the MestReNova software (Mesterlab Research, Spain).

During succinic acid fermentations reducing sugars were analyzed by the phenol-sulfuric acid method (Dubois et al., 1956) to

reduce the time required for analyses. This method is based on the phenol-sulfuric acid reaction and it is useful for the determination of simple sugars, oligosaccharides, polysaccharides and their derivatives.

2.5.3. Elemental analysis

An inductively coupled plasma mass spectrometer (Thermo X-Series II, Germany) was used for elemental determination of hydrolyzates obtained from dilute acid hydrolysis (conditions: 109 °C, 10 min, 5% of dry solids) and a combination of acid/enzyme hydrolysis (109 °C, 10 min, 5% of dry solids/20 IU crude cellulase solution g_{drm}^{-1}) as well as of the crude cellulase solution. Calibration curves with at least 6 points in the range of 5–100 μg L^{-1} were prepared for 21 trace elements: As, Be, Ca, Cd, Co, Cu, Cr, Fe, Li, Mo, Mn, Mg, Ni, P, Pb, Sb, Se, Sr, Ti, Tl and V. The calibration curve with the highest correlation coefficient was used for each element, while for the preparation of working standards a multi-analyte calibration standard (Thermo Scientific, Germany) was applied. All samples (15 mL) were acidified with 2% HNO_3 and 30 μL of an internal standard mixture of Ga, Lu and In were added prior to analysis. Each sample was analyzed in duplicate and for each duplicate at least 30 mass scans were performed. The concentration of trace elements in the samples was based on monitoring the analyte and its corresponding internal standard. A quality control (QC) in which a recovery of 80–120% of spikes and standards was used.

3. Results and Discussion

3.1. Experimental rationale

CPW was treated through a series of physicochemical and biochemical processes constituting the biorefinery depicted on Fig. 1. Essential oils (0.43% w/w) were first extracted from the waste while the solid residue was dried and applied to dilute acid hydrolysis. The choice of conditions for acid hydrolysis aimed at generating a hydrolyzate rich in carbohydrates assimilable by *Actinobacillus succinogenes* based on existing literature. Talebnia et al. (2008) demonstrated that the optimal conditions for dilute acid hydrolysis of CPW, based on the maximum total sugar yield and the minimum yield of hydroxymethylfurfural (HMF) constitute the use of 0.5% (v/v) H_2SO_4 at 116 °C for 13 min. Furthermore, the structures of arabinose and galactose are more stable at temperatures higher than 120 °C, while fructose is more stable between 100 °C and 120 °C as compared to higher temperatures (Grohmann et al., 1995). Thus, since *Actinobacillus succinogenes* demonstrates lower succinate yields when fed with galactose and arabinose as

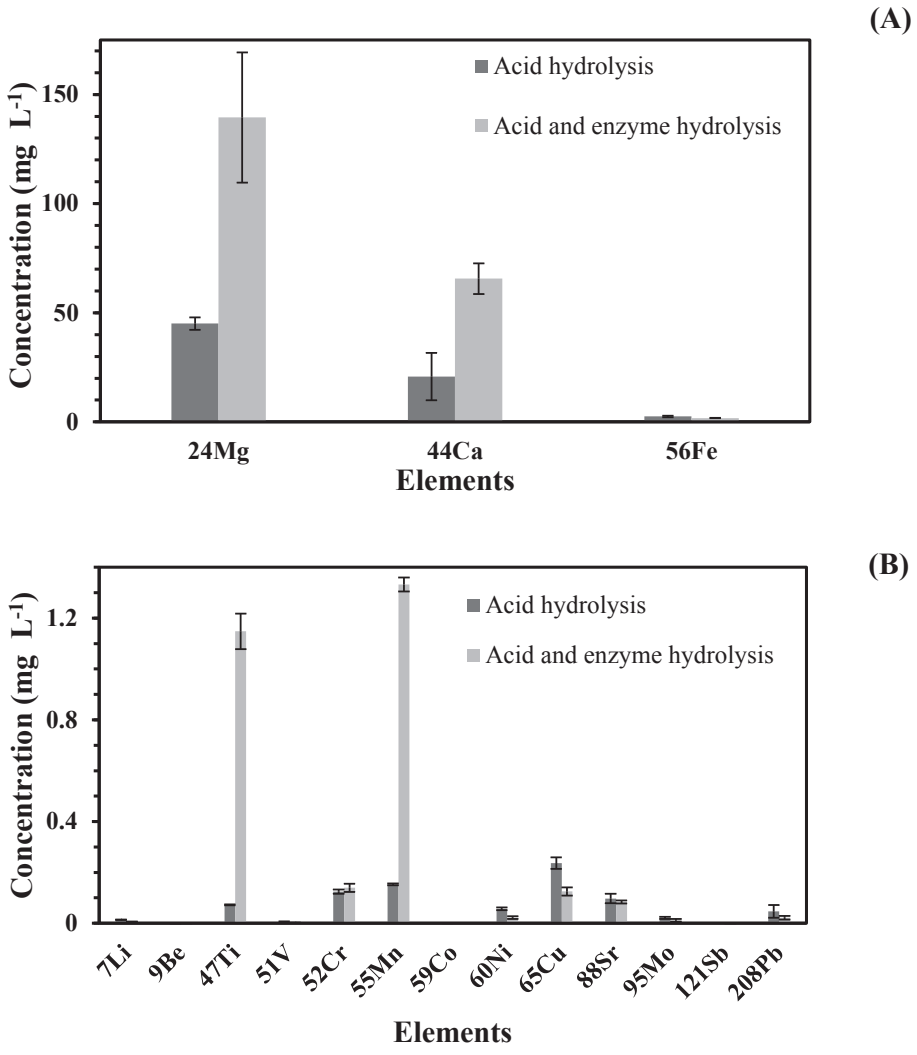

Fig. 2. Concentration of metal ions generated using dilute acid hydrolysis and a combination of sequential acid and enzyme hydrolysis. (A) Metal ions with concentrations higher than 1.5 mg L^{-1}; (B) Metal ions with concentrations lower than 1.5 mg L^{-1}. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

compared to fructose (Pateraki et al., 2016), the range of selected temperatures for dilute acid hydrolysis was limited below 120 °C. Dilute acid hydrolysis of CPW was tested with the use of the conditions specified in section 2.2, which were screened based on measurement of the total sugars yield as well as the formation of the final fermentation product. Pectin (23.25% w/w) was extracted from the hydrolyzate through ethanol addition according to the protocol presented in Pourbafrani et al. (2010), while distillation was applied at 80 °C for the removal of ethanol from the hydrolyzate. Subsequently, the hydrolyzate was mixed with the solid residues from dilute acid hydrolysis and it was enzymatically hydrolyzed with cellulases and β-glucosidases applied in different ratios aiming to enhance the release of fermentable sugars. All the hydrolyzates generated through dilute acid treatment or with a combination of acid/enzyme hydrolysis were tested as feedstocks for the production of succinic acid by *Actinobacillus succinogenes*.

3.2. Elemental analysis of hydrolyzates

Hydrolyzates obtained through acid hydrolysis (conditions: 109 °C, 10 min, 5% of dry raw material) as well as a combination of sequential acid and enzyme hydrolysis (109 °C, 10 min, 5% of dry raw material/20 IU crude cellulase solution g_{drm}^{-1}) of CPW, were analyzed through ICP-MS to evaluate the release of metal ions by each process. Additionally, the analysis was also performed for the crude cellulase solution to determinate the ion content contributed through enzymes' addition. ICP-MS analysis demonstrated the release of Mg^{2+}, Ca^{2+} and Fe^{2+} in substantially higher concentrations as compared to other detected metals ions such as Li^{1+}, Be^{2+}, Ti^{4+}, V^{5+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Sr^{2+}, Mo^{6+}, Sb^{5+} and Pb^{2+} (Fig. 2) for both hydrolyzates obtained through acid and the combined acid/enzyme hydrolysis. Specifically, 45.03 mg L^{-1} Mg^{2+}, 20.75 mg L^{-1} Ca^{2+} and 2.51 mg L^{-1} Fe^{2+} were measured in hydrolyzates obtained with the use of acid hydrolysis, while the rest of the metal ions detected were present in trace concentrations which were lower than 0.2 mg L^{-1}. Furthermore, the hydrolyzate obtained via a combination of sequential acid and enzyme hydrolysis (Fig. 2) comprised substantially higher concentrations of Mg^{2+} (139.46 mg L^{-1}), Ca^{2+} (65.65 mg L^{-1}), Ti^{4+} (1.14 mg L^{-1}) and Mn^{2+} (1.33 mg L^{-1}) as compared to the hydrolyzate generated through acid hydrolysis, while the rest of the substances detected did not demonstrate notable concentration difference between the two treatments. Mg^{2+} was detected in relatively high concentrations in the crude cellulase solution applied, demonstrating that following subtraction of the enzymatic solution's content in ions the net liberation of Mg^{2+} during enzyme hydrolysis constituted approximately 22 mg L^{-1}. However, the rest of the ions tested in the cellulase media included concentrations below the detection limit and did not affect the ionic composition of hydrolyzates.

Metal ions can be important factors affecting the efficiency of microbial fermentations. The concentrations of Cu^{2+}, Pb^{2+} and Ni^{2+} released from CPW by both pretreatment approaches (Fig. 2) were substantially lower as compared to the inhibitory levels previously identified for rumen microflora (Forsberg, 1978). Specifically, 150 mg L^{-1} of Pb^{2+} and 200 mg L^{-1} of Ni^{2+} did not present any negative effect on bacterial growth, while 21 mg L^{-1} of Cu^{2+} was required to inhibit cells by 50%. Thus, based on the release of two orders of magnitude lower ion concentrations, the hydrolyzates generated are not expected to include metal ions at inhibitory levels for *Actinobacillus succinogenes*, which is a strain known to have been isolated from rumen microflora. Furthermore, Mg^{2+} and Ca^{2+} ions serve as important cofactors for *Actinobacillus succinogenes* fermentations. Ca^{2+} are necessary for preserving the fluidity and permeability of the cell membrane, thus facilitating energy and transfer regulation (Norris et al., 1996; Li et al., 2011). Moreover, apart from the positive influence of $MgCO_3$ as a neutralizing agent for succinic acid production, which needs to be supplemented in *Actinobacillus succinogenes* fermentations for optimal performance, Mg^{2+} ions do not interrupt the stability of the membrane and cell flocculation is not observed (Pateraki et al., 2016). The results presented on Fig. 2 demonstrate that during acid/enzyme hydrolysis, Mg^{2+} and Ca^{2+} ions were liberated at substantially higher concentrations as compared to acid hydrolysis highlighting the favorable effect of combining the two hydrolysis methods for the release of ions that usually need to be supplemented to the fermentation medium.

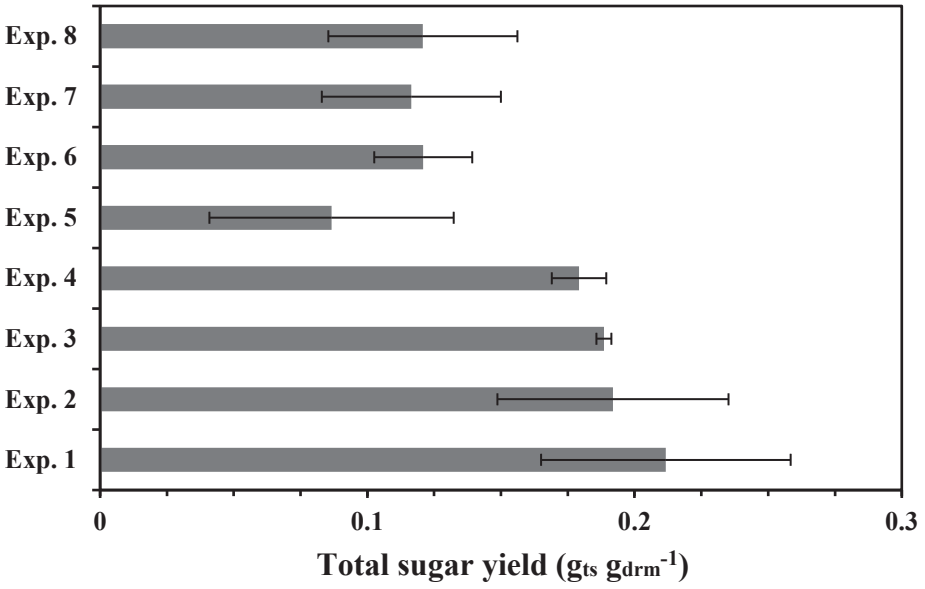

Fig. 3. Yields of sugars obtained through acid hydrolysis of CPW. NMR was used for detection of monosaccharides and disaccharides in the hydrolyzates generated, the concentration of which has been converted into glucose equivalents (yield = g_{ts} g_{drm}^{-1}, ts: total sugars, drm: dry raw material). Exp. 1: 116 °C, 10 min, 5% (w/v) drm; Exp. 2: 116 °C, 20 min, 5% (w/v) drm; Exp. 3: 109 °C, 10 min, 5% (w/v) drm; Exp. 4: 109 °C, 20 min, 5% (w/v) drm; Exp. 5: 116 °C, 10 min, 10% (w/v) drm; Exp. 6: 116 °C, 20 min, 10% (w/v) drm; Exp. 7: 109 °C, 10 min, 10% (w/v) drm; Exp. 8: 109 °C, 20 min, 10% (w/v) drm. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

3.3. Succinic acid production using hydrolyzates of CPW obtained through acid treatment

Dilute acid hydrolysis was applied to break down cellulose and hemicellulose into simple sugars, constituting also a necessary processing step for extracting pectin from the waste (Kaya et al., 2014). Acid hydrolysis conditions were selected based on the relevant literature as described in section 2.1 and the conditions tested in the experiments are listed on Table 1. The yields of reducing sugars (Fig. 3) released through the process were obtained using NMR. The highest sugar yields were achieved using 5% (w/v) of dry CPW and ranged between 0.17 and 0.21 $g_{ts}\ g_{drm}^{-1}$. Thus, the maximum yield of glucose equivalents liberated from the raw material was achieved at 116 °C for 10 min reaction time with 5% (w/v) of dry CPW, through the addition of 0.5% (v/v) sulfuric acid. However, the use of 10% (w/v) of dry CPW substantially reduced the yields obtained to a level of 0.08–0.12 $g_{ts}\ g_{drm}^{-1}$ demonstrating that low material contents enhanced the effect of acid hydrolysis. This conclusion was clarified by the *t*-test ($p < 0.05$) performed to identify statistically significant differences in the mean values obtained for the various acid hydrolysis conditions stated above. Pourbafrani et al. (2010) achieved a maximum sugar yield of 0.41 g g^{-1} of total dry citrus waste optimizing hydrolysis conditions through application of central composite rotatable experimental design that involved dilute acid hydrolysis of CPW at a substantially higher temperature (150 °C) for 6 min as compared to the current work. However, although higher sugar yields were obtained the considerably elevated temperatures employed are expected to raise processing costs as well as to potentially increase the content of inhibitors in the feedstock.

The presence of HMF, a common product formed during acid hydrolysis of lignocellulose due to dehydration of hexoses, was also investigated by NMR aiming to assess the influence of process conditions on the formation of fermentation inhibitors in the hydrolyzate (Fig. 4). Concentrations of HMF lower than 0.35 g L^{-1} have been previously demonstrated not to impose an inhibitory effect on *Actinobacillus succinogenes* fermentations (Gunnarsson et al., 2015). HMF analysis performed in all the hydrolyzates generated involved concentrations lower than 0.038 g L^{-1}, highlighting that the content of the inhibitor in the feedstocks produced was an order of magnitude lower than the non-inhibitory concentrations reported in the literature. The influence of hydrolysis temperature, time and acid concentration on the generation of fermentation inhibitors (Palmqvist and Hahn-Hagerdal, 2000) has been previously explored demonstrating that the concentration of HMF could continuously increase up to 120 h and 240 °C under dilute acid hydrolysis conditions (Asghari and Yoshida, 2006). Thus, it is expected that experiment 2 would exhibit the highest concentration of HMF since it was conducted at the highest temperature tested (116 °C) for 20 min (longest reaction time explored), while containing the lowest quantity of raw material employed (5% w/v). The *t*-test analysis performed demonstrated that the concentration of HMF in experiment 2 was the highest achieved exhibiting statistical difference as compared to the rest of the results including p-values that ranged between 0.02 and 0.04.

The CPW hydrolyzates formed through the acid hydrolysis experiments described above were applied following pectin recovery for the production of succinic acid in *Actinobacillus succinogenes* fermentations (Fig. 5). Biomass production was maintained at the same levels during all fermentations, while the total consumption of sugars as well as acids' production are presented on Table 3. *Actinobacillus succinogenes* could not completely metabolize the total carbohydrate content at the end of fermentation and apart from the formation of succinic acid, other organic acids such as formic, lactic and acetic acid were also generated in lower concentrations. The concentration of formic and lactic acid (reported as a combined concentration of the two metabolites) remained lower than 2.4 g L^{-1} in all experiments. However, the concentration of acetic acid was overall higher and reached 4.86 g L^{-1} in experiment 5. Although the concentration of succinic acid in the specific experiment was 9.11 g L^{-1}, the additional production of other organic acids was 7.26 g L^{-1} highlighting that following future optimization of fermentation conditions, aiming to minimize the generation of by-products, succinic acid yields could be substantially improved.

In line with the above, the succinic acid yields presented (Fig. 5)

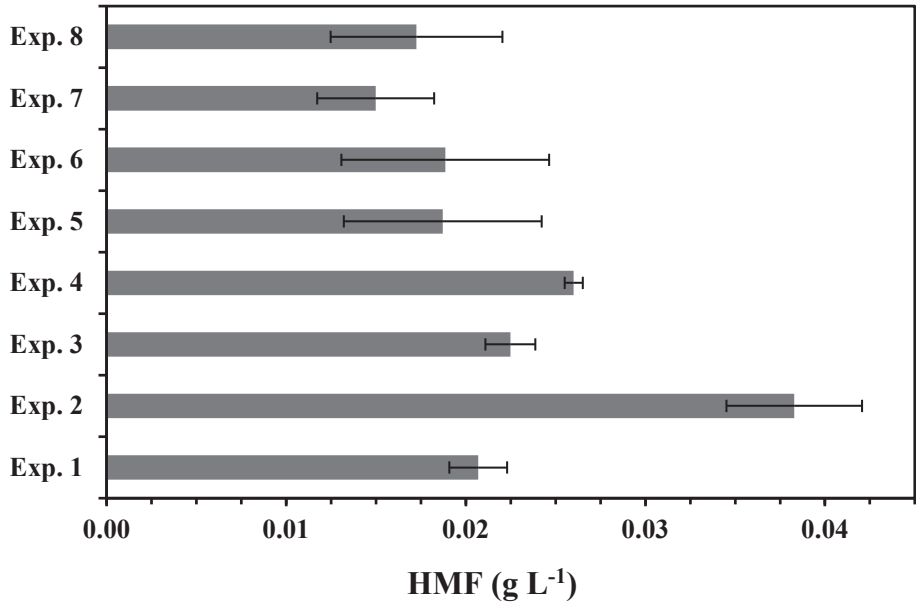

Fig. 4. Concentration of HMF in dilute acid hydrolyzates. Exp. 1: 116 °C, 10 min, 5% (w/v) drm; Exp. 2: 116 °C, 20 min, 5% (w/v) drm; Exp. 3: 109 °C, 10 min, 5% (w/v) drm; Exp. 4: 109 °C, 20 min, 5% (w/v) drm; Exp. 5: 116 °C, 10 min, 10% (w/v) drm; Exp. 6: 116 °C, 20 min, 10% (w/v) drm; Exp. 7: 109 °C, 10 min, 10% (w/v) drm; Exp. 8: 109 °C, 20 min, 10% (w/v) drm. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

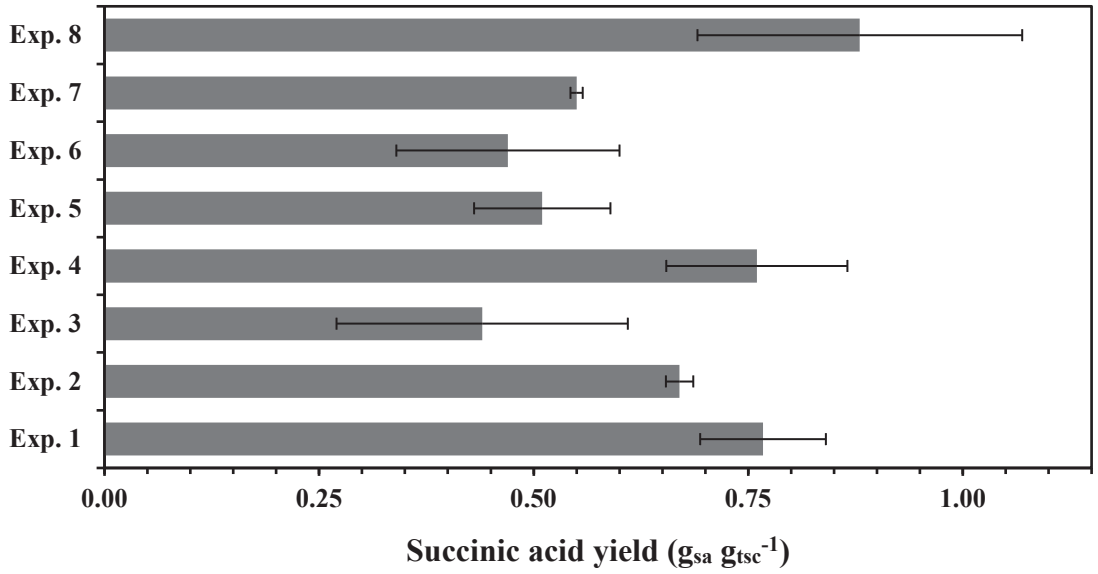

Fig. 5. Succinic acid yields achieved in *Actinobacillus succinogenes* fermentations of hydrolyzates obtained through dilute acid treatment of CPW (yield = g_{sa} g_{tsc}^{-1}, sa: succinic acid, tsc: total sugars consumed). Exp. 1: 116 °C, 10 min, 5% (w/v) drm; Exp. 2: 116 °C, 20 min, 5% (w/v) drm; Exp. 3: 109 °C, 10 min, 5% (w/v) drm; Exp. 4: 109 °C, 20 min, 5% (w/v) drm; Exp. 5: 116 °C, 10 min, 10% (w/v) drm; Exp. 6: 116 °C, 20 min, 10% (w/v) drm; Exp. 7: 109 °C, 10 min, 10% (w/v) drm; Exp. 8: 109 °C, 20 min, 10% (w/v) drm. Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

Table 3
Total sugars' consumption and final titre of products following fermentation of acid hydrolyzates.

Experiment	Concentration of sugars (g L^{-1})			Final product titres (g L^{-1})		
	ISC[b]	FSC[b]	TSC[b]	SA[a]	FA & LA[a]	AA[a]
1	12.00	4.01	7.99	6.13	1.29	2.17
2	11.91	3.73	8.18	5.48	1.39	2.67
3	15.79	2.15	13.64	5.95	1.73	2.79
4	12.94	4.85	8.09	6.17	1.42	2.60
5	21.83	4.08	17.75	9.11	2.40	4.86
6	15.84	3.09	12.75	5.95	1.73	2.79
7	9.47	2.12	7.35	4.05	1.27	1.59
8	16.15	5.26	10.89	9.57	1.13	3.03

[a] SA: Succinic acid, FA & LA: Formic and lactic acid, AA: Acetic acid.
[b] ISC: Initial sugars concentration, FSC: Final sugars concentration, TSC: Total sugars consumed.

were calculated as the final concentration of succinic acid generated over the total concentration of sugars consumed. The highest yield reached was 0.88 g_{sa} g_{tsc}^{-1} and it was obtained through fermentation of the hydrolyzate obtained at 109 °C for 20 min using 10% of dried CPW. However, Fig. 3 indicates that the highest yield of total sugars released was achieved in the dilute acid hydrolysis performed at 116 °C for 10 min reaction time using 5% (w/v) of dry CPW (presenting a yield of 0.77 g_{sa} g_{tsc}^{-1}). Thus, although higher succinic acid yields would be expected for process conditions generating elevated sugar yields, the slightly improved generation of succinic acid at 109 °C could be due to the release of higher contents of sugars assimilable by *Actinobacillus succinogenes* at lower temperatures (Grohmann et al., 1995). However, the *t*-test analysis performed between the results of Fig. 5 did not present statistical difference between the mean values of experiments 1 and 8 ($p = 0.44$) demonstrating that either of the two conditions could be used.

Similarly to the present work, different methods of CPW pretreatment have been employed for generation of hydrolyzates applied as feedstocks in microbial fermentations. Steam explosion, hydrothermal sterilization, dilute acid hydrolysis and enzymatic hydrolysis (through application of pectinases, cellulases and β-glucosidases) have been used for the release of sugars from the cellulose, hemicellulose and pectin content of the waste (Grohmann and Baldwin, 1992; Grohmann et al., 1995; Wilkins et al., 2007a). Thus, the production of bioethanol in *Saccharomyces cerevisiae* fermentations has been investigated in a citrus peel derived hydrolyzate generated through steam explosion (Wilkins et al., 2007b) followed by dilute acid hydrolysis and pectin recovery (Pourbafrani et al., 2010), where the yields obtained reached 0.43 $g_{ethanol}$ g_{ts}^{-1} for both pretreatment approaches applied. Moreover, acid pretreated orange peel waste have been tested for the production of single cell protein that contained 35–40% of crude protein including high *in vitro* digestibility (73–88%) with the use of *Geotrichum candidum* (Vaccarino et al., 1989).

3.4. Succinic acid production with the use of CPW hydrolyzates obtained through combined acid and enzyme treatment

A combination of sequential acid and enzyme hydrolysis was tested aiming to produce higher amounts of fermentable sugars as well as to enhance the formation of succinic acid. The dilute acid hydrolyzate obtained at 116 °C for 10 min treatment of 5% (w/v) dried CPW was chosen for further enzymatic treatment since the yield of total carbohydrates liberated was high, while the lower initial raw material applied would be expected to enhance the enzymatic conversion of polysaccharide molecules into fermentable sugars. Thus, enzyme hydrolysis was performed as described in section 2.3 and the enzyme load applied in each experiment is given on Table 2. The hydrolyzate employed for enzyme treatment contained an initial concentration of total carbohydrates of 16.6 g L^{-1} generated through the previous step of dilute acid hydrolysis (Fig. 6). The net release of total sugars through enzyme hydrolysis in all experiments conducted ranged between 10 and 15 g L^{-1}. The highest release of total sugars was obtained in experiments B and F were following 40 h of incubation the final total sugar concentration was 29 g L^{-1} for both experiments, while the total sugar yield reached 0.58 g_{ts} g_{drm}^{-1}. The specific yield was substantially higher as compared to the yield achieved through acid

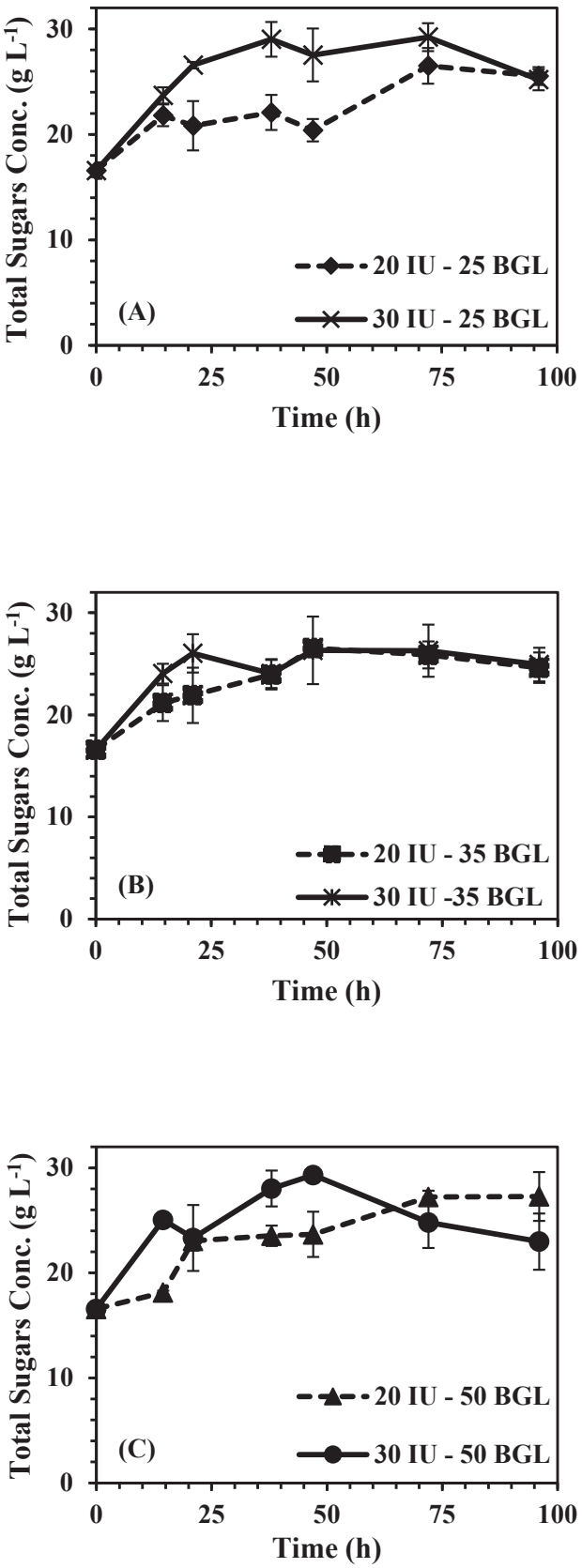

Fig. 6. Concentrations of total liberated sugars during enzyme hydrolysis of CPW through addition of a combination of cellulases and β-glucosidases supplied at a content of 25 BGL (A), 35 BGL (B) and 50 BGL (C). Enzymatic hydrolyses was performed using hydrolyzates generated through the conditions of acid treatment specified in experiment 1 (Table 1). Error bars represent standard deviation of 4 samples obtained from duplicate experiments.

hydrolysis applied as a single treatment (0.21 $g_{ts}\ g_{drm}^{-1}$). Thus, the highest yields were achieved with the use of 30 IU of cellulases as well as 25 BGL (experiment B) and 50 BGL (experiment F) of β-glucosidases, respectively. In an attempt to reduce the cost of the developed process a combination of 30 IU of cellulases and 25 BGL of β-glucosidases was chosen as the most suitable enzyme load combination between the enzyme hydrolysis conditions tested for application in the proposed biorefinery. Various, pretreatments of citrus waste have been previously explored for their capacity to release fermentable sugars (Table 4). Wilkins et al. (2005) and Lennartson et al. (2012) have evaluated enzyme hydrolysis as the sole pretreatment applied for orange peel waste hydrolysis through the use of a mixture of 1.4 $mg_{cellulase\ protein}\ g_{drm}^{-1}$ and 1.7 $mg_{\beta\text{-glucosidase protein}}\ g_{drm}^{-1}$ as well as 0.24 FPU g_{drm}^{-1} of cellulase, 3.9 IU g_{drm}^{-1} of β-glucosidase and 1163 IU g_{drm}^{-1} of pectinase, respectively. Although similar conditions (50 °C and pH of 4.8) were applied as compared to the current study the total sugar yield reached a lower level in both studies that ranged between 0.25 and 0.32 $g_{ts}\ g_{drm}^{-1}$. However, various researchers have previously suggested application of pretreatment in two sequential steps as a more efficient approach for CPW valorization. Thus, two stage acid hydrolysis (Oberoi et al., 2010), dilute acid hydrolysis and pectin recovery (Pourbafrani et al., 2010) as well as popping and enzyme hydrolysis (Choi et al., 2013) have been explored for citrus waste pretreatment and the total sugar yields achieved were 0.23 $g_{ts}\ g_{drm}^{-1}$, 0.41 $g_{ts}\ g_{drm}^{-1}$ and 0.63 $g_{ts}\ g_{rm}^{-1}$ respectively. The present work demonstrates that the yield of sugars released from the raw material could approximately double when a combination of acid and enzyme treatment is applied in CPW for the production of a carbohydrate-rich fermentation supplement.

The hydrolyzate generated through the two sequential steps of dilute acid (116 °C, 5% (w/v) for 10 min) and enzyme (30 IU of cellulases and 25 BGL of β-glucosidases) hydrolysis demonstrating the highest sugar yield was applied as feedstock for the production on succinic acid by *Actinobacillus succinogenes* achieving a maximum succinic acid concentration of 8.25 g L^{-1} (0.70 $g_{sa}\ g_{tsc}^{-1}$). Based on the data generated, without optimization of fermentation conditions the product yield achieved reached 62.5% of the theoretical yield considering that the maximum glucose to succinic acid conversion yield for *Actinobacillus succinogenes* corresponds to 1.12 $g_{sa}\ g_{glucose}^{-1}$ (Pateraki et al., 2016). *Fibrobacter succinogenes* S85 has been previously employed for the production of succinic acid using pretreated CPW following D-limonene removal and drying (Li et al., 2010b). However, the succinic acid yield obtained reached only 0.12 $g_{sa}\ g_{pre\text{-treated citrus peel}}^{-1}$ highlighting that the material should have been further hydrolyzed to generate a sugar-rich feedstock prior to fermentation. Previous studies demonstrated the release of reducing sugars through various technologies such as acid or enzyme hydrolysis as well as popping (Table 4). The results presented show that CPW treatment with relatively high reducing sugar yields entail higher energy demand. Acid hydrolysis experiments, including both two stage acid (Oberoi et al., 2010) and dilute-acid hydrolysis (Pourbafrani et al., 2010) as well as popping were performed at temperatures that ranged between 121 and 150 °C. Nevertheless, the current approach of CPW pretreatment integrates lower energy and environmental friendly technologies, due to the use of reduced acid hydrolysis temperatures (116 °C) combined with enzyme hydrolysis, while approximately reaching the maximum yield that exists in the literature (Table 4).

Actinobacillus succinogenes is predicted to be an industrially important microorganism because of its high efficiency in succinic acid production, while the use of CO_2 in fermentations constitutes a very promising aspect contributing a series of environmental benefits. Wheat, bread, cotton stalk and macroalgal hydrolyzates, as well as glycerol, rapeseed meal and whey have been previously

Table 4
Yields of total reducing sugars released through citrus waste pretreatment.

Raw material	Pretreatment method	Yield of total reducing sugars	References
Orange peel waste	Enzyme hydrolysis	0.25–0.30 (g g_{drm}^{-1})	Wilkins et al., 2005
Mandarin waste and banana peels	Steam depressurization	0.17 (g g_{drm}^{-1})	Sharma et al., 2007
Orange peel hydrolyzate	Two stage acid hydrolysis	0.23 (g g_{drm}^{-1})	Oberoi et al., 2010
Citrus waste	Dilute-acid hydrolysis and pectin recovery	0.41 (g g_{drm}^{-1})	Pourbafrani et al., 2010
Orange peel waste	Enzyme hydrolysis	0.32 (g g_{drm}^{-1})	Lennartson et al., 2012
Mandarin peel waste	Popping and enzyme hydrolysis	0.63 (g $g_{raw\ material}^{-1}$)	Choi et al., 2013
Citrus peel waste	Dilute acid hydrolysis and enzyme hydrolysis	0.58 (g g_{drm}^{-1})	Current study

Table 5
Succinic acid production in fermentations utilizing different raw materials.

Raw material	Nitrogen source	Fermentation	Succinic acid (g L^{-1})	Yield (g_{sa} g_{tsc}^{-1})	Reference
Glycerol	YE (10 g L^{-1})	Fed-batch, bioreactor	49.6	0.64	Carvalho et al., 2014
Wheat hydrolyzate	YE (5 g L^{-1})/Vit	Batch, bioreactor	62.1	1.02	Dorado et al., 2009
Bread hydrolyzate	BH (200 mg L^{-1} FAN)	Batch, bioreactor	47.3	n.d.	Leung et al., 2012
Cotton stalk hydrolyzate	YE (30 g L^{-1})/Urea (2 g L^{-1})	Batch SSF[a], shake flasks	63.0	0.64	Li et al., 2013
Macroalgal hydrolyzate	YE (16.7 g L^{-1})	Batch, bioreactor	33.8	0.63	Morales et al., 2015
Rapeseed meal	YE (15 g L^{-1})	Fed-batch SSF[a], bioreactor	23.4	0.115	Chen et al., 2011
Whey	YE (5 g L^{-1})/Pep (10 g L^{-1})	Batch, bioreactor	22.2	0.57	Wan et al., 2008
CPW hydrolyzate	YE (5 g L^{-1})	Batch, shake flasks	8.3	0.70	Current study

YE: yeast extract, Vit: vitamins, BH: bread hydrolyzate, Pep: peptone.
[a] Simultaneous saccharification and fermentation.

tested for the production of succinic acid by *Actinobacillus succinogenes* through supplementation of CO_2, obtaining a range of yields between 0.115 and 1.02 g_{sa} g_{ts}^{-1} (Table 5). Pateraki et al. (2016) underlined the importance of CO_2 on the environmental impact of the process emphasizing that anthropogenic energy-related CO_2 emissions could be employed for the production of succinic acid. Therefore, not only carbon assimilation and CO_2 recycling is targeted, but also the consumption of CO_2 during fermentation maintaining the reaction equilibrium. Furthermore, the use of CO_2 constitutes a crucial factor in microbial fermentations as it is inhibitory for the growth of a number of microorganisms (Dixon and Kell, 1989). Thus, the use of *Actinobacillus succinogenes* contributes two major advantages including the high yield of succinic acid production as well as the consumption of CO_2, which make it competitive in succinic acid fermentations. The results of the current study demonstrate the ability of *Actinobacillus succinogenes* to valorize CPW with high product yields (0.77 g_{sa} g_{tsc}^{-1} and 0.70 g_{sa} g_{tsc}^{-1} for acid hydrolysis and the combined pretreatment approach respectively) demonstrating the potential for future optimization of this novel CPW valorization route under environmental friendly conditions.

3.5. Economic analyses

The bio-based succinic acid production from renewable feedstocks holds the potential for sustainable replacement of its petroleum-based manufacturing (Jansen and Gulik, 2014). The raw material (maleic anhydride) costs of the chemical process constitute 1.027 \$ kg^{-1} of succinic acid exhibiting an overall conversion yield of 95% (w/w) (Song and Lee, 2006). However, glucose costs 0.39 \$ kg^{-1} of succinic acid including an overall conversion of 91% (w/w). Moreover, lignocellulosic biomass constitutes a low-cost alternative to the use of glucose as raw material for the production of succinic acid (Akhatar et al., 2014). Thus, the biochemical production of succinic acid from renewable resources could be a competitive alternative to petroleum-based processes saving fossil reserves and contributing environmental benefits as well as economic feasibility (Pinazo et al., 2015).

The economic feasibility of the biorefinery with the combined

Table 6
Overall annual revenue generated from the succinic acid production facility for Scenarios 1 and 2 as well as the total fixed-capital investment and total production cost of enzyme hydrolysis.

Revenue

		Scenario 1	Scenario 2
Succinic acid	9 \$ kg^{-1} (Lam et al., 2014)	**33,102 \$**	**44,820 \$**
Pectin	12 \$ kg^{-1} (Davila et al., 2015)	**83,700 \$**	**83,700 \$**
Essential oils	10 \$ kg^{-1} (Vlysidis et al., 2017)	**12,900 \$**	**12,900 \$**

Total fixed-capital investment for enzyme hydrolysis

Type	Component	Percentage of FCI[a] (%)	Cost (\$)
Direct	Purchased equipment	30	22,307.50
	Purchased-equipment installation	10	7435.83
	Instrumentation and controls (installed)	4	2974.33
	Piping (installed)	6	4461.50
	Electrical systems (installed)	4	2974.33
	Building (including services)	10	7435.83
	Yard improvements	2	1487.16
	Service facilities (installed)	5	3717.91
	Land	0	0
Indirect	Engineering and supervision	6	4461.50
	Construction expenses	8	5948.66
	Legal expenses	2	1487.16
	Contractor's fee	3	2230.75
	Contingency	10	7435.83
	Total FCI		**74,358.33**

Total production and depreciation cost of enzyme hydrolysis

Component	Description	Cost (\$ $year^{-1}$)
Raw materials	enzymes based on Olofsson et al., 2017	5325
Utilities	nd	nd
Operating labor cost	0.25 × 30,000 \$ based on Vlysidis et al., 2017	7500
Maintenance & repair	1% of FCI	743.58
Laboratory charges	8% of operating labor cost	600
Depreciation	5% of FCI	3717.92
	Total production and depreciation cost	**17,886.50**

nd: no data.
[a] The percentages were estimated based on Lam et al. (2014).

use of the enzymatic and the chemical treatment of CPW was assessed through comparison of the additional revenue contributed through enzyme hydrolysis and the costs incurred. The annually cost of the fixed capital investment (FCI) as well as the total production cost were estimated (based on Lam et al., 2014), considering treatment of 1 ton CPW day^{-1} for 300 days per year (Table 6). The annual revenue of products was estimated based on two scenarios. Apart from essential oils and pectin extraction, Scenario 1 included acid treatment as the sole hydrolysis process applied exhibiting production of 6.13 g L^{-1} (0.77 g_{sa} g_{tsc}^{-1}) of succinic acid, while Scenario 2 involved combination of acid and enzyme hydrolysis where the concentration of succinic acid reached 8.30 g L^{-1} (0.70 g_{sa} g_{tsc}^{-1}). 4.3 kg of essential oils and 23.25 kg of pectin could be extracted from pretreatment of 1 ton of CPW in both Scenarios, including annual revenues of 83,700 $ and 12,900 $ respectively (Table 6). 1 ton of CPW could yield 12.26 kg and 16.6 kg of succinic acid in Scenarios 1 and 2 respectively, while the addition of enzymatic treatment increased the revenue by 11,718 $. However, the total production cost (excluding utilities) and the depreciation of the FCI calculated using the 20-years straight-line method constituted 17,886.50 $, indicating that the use of enzyme hydrolysis cannot be competitive given that the additional cost involved in enzyme hydrolysis is substantially higher compared to the revenue incurred by the increase in succinic acid production.

4. Conclusions

In this work, a preliminary study for the development of a CPW biorefinery has been proposed and applied for valorization of the waste with the use of *Actinobacillus succinogenes*. Following extraction of essential oils and pectin, the residue was hydrolyzed to fermentable sugars via dilute acid and enzyme hydrolysis. The release of metal ions was enhanced through applying a combination of sequential acid and enzyme hydrolysis demonstrating that the concentrations of ions released were substantially lower to the inhibitory levels identified for rumen microflora, while Mg^{2+} and Ca^{2+} ions were formed at substantially higher concentrations in the combined treatment reducing the requirement for their addition to the fermentation medium. The most suitable conditions for the release of fermentable sugars through dilute acid and enzyme hydrolysis included 116 °C for 10 min and 5% (w/v) of dry CPW followed by the addition of 30 IU of cellulases and 25 BGL of β-glucosidases respectively, resulting in a substantially high total yield of 0.58 g_{ts} g_{drm}^{-1}. The hydrolyzate generated based on the combined pretreatment was fermented by *Actinobacillus succinogenes* for the production of succinic acid at high yields. Nevertheless, economic analysis confirmed that the combined CPW preprocessing approach could not be competitive due to the elevated cost of enzymatic treatment. Future research should include characterization and quantification of the essential oil and pectin fractions generated in the biorefinery, optimization of fermentation conditions and biogas production from the remaining organic residues.

Acknowledgements

We would like to thank KEAN Soft Drinks Ltd. for the provision of citrus peel waste.

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