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A Sustainable Strategy for the Conversion of Industrial Citrus Fruit Waste into Bioethanol

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Abstract: The present study focused on the development of a methodology for the production of ethanol from the fermentation of waste resulting from citrus fruit processing. The experimental design included a number of steps, each optimized to make the whole process cost-effective, energy-saving, and ecofriendly. Particular emphasis was given to the pretreatment of citrus waste (CW), which was carried out through a combination of physical means, namely milling, heating, sonication, and microwave irradiation. Following this, an enzymatic hydrolysis was performed by loading a mix of enzymes, i.e., cellulase, pectinase, and β-glucosidase. Different combinations and concentrations were assayed with respect to the effective degree of saccharification. Afterwards, the hydrolysate was transferred to a bioreactor, added with nutrients and inoculated with two yeast strains, i.e., *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. Fermentation lasted 48 h, leading to an amount of 40.1 $g \cdot L^{-1}$ ethanol. The process involved an extra step of fed batch that allowed the entire potential productivity of CW to be exploited by yielding 52.3 $g \cdot L^{-1}$ ethanol at a rate of 1.09 $g \cdot L^{-1} \cdot h^{-1}$. In accord with previously published data, this investigation has proven to be successful in reaching its prefixed objectives of sustainability.

Keywords: bioethanol; citrus waste; waste management; fermentation; cellulase; pectinase; β-glucosidase; saccharification; *Saccharomyces* spp.

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1. Introduction

World annual production of citrus fruit currently exceeds 143 million tonnes [1]. Approximately 50–60% of processed fruits consists of wasted material, namely peels, exhausted pulp, and seeds that are inedible and are, therefore, trashed away. These data make understandable how the improper management of citrus waste (CW) can exacerbate the present scenario of environmental decay. Recent attempts to valorize and give new life to CW include food and non-food applications, such as employment as a source of bioactives for nutraceutical formulations, animal feed, edible packaging, cosmetics, pharmaceuticals, biogas, and fertilizers [2,3]. However, not all the applications designed for CW reutilization have been demonstrated to be genuinely sustainable and advantageous. For instance, pelletizing CW for feedstuff manufacturing is an expensive process, accompanied by emission of gases, such as sulfur, nitrogen, and carbon dioxides, particulate matter, and, in general, pollutants derived from the decomposition of organic matter and from the dehydration process [4]. Not least, it should be pointed out that the low nutritional value of such feedstuff is often a cause of certain diseases, moreover, being unattractive—bitter taste—to cattle [5]. Conversely, the obtainment of biofuels from CW has demonstrated to be an appealing and timely strategy [6]. Among the alternative fuels for combustion engines, ethanol has been widely investigated in the last years, leading to an increasing demand for this chemical, especially when originating from ecofriendly sources [7,8]. An overview of literature highlights that CW biomass

pretreatment is a fundamental step for the obtainment of a good-quality bioethanol at an effective yield [9,10]. Such pretreatment includes a number of physical, chemical, physicochemical, and biological procedures. The goodness of pretreatment is a pivotal factor that controls the duration of fermentation and the ethanol production in its entirety [4,11,12]. Transformation of CW into biofuel is made possible by its content of carbohydrates that can be broken down to sugars upon hydrolysis and used as feedstock. The carbohydrate fraction of CW amounts to 70% ca. of total solid content; hence, the correspondent global potential of bioethanol production equals 1.2 billion liters [13]. The ethanol derived from the fermentation of vegetable sugars is commonly used as a blend with gasoline, after transformation into ethyl tert-butyl ether (ETBE), or 100% pure as motor fuel [14,15]. However, the bioethanol production technology from CW is not yet industrially mature. Conventional processes show clear weaknesses with regard to final yield and sustainability. In particular, the acidic hydrolysis that is conducted to minimize the degradation of sugars is an energy-expensive step; additionally, the acids or bases utilized dramatically reduce the ecofriendly aspect of the procedure. Also, the enzymatic hydrolysis, if not preceded by an effective physical pretreatment (i.e., microwave, sonication, high pressure, and hydrothermal), makes the whole process time-consuming and expensive [16].

In this scenario, the present work aimed to develop, on a lab scale, a sustainable strategy for the obtainment of bioethanol (BioEt) by fermentation of CW. The experimental activities that were oriented to maximize both the eco-efficiency and the yield of the process were applied to limonene-free orange and mandarin wastes coming from Sicilian processing plants. The conventional process was redesigned with a "green vision" to overcome some critical issues of conventional procedures. In particular, key steps of our approach were (i) selection of optimal yeast strains; (ii) substitution of acidic hydrolysis with physical and mechanical pretreatments; and (iii) increase in final bioethanol yield through circular recovery of residual dry matter.

2. Materials and Methods

2.1. Sampling

Citrus (orange and mandarin) wastes were obtained via the cold chain from a processing plant located in Messina, Italy. Citrus waste was limonene-free due to a preliminary deterpenation carried out by cryomilling, followed by distillation. Samples were kept at −20 °C until further processing.

2.2. Chemicals

Glycerol, magnesium sulphate, and calcium pantothenate were provided by Fisher Scientific (UK Ltd., Loughborough, UK). Commercial enzymes were as follows: cellulase from *Trichoderma* sp., 3–10 units/mg solid (Sigma-Aldrich, Saint Louis, MO, USA), with xylanase activity ≥3 units/g solid; pectinase from *Aspergillus niger*, 300,000 U/g (Tokyo chemical industry, Tokyo, Japan); β-glucosidase from *Aspergillus niger* (Novozyme 188) provided by Sigma-Aldrich, 665 CBU/mL, protein content 140 ± 5 mg protein/mL. MBTFA and pyridine silylation grade solvent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Helium of 99.9995% purity was supplied by Rivoira gases (Milan, Italy). The following standards were provided by J.T. Baker Fisher (Milan, Italy): sorbitol, glucose, sucrose, mannose, rhamnose, arabinose, galacturonic acid, xylose, galactose, and fructose. Chemicals for the preparation of Fehling's reagents were supplied by Merck (Milan, Italy), including potassium sodium tartrate tetrahydrate, copper sulfate, sodium sulfate, sodium hydroxide, and methylene blue indicator.

2.3. Proximate Composition

The determinations of crude proteins and moisture were performed on citrus waste according to the official analysis protocol AOAC 976.05 [17]. Briefly, the residue was digested with sulfuric acid (98%), copper (II) selenite dihydrate, and potassium sulfate

by SpeedDigester K-439 (Büchi, Switzerland) and then analyzed by KjelMaster System K-375 (Büchi, Switzerland) equipped with gas and steam scrubber (Scrubber K-415, Büchi, Switzerland). The resulting solution was treated with sodium hydroxide (40%) to develop ammonia, which was subsequently distilled and collected in 50 mL of boric acid (1%). Then, the amount of nitrogen was determined by titration with hydrochloric acid (0.1 N). The percentages of proteins were obtained from nitrogen percentage through a conversion factor of 6.25 [18]. For the evaluation of humidity and dry matter contents, 2 g of sample were weighed in a thermal balance at a constant temperature of 110 °C for 60 min. Pectin was extracted from CW and quantified according to the procedure reported elsewhere and based on acetic acid and alcohol precipitation [19]. Cellulose and hemicellulose were quantified in accord with Van Soest et al., 1991 [20].

2.4. Microrganisms

Saccharomyces cerevisiae ATCC 9763 and *Saccharomyces bayanus* BCS103 (Manassas, VA, USA) were cultured and maintained on yeast medium (YM) agar (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L, agar 20 g/L; Oxoid, Basingstoke, UK) at 4 °C. *Saccharomyces cerevisiae* and *Saccharomyces bayanus* were separately cultured overnight at 30 °C on a rotary shaker (INNOVA 44, Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA) at 250 rpm in tubes containing 20 mL YM. After overnight incubation, the cell suspensions were aseptically harvested by centrifugation at 3000 rpm for 5 min (Centrifuge 5810 R, Eppendorf UK Ltd., Stevenage, UK). The supernatant (YM media) was discarded and the yeast cells were washed twice in 5 mL 0.9% (*w*/*v*) NaCl to minimize nutrient transfer from seed culture to fermentation medium. The total viable yeast cells were measured by using a cell count reader (Nucleocounter® YC 100™, Chemo Metec, Allerød, Denmark). The standard yeast culture contained 10^8 cells·mL^{-1} of *S. cerevisiae* ATCC 9763 and 10^7 cells·mL^{-1} of *S. bayanus* BCS103.

2.5. Pretreatment of Citrus Waste (CW)

2.5.1. Physical Methods

Mandarin and orange CWs were preliminarily mixed in a 50:50 *w*/*w* ratio. Deterpenated samples of CW were milled to achieve particle sizes of around 500 µm and subsequently homogenized for 10 min to reduce further the particle size to around 200 µm. Afterwards, they were diluted with distilled water up to 17% dry matter (dm). Successively, the mixture was heated to 120 °C in autoclave for 15 min. Afterwards, sonication took place for 30 min at 50 Khz. Finally, microwave treatment occurred at 450 W for 8 min.

2.5.2. Enzymatic Hydrolysis

In order to breakdown carbohydrates into simple sugars, an enzymatic hydrolysis was carried out. The pH of the partially hydrolyzed mixture from a previous step was adjusted from 3 to 5 by means of ammonium hydroxide addition. The latter substance avoids the formation of harmful salts in the final product and provides nitrogen for microbial strains. Aliquots of enzymes per gram of pretreated CW were added as detailed: 1.9 mg cellulase, 1.6 mg pectinase, and 0.8 mg β-glucosidase. The whole mixture was incubated at 50 °C under stirring (300 rpm) for 48 h.

2.6. Determination of Sugars

Total reducing sugars were determined by means of back-titration with Fehling's reagents. Also, the single sugar species were detected and quantified through GC-MS technique. Samples were derivatized by an acylation reaction with MBTFA (acylation reagent) to remove hydroxyl active sites, therefore increasing volatility and improving detectability. The acylation step consisted of the conversion of hydroxyl groups into trifluoro esters via a carboxylic derivative. Sugars were isolated from hydrolysates by using liquid–solid extraction. Briefly, 1 g of hydrolysate was mixed with 20 mL

of methanol and 1 mL of a 1000 mg/L sorbitol solution (internal standard), and then ultrasound-assisted extraction was performed for 30 min. The obtained extract was filtered over anhydrous sodium sulfate and then evaporated to dryness on a rotary evaporator. To the obtained dry residue 0.25 mL of MBTFA and 0.25 mL of pyridine were added and stirred for 1 h at 65 °C. Finally, 1 µL was injected into the GC Dani Master GC1000, equipped with a split/splitless injector and a flame ionization detector (FID) (Dani, Instrument, Milan, Italy). A Restek (Rtx-1701) capillary column (30 m × 0.32 mm ID, 0.25 µm film thickness) was used. The oven temperature was programed as follows: from 40 °C (1 min hold) to 260 °C (5 min hold) at 10 °C·min^{-1}. Injector and detector were, respectively, set at 200 °C and 250 °C. Column flow was 1.2 mL min^{-1}. The injection volume was 1 µL, with a split ratio of 1:75. The individual peaks were identified on the basis of retention times and by comparison with the mixture of sugar derivatives, analyzed under the same conditions. Sugars were quantified with an external calibration curve with internal standard normalization by using sorbitol.

2.7. Fermentation

The experimental design for the fermentation process was based on previous experience with similar matrices [21]. After a period of 15 min at 120 °C in autoclave, the hydrolysates (3000 mL) were supplemented with urea phosphate (2.3 g/L), KCl (0.2 g/L), $MgSO_4 \cdot 7\ H_2O$ (3.8 g/L), Ca-pantothenate (0.0833 mg/L), and biotin (0.0833 mg/L). Afterwards, they were inoculated with microorganisms (250 mL inoculum seed of each strain). Experimental setup fermentation tests were carried out in a 5 L batch fermenter (Biostat Biotech B, Sartorius Stedim Biotech, Goettingen, Germany). The fermenter was equipped with one four-bladed Rushton turbine and control systems. Fermentation parameters were: temperature, 30 °C; pH 5; constant stirring, 300 rpm; aeration velocity, 3 L·min^{-1}; duration, 48 h. CO_2 evolution was measured during all fermentation tests using a BioPAT® Xgas 1 analyser for BIOSTAT® B-DCU II system (Sartorius Stedim Biotech, Goettingen, Germany). All fermentations were carried out until no further CO_2 fluctuations were observed. The pH was controlled by the addition of ammonium hydroxide during fermentation. For the fed-batch procedure, the fermentation broth was added with a 50% freshly pretreated CW at 24 h fermentation.

2.8. Quantification of Ethanol

Samples from the fermenter were collected at times 0 h, 24 h, and 48 h. After a centrifugation step (4000 rpm for 15 min), the supernatant was filtered through 0.45 µm nylon filter (Whatman, Merck, Germany) and analyzed by means of the same GC-FID system as for the analysis of sugars. A Supelcowax-10 column (30 m × 0.32 mm i.d. × 0.25 µm d_f) from Supelco (Merck, Germany) was employed. Oven temperature was initially set at 50 °C and then elevated at the rate of 7 °C/min to 100 °C. Samples were run in triplicate. Injector temperature was 250 °C, while detector temperature was 280 °C. Ethyl alcohol was quantified by means of a calibration curve preliminarily built by injecting serial dilutions of ethanol in acetonitrile (external standardization).

2.9. Statistical Analysis

The dataset has been subjected to one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. In particular, significant differences ($p < 0.05$) within means were analyzed. The dataset is expressed as mean ± standard deviation of triplicate measurements. XLStat statistical software (Excel 2016, ver 16.0) for Microsoft excel was used (Microsoft corporation, Redmond, WA, USA).

3. Results and Discussion

3.1. Proximate Composition of Citrus Waste

The deterpenated CW was preliminarily characterized as concerns its proximate composition. As can be seen from Table 1, no relevant differences could be caught

from the analyses of the two types of CW, namely orange- and mandarin-derived biomass. For this reason, it was decided to work indiscriminately on a mixture of the two CW types. Furthermore, the carbohydrate composition of CW before physical pretreatment was assessed, highlighting as predominant components pectin, cellulose, and hemicellulose.

Table 1. Proximate composition of citrus waste. N = number of samples. Each sample was analyzed in triplicate.

CW	Crude Protein (%)	Moisture (%)	Density (g/cm^3)	Viscosity (Pa·s)	Pectin (wt%)	Cellulose (wt%)	Hemicellulose (wt%)
Orange Mean ± s.d. (N = 10)	1.15 ^{a} ± 0.09	74.19 ^{a} ± 1.16	0.95 ^{a} ± 0.09	0.3 ^{a} ± 0.01	28.57 ^{a} ± 0.63	18.13 ^{a} ± 0.45	13.10 ^{a} ± 0.62
Mandarin Mean ± s.d. (N = 10)	0.93 ^{a} ± 0.09	73.47 ^{a} ± 1.21	0.93 ^{a} ± 0.07	0.4 ^{a} ± 0.02	27.35 ^{a} ± 0.78	17.29 ^{a} ± 1.02	11.38 ^{a} ± 0.98

Different superscript letters in the same column indicate significantly different values ($p < 0.05$).

3.2. Pretreatment

A fundamental requirement for a successful bioethanol production is sample pretreatment. The main purpose of this step is to make the CW matrix accessible to the attack of enzymes necessary for its conversion into fermentable sugars. Indeed, the hydrolysis of cellulose and hemicellulose is hindered by the degree of porosity, hence, by the accessible surface area, although other factors such as fiber crystallinity and amount play a crucial role [22]. Therefore, an effective pretreatment should remove structural barriers in order to facilitate hydrolysis and the formation of sugars. Additionally, pretreatment has to be cost-effective with no formation of by-products that could inhibit hydrolysis. To this end, a variety of methodologies have been employed, including (i) physical (milling, pyrolysis, and microwaves); (ii) physico-chemical (steam explosion, ammonia fiber explosion, and CO_2 explosion); (iii) chemical (acid, alkaline, wet oxidation, and organic solvents); and (iv) biological (cellulase-producing fungi and microorganisms) [23]. In this work, in order to make the pretreatment process environmentally sustainable, it was chosen to prepare the sample by applying a set of three physical methods, namely autoclave, sonication, and microwave irradiation. Pretreated CW was again analyzed with respect to its carbohydrate content, reportedly pectin (12.43%), cellulose (17.15%), and hemicellulose (12.32%). The physical pretreatment of CW is less effective in breaking down cellulose due to its strong crystalline structure and the presence of other resistant components in the cell walls. Hemicellulose further contributes to cellulose resistance. Unlike cellulose, pectin has a higher sensitivity to physical pretreatment due to its disordered and amorphous structure. Also, pectin surrounds cellulose and hemicellulose fibers, with low cross-linking regions. The effectiveness of ultrasonic pretreatment has been widely demonstrated for biofuel production from lignocellulosic material [24]. In particular, low-frequency sonication is advised for such applications in order to break down cell walls, release cell contents, alter the surface structure, and produce oxidizing radicals that chemically attack lignocellulose. With regards to microwaves, the literature reports numerous applications of this physical methodology for isolation or preparation of samples with vegetable origin [25,26]. Microwave irradiation is capable of providing two effects: thermal, with release of acetic acid that triggers a moderate autohydrolysis, and nonthermal, which causes vibrations of polar bonds within vegetable cells. As a unique feature of this methodology, an internal explosion occurs with disruption of the most resistant structures. Overall, the combined effects of the three methodologies applied were increase in surface area, partial depolymerization and hydrolysis, and increase in the total reducing sugars. Preliminarily, some trials were carried out by adding cellulase to CW in a ratio of 20 units cellulase per gram of CW. This ratio was

defined as optimal only in correlation to the experimental step aimed at investigating the influence of the pretreatment methodologies on the conversion yield of cellulose to glucose. Once established that the best performance was obtained with the triple combination, the amount of cellulase, namely the ratio cellulase:CW, was adjusted to the general process. In other words, a high amount of enzyme was employed only at a small scale.

Figure 1 shows the effects of different pretreatments on the conversion yield of cellulose to glucose after cellulase addition. The three physical methods were tested both separately and combined. As can be seen, the highest yield was obtained from the triple combination, with a 100% yield observed after 36 h. When considering each pretreatment type, the least effective proved to be the ultrasound method, with a maximum conversion yield of only 35%. Heating and milling demonstrated their fundamental role with a good yield (86%) after 36 h, as well as microwave irradiation (88%). After this trial, it was chosen to pretreat CW with the multicombined procedure and to set at 48 h the duration of the enzymatic hydrolysis.

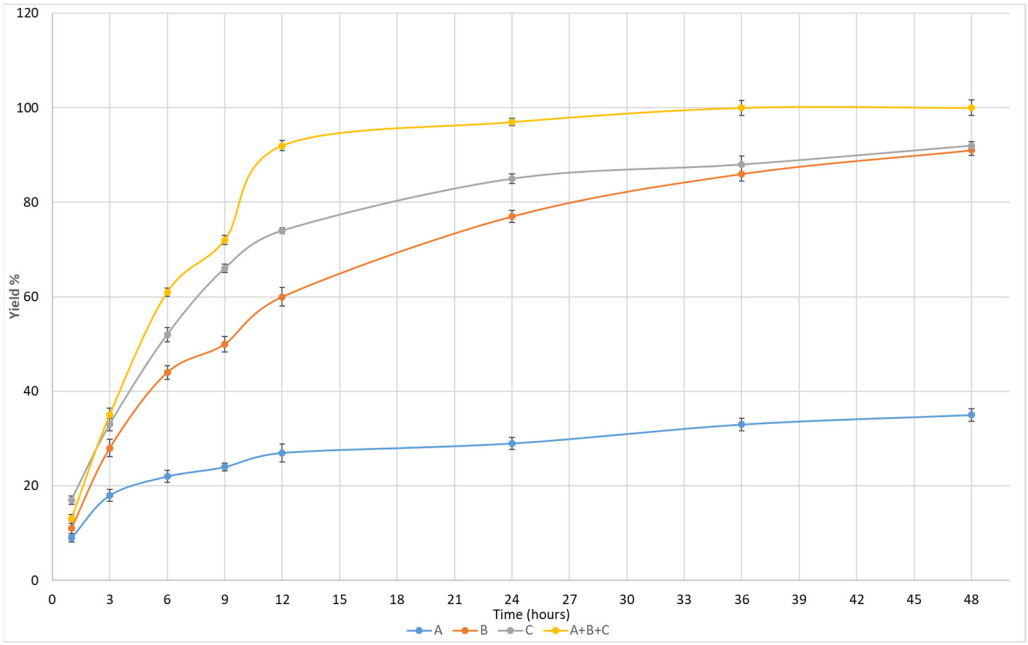

Figure 1. Conversion yield (%) of cellulose to glucose. Type of pretreatment: A, sonication; B, thermomechanical; C, microwaves.

3.3. Enzymatic Hydrolysis

Low loadings of cellulase and pectinase allowed high yields of reducing sugars to be obtained. The enzyme loading was expressed as mg of enzymes per g of hydrolysate. Different concentrations of enzymes were tested in order to define the most convenient loading for optimization of sugar production. Enzyme loadings were studied in accord with the activities declared by the manufacturer. Furthermore, a fixed amount of β-glucosidase was added to cellulase and pectinase to decrease inhibition of cellulase in case of the presence of cellobiose [27]. The results related to this step of method development have been summarized in Figure 2.

The quantitative composition of enzyme preparations has been suitably established after several trials (see Figure 2) carried out on the pretreated CW and having in mind two issues: (i) ratio of minimum quantity—hence, minimum costs—to maximum yield of sugars; (ii) literature data and enzymatic activity of the strains at our disposal. The conversion percent of dry matter into sugars was 72–73% ca. both for 1.9/0.8/1.6 mg and for 3.8/0.8/3.2 mg loadings. In consideration of the halved quantity of enzymes, the chosen loading was 1.9 mg cellulase/0.8 mg β-glucosidase/1.6 mg pectinase that

yielded 71.8% of conversion to reducing sugars. During the enzymatic saccharification, a good increase in sugar monomers was observed as a result of cleavage of cellulose and hemicelluloses. Beyond glucose, other monomers have been identified that are typical products of hemicellulose breakdown, such as xylose, mannose, galactose, and arabinose. On the other hand, galacturonic acid has been produced by the breakdown of pectin.

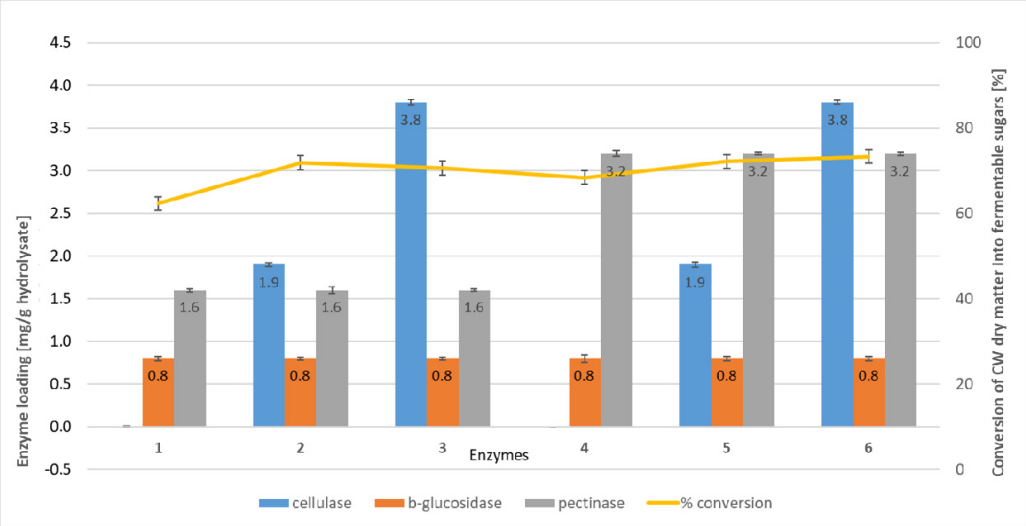

Figure 2. Effects of different enzyme loadings on the conversion of CW dry matter into fermentable sugars.

3.4. Reducing Sugars and Fermentation

Two different procedures were used for the quantification of total reducing sugars (TRS) and single sugar species, respectively. In particular, TRS were quantified by means of Fehling's reagents titration. Samplings occurred before enzyme addition and every 12 h since the fermentation started. Due to the effectiveness of physical pretreatment, the initial concentration of sugars was quantified as 56.6 ± 2.3 g·L^{-1}. This value further increased after enzymatic hydrolysis by reaching a maximum of 73.3 ± 2.7 g·L^{-1}. On the other hand, GC-MS analysis allowed identity to be assigned to nine different sugars. Major products of hydrolysis were glucose, fructose, and galacturonic acid; whereas minor products included the pentoses arabinose and xylose, the hexoses galactose, rhamnose, and mannose, and the disaccharide sucrose. All their quantities have been reported as g·L^{-1} in Table 2, along with their changes during fermentation. Glucose and fructose were promptly metabolized by the yeasts. Fructose, preliminarily quantified as 4.7 g·L^{-1} in CW, is mainly produced by the breakdown of sucrose, whose amounts were 11.28 g·L^{-1} and 1.66 g·L^{-1} in fresh and hydrolyzed CW, respectively [28]. As can be seen, arabinose and xylose remained approximately unchanged during the whole fermentation process. This finding can be attributed to the inability of Saccharomyces spp. to metabolize pentoses, although xylose is one of the most important contributors to fermentation present in cellulosic biomass [29]. A short remark has to be given on the choice of *S. bayanus* as an additional yeast strain. *S. bayanus* has been reported as a consumer of fermentation by-products, which, in turn, can cause stress to *S. cerevisiae*, thus compromising the success of fermentation. It also appears as a robust yeast able to metabolize sugars even under less favorable conditions [30].

Table 2. Concentrations ($g \cdot L^{-1}$) of sugars at different stages of the fermentation process ($n = 3$).

Sugar	Sampling Time		
	0 h	24 h	48 h
Arabinose	3.63 ± 0.08 [a]	3.09 ± 0.42 [a]	2.52 ± 0.13 [a]
Xylose	1.82 ± 0.05 [a]	1.57 ± 0.08 [a]	1.29 ± 0.06 [a]
Fructose	13.28 ± 0.08 [b]	2.72 ± 0.12 [a]	1.42 ± 0.18 [a]
Glucose	30.01 ± 0.26 [c]	3.48 ± 0.15 [a]	1.04 ± 0.21 [a]
Galactose	4.22 ± 0.61 [a]	2.18 ± 0.09 [a]	0.99 ± 0.08 [a]
Galacturonic acid	12.8 ± 0.12 [a]	7.72 ± 0.08 [a]	0.81 ± 0.03 [a]
Rhamnose	1.92 ± 0.09 [a]	0.85 ± 0.03 [a]	0.46 ± 0.08 [a]
Mannose	1.62 ± 0.07 [a]	0.97 ± 0.01 [a]	0.39 ± 0.05 [a]
Sucrose	1.66 ± 0.12 [a]	0.89 ± 0.11 [a]	0.56 ± 0.05 [a]
Total	70.96 ± 1.48	23.47 ± 1.09	9.48 ± 0.87

Different superscript letters in the same column indicate significantly different values ($p < 0.05$).

3.5. Production of Bioethanol

The liquid effluent from the bioreactor was roughly analyzed for its volatile composition by means of GC-MS. After a liquid–liquid extraction with n-hexane, the organic layer was injected into the GC-MS system—the mass spectral matching highlighted the presence of compounds mainly belonging to furans and phenols (i.e., furfural and guaiacol). Parameters influencing fermentation, such as temperature, feedstock amount, pH, and yeasts concentrations, were all tuned during method development in accord with the literature and previous experience [21,31]. The results presented in Figure 3 show that ethanol concentration at 12 h was 12.5 ± 1.5 $g \cdot L^{-1}$. This amount gradually increased until reaching the maximum volumetric productivity in the range from 0.8 to 1.0 g/L/h. Consumption of nutrients and formation of inhibitors cause a stop in the fermentation process. In fact, already in the pretreatment phase, inhibitors such as hydroxymethylfurfural can be formed. The GC-MS analysis confirmed the presence of furans, weak acids, and phenolics in the fermented mash that are known to severely inhibit yeast fermentation.

As can be seen from Figure 3, the fermentation was almost completed in 36 h, during which the fermentable sugars were consumed and bioethanol was produced. The final yield of ethanol at 48 h of fermentation was 40.1 $g \cdot L^{-1}$.

After fermentation, the liquid effluent was put into a distillation unit set at 78.5 °C to strip off ethanol. The ethanol yield of the present study is comparable to that reported for mandarin peel waste using popping pretreatment and simultaneous saccharification and fermentation (SSF), namely 46.2 $g \cdot L^{-1}$ [11]. Lower yields (22.7 $g \cdot L^{-1}$) were achieved from orange peels hydrolyzed by using sulfuric acid [32]. The fermentation with *S. cerevisiae* and *Candida parapsilosis* of orange pulp of floater led to the production of 24 $mg \cdot g^{-1}$ bioethanol after six hours; enzymatic hydrolysis was carried out by adding a protein extract [33]. Another SSF procedure applied to pomelo CW succeeded in the production of 36 $g \cdot L^{-1}$ ethanol (73.5% yield) [34]. In this study, to maximize ethanol yield, a fed-batch operation was carried out by feeding the bioreactor with 50% of new substrate, consisting of pretreated CW—the one obtained before the enzyme addition step. This procedure allowed the recovery of an extra amount of ethanol, as shown in Figure 4. The fed-batch fermentation increased both the amount of ethanol (from 40.1 $g \cdot L^{-1}$ to 52.3 $g \cdot L^{-1}$) and its productivity (from 0.83 $g \cdot L^{-1} \cdot h^{-1}$ to 1.09 $g \cdot L^{-1} \cdot h^{-1}$ at 48 h). This strategy made the whole process more sustainable considering that the productivity of ethanol from CW was almost completely depleted. It is worth mentioning that ethanol itself acts as an inhibitor of the fermentation process. Therefore, the addition of new substrate, although not rich in fermentable sugars, boosts fermentation. Also, the extra-substrate added at 24 h is not

enzymatically treated, meaning that no costs can be ascribed to this step with the exception of the energy spent for physical pretreatment.

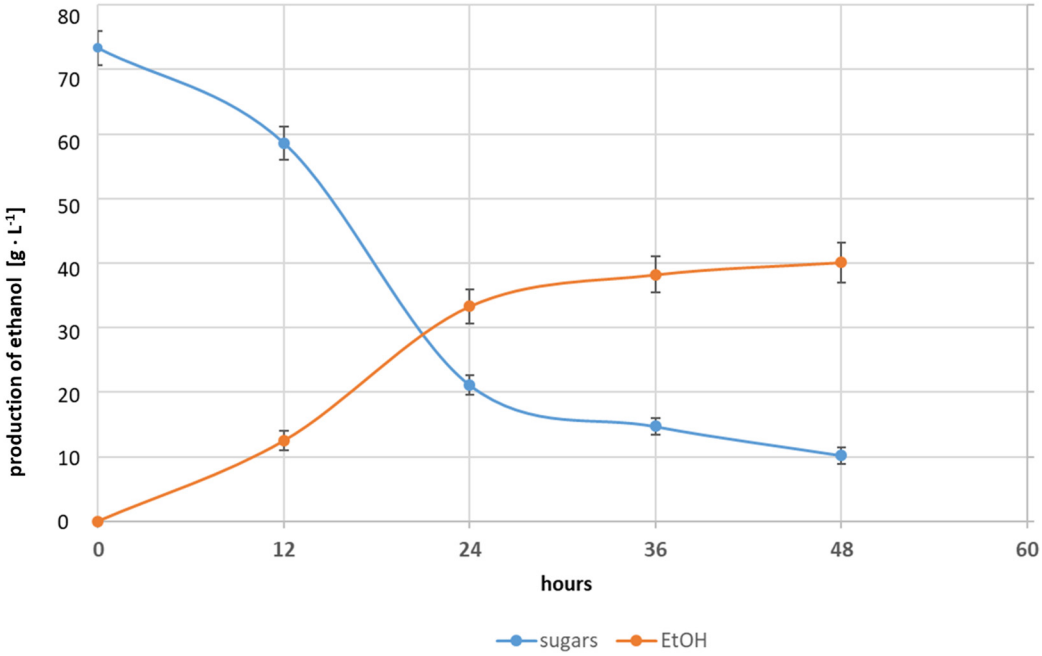

Figure 3. Correlation between sugar consumption and production of ethanol during fermentation by *Saccharomyces cerevisiae* and *Saccharomyces bayanus*.

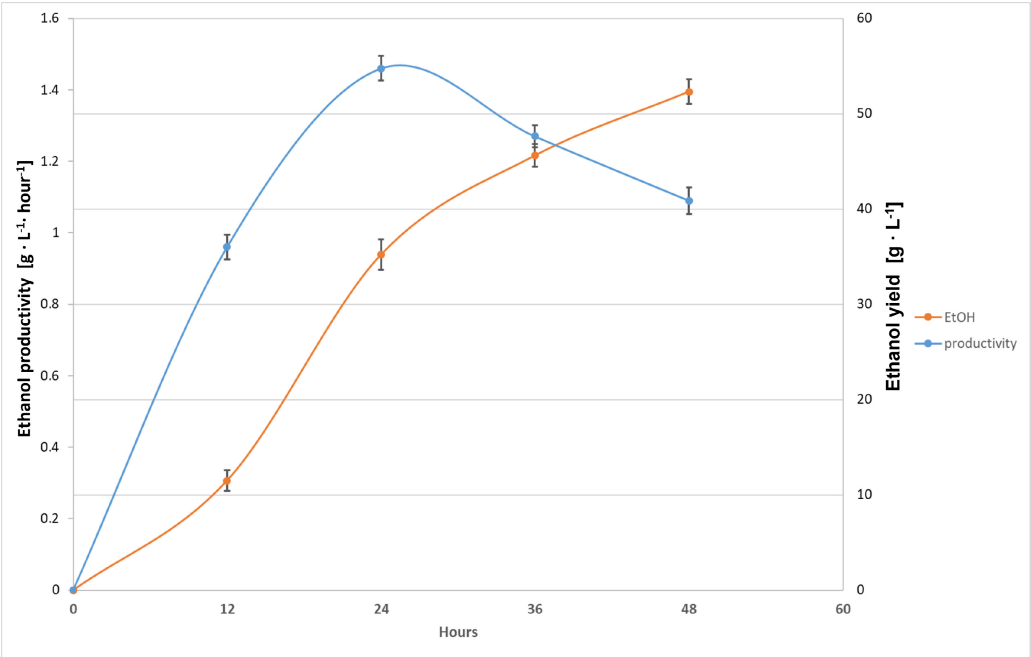

Figure 4. Maximization of bioethanol production through fed-batch operation by adding 50% pretreated CW at 24 h fermentation.

4. Conclusions

In this study, a sustainable strategy has been developed and applied to citrus waste material with the scope of producing ethanol to be used as biofuel. A combination of tools and the experimental setup make this approach innovative and sustainable. Compared to conventional processes of biofuel production through fermentation of waste, no acids were used for hydrolysis. Instead, a number of physical treatments (i.e., heating, sonication,

and microwave irradiation) were implemented to make the CW texture more accessible to enzyme attack. Various concentrations and types of enzymes were tested in order to establish the most suitable cocktail producing the highest amount of fermentable sugars.

The enzymatic saccharification of CW biomass gave relatively good yields of fermentable sugars, which were the substrate for yeast strains to produce bioethanol. In order to enhance the ethanol production, a fed-batch procedure was carried out by adding freshly pretreated CW during fermentation, achieving 52.3 $g \cdot L^{-1}$ ethanol and 1.09 $g \cdot L^{-1} \cdot h^{-1}$ productivity.

The experimental model here presented (see Figure S1) has shown considerable success on a laboratory scale. Its upgrading to an industrial scale opens up interesting scenarios for the exploitation of processing waste for energy production. This investigation largely meets at least two goals of the 2030 agenda for sustainable development, namely goal #7 (Affordable and Clean Energy) and goal #12 (Responsible Consumption and Production).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15129647/s1, Figure S1: scheme of the entire process for the production of bioethanol from citrus fruit waste.

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